Oestrogen Binding Species in the Rat Granulosa Cell

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To Phyllis and our children

ABSTRACT

This thesis describes the characterisation and ontogeny of oestrogen binding species of the rat granulosa cell together with a histological study of sections of the ovary during postnatal development of the follicle.

The preparation of pure rat granulosa cell suspension, cell fractionation and quantitative assays of oestrogen binding species are described. The oestrogen binding species have been characterised with respect to their dissociation constants, ligand dissociation and association rates, ligand specificities, thermal stabilities and binding capacity. The molecular sizes of these species have been determined using sucrose density gradient centrifugation and gel filtration. The intracellular distribution of oestrogen receptor between the cytoplasmic and nuclear compartments has been studied in the immature rat from 8 days after birth until commencement of the oestrous cycle. The in vivo effect of gonadotrophins on the intracellular distribution of oestrogen receptor in the granulosa cells has been studied using immature rats aged 20 days and over to establish the interaction of gonadotrophins and oestrogen in governing the biological activity of the granulosa cell.

These studies indicate the presence of a novel oestrogen binding species found only in the cytosol of granulosa cells which is distinct from the classical oestrogen receptor found in both the cytosol and the nuclear compartments. The oestrogen receptor is a constitutive property of granulosa cells which undergoes re-distribution during cell division.

The discussion of the thesis compares the characteristics of the granulosa cell oestrogen receptor and the putative novel oestrogen binding species with that of other tissues and examines the possible physiological roles of both oestrogen binding species in the granulosa cell function. The role that oestrogen receptor distribution might play in follicular development is also discussed.

LIST OF ABBREVIATIONS

OE_2	Oestradiol-17β
DES	Diethylstilboestrol
DHT	5α-dihydrotestesterone
R1881	Methyltrienolone
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra-acetic acid
TED	10mM Trizma, 1.5mM EDTA, 1mM DTT buffer
DNA	Deoxyribonucleic acid
PCA	Perchloric acid
dT	Deoxythymidylate
DCC	Dextran-coated charcoal
BSA	Bovine serum albumin

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CHAPTER 1

INTRODUCTION

1.1 Ovarian Development

The mammalian ovary is a complex heterogeneous organ which exhibits constant growth and development and paradoxically at the same time a continuous process of degeneration. As a result of the continuous differentiation the morphology of the ovary changes considerably between birth and maturity of the animal. The development of the mammalian ovary has been extensively studied. The mouse ovary, like that of most mammals, has been found to be a solid mass at birth (Peters, 1969) and consists of basically two types of cells, (Fig 1.1 I); these are (a) morphologically undistinguished cells or Stroma and (b) the specialised primordial oocytes whose meiotic cell division has been arrested at the dictyate stage. The meiotic process is only resumed shortly before ovulation.

During the period of postnatal development, the ovary grows in size and the stroma becomes differentiated in association with the primordial oocytes to form complex bodies called follicles which are embedded within the ovary.

The hallmark of follicular growth is the appearance and proliferation of granulosa cells. There are no

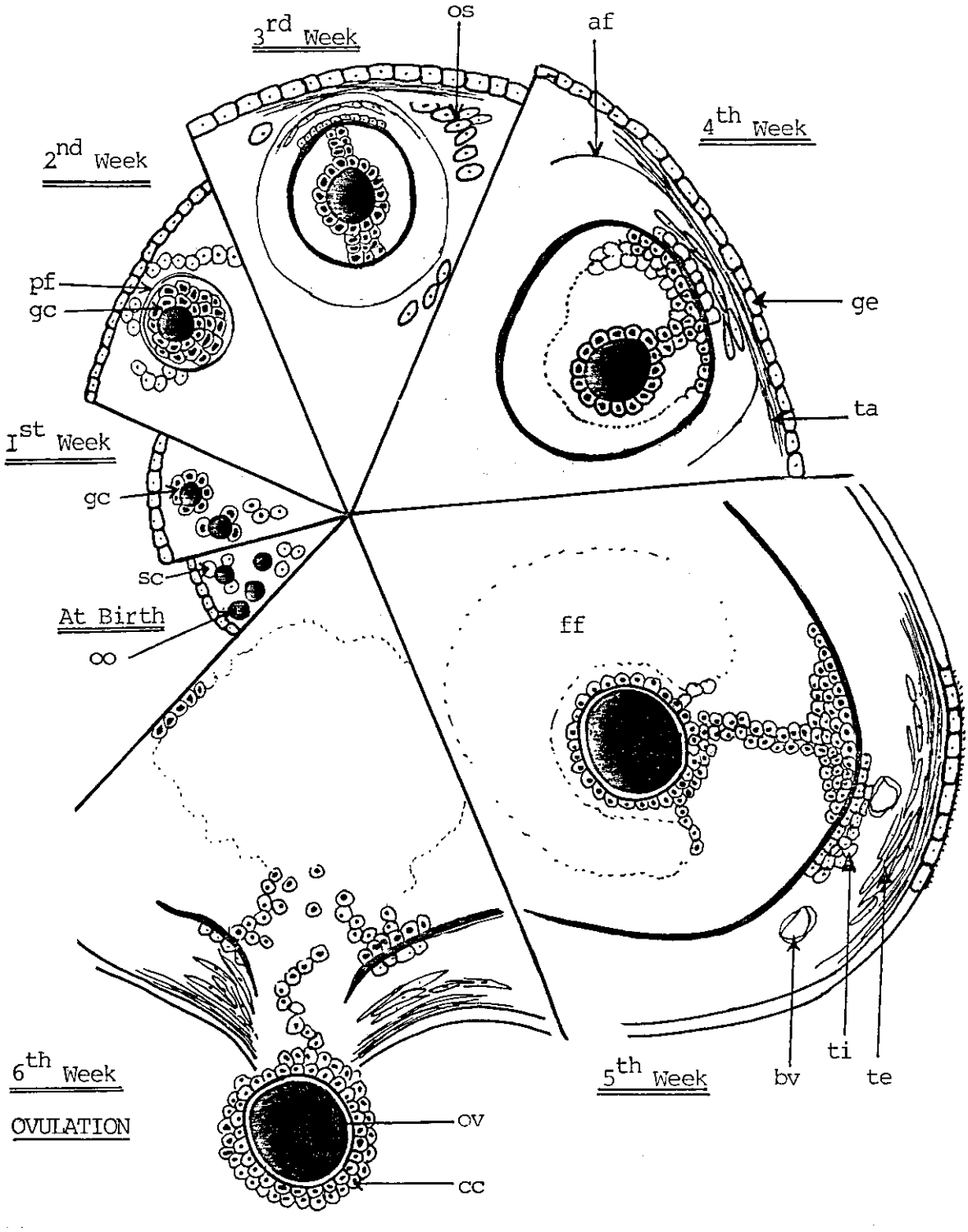


Fig.1.1. Ovarian development in the rodent. (oo)oocyte (sc)stromal cell(gc)granulosa cell(pf)preantral follicle (os)ovarian stroma(af)antral follicle(ge)germinal epithelium (ta)tunica albuginea(ti)theca interna(te)theca externa (ov)ovum(cc)cumulus cells(ff)follicular fluid.

granulosa cells in the ovary at birth, and in the mouse ovary they only appear at the end of the first week of life (Pederson, 1969). The exact origin of the granulosa cells is not known but many theories have been advanced. Odor & Blandau (1969) postulated that cells found attached to oogonia before meiosis started in the foetal mouse ovary gave rise to future granulosa cells while Zuckerman and Baker (1977) indicated that granulosa cells were derived from the epithelial cells that surround the oocytes in early life. This latter view was disputed by Byskov et al.,(1977) who showed that removal of the rete ovarii from the ovarian tissue before follicles were formed prevented follicular organization, while the absence of the surface epithelium had no effect on follicular growth. The best accepted theory however has been derived from autoradiography. After pulse-labelling stromal cells with [^{3}H]-thymidine ([^{3}H]-TdR) in the new-born mouse ovary Peters (1969) found that several days later the marker had been incorporated into granulosa cells and therefore deduced that granulosa cells may have arisen from the pool of stromal cells (Fig 1.1 II). Concomitant with the appearance of the granulosa cells is the formation of an elastic basement membrane which encloses the granulosa cells and then the theca cell layer which borders the developing follicle. By the end of the second week of life the rodent ovary acquires two to three rows of granulosa cells, the

basement membrane and the theca cell layer become well developed but the oocytes are still very small (Fig 1.1 III). During the third and fourth weeks of life the follicles develop rapidly passing through the preantral and antral stages with increasing granulosa cell proliferation (Fig 1.1 IV, V). Some of these follicles are however observed to undergo degeneration after reaching a certain size and Pederson (1969) estimated that on average between one and two large follicles started to degenerate every hour during the late immature period (from day 21 to 35) in the mouse ovary.

Measuring the rate of follicular degeneration by oocyte atresia, Arai (1920) estimated that out of about 35,000 ova present in the rat ovary at birth, only 11,000 were still present by day 21 post-partum. This represents a degeneration rate of about 1,000 follicles a day. In the human, it is estimated that 99.9% of all follicles present at birth are destined to degenerate (Hodgen, 1982).

Peters & Levy (1964) have suggested some mechanisms which transform the atretic follicles or remove them from the ovary : (a) the follicle might be surrounded by blood vessels, their cells lysed and the debris carried out by the capillary system or (b) the follicles might be transformed back into stroma. This latter conclusion has arisen from autoradiographic

studies. Radioactive thymidine ([^{3}H]-TdR) when injected into juvenile ovaries was quickly incorporated into granulosa cells of large follicles but after several days, the marker was found in the intrafollicular stroma. Thus the cells in the follicle of the younger ovary might have arisen from the undistinguished stroma cell pool and the cells in the stroma of the older ovary might well be cells which used to be part of the follicle.

1.2 Hormonal Control of Follicular Growth

The growing ovarian follicle is a differentiating system that requires the interaction of polypeptide and steroid hormones for its functions. The studies of Greep et al.(1942) are fundamental to our understanding of this system. These workers showed that

(1) at least two gonadotrophins can be isolated from the extract of pituitary glands : the Interstitial Cell Stimulating Hormone (ICSH)/Luteinising Hormone (LH) then called metakentrin and the 'gametogenic' hormone' Follicle Stimulating Hormone (FSH) then called thylakentrin.

(2) Hypophysectomy produced atrophy of the rat ovary but administration of LH to these animals produced hypertrophy of the interstitial cells and caused the formation of corpora lutea.

(3) FSH adminsitration to hypophysectomised rats produced growth and maturity of follicles without signs of luteinisation or atresia. Moreover, FSH-

induced growth was restricted to only medium-sized follicles so that follicular growth prior to this must have been due to 'extra-pituitary factors' and (4) given alone, neither LH or FSH could stimulate oestrogen secretion but in combination there was marked evidence of oestrogen secretion assessed histologically as well as by uterine weight increase and vaginal epithelial cell stratification and cornification.

Prior to these studies, Pencharz (1940) and Williams (1940) had also been able to reverse the effects of hypophysectomy in female rats by direct oestrogen treatment. By administering oestradiol benzoate, de Wit (1953) confirmed the reversal of ovarian follicular atrophy and showed that the steroid stimulated mitosis and enlargement of granulosa cells which resulted in total ovarian weight gain. Overall then it was firmly established that the cooperative action of the polypeptide hormones LH and FSH were required for the production of oestrogen which then stimulated ovarian follicular growth. However, the relationship between the peptide hormones and the production of steroid as a result of their combined action was not clarified until Ryle et al. (1974) incubated whole mouse ovaries from intact animals in the presence of FSH or LH or both. They found that all the hormone treatments induced follicle growth. LH had least effect but a combination of FSH and LH acted synergistically to produce the greatest

amount of oestrogen. The earlier studies of Goldenberg et al., (1972) had shown the interaction of FSH and oestrogen. They found that at every dose level of FSH administered, pretreatment of the animals with the oestrogen, diethylstilboestrol (DES) resulted in an increase in ovarian weight and an increase in the percentage of follicles with antra. These workers demonstrated that FSH caused an enhanced uptake of radioactive thymidine into granulosa cell DNA following DES treatment. These experiments suggested that at some stages during follicular development, oestrogen was required to prime the granulosa cells whereupon they became more responsive to FSH. The signal that triggers off the initiation of follicular growth is still not known but once it is received, a group of follicles do begin to grow in response to LH, FSH and oestradiol. Some eventually ovulate but the majority degenerate.

1.3 Steroid Biosynthesis in the Ovary

The experiments which established that oestrogen stimulated ovarian growth and was synthesized in the ovary (Greep et al., 1942; Pencharz, 1940; Williams 1940; de Wit, 1953; Ryle et al., 1974) did not identify the compartments within the ovary where the two gonadotrophins acted, where oestrogen was produced or where it acted. In attempting to elucidate these points, Falck (1953) measured the occurrence of cholesterol and assessed oestrogen

production in the rat ovary. He found that oestrogen did not occur in the ovary until the rats were 7 to 9 days old. Whilst initially occurring in scanty patches, cholesterol increased in abundance from day 12 onwards and was restricted to only interstitial glands. Falck also observed that until the rats were 8 days old, their ovaries did not respond to gonadotrophic (HCG) stimulation. Older ovaries responded to HCG and vaginal cornification was seen. Because the transformation of the rat ovary from a non-oestrogen producing to an oestrogen producing state on days 7 to 11 post-partum was accompanied by the appearance and storage of cholesterol, these experiments indicated that cholesterol could be the precursor for oestrogn formation. Since Greep et al., (1942) had shown that ICSH/LH acted predominantly on interstitial cells it could be deduced that the gonadotrophin was involved in cholesterol conversion into a form which was then processed into oestrogen. Contrary to prevailing evidence that the theca cells were the source of oestrogen in the follicle, Falck (1959) demonstrated that when pure samples of theca cell layers were grafted onto the rat vagina, there was no oestrogenic response. Instead microtransplants containing theca in combination with granulosa cells caused vaginal epithelium to cornify. This experiment demonstrated that oestrogen production in the follicle depended on the cooperation of the theca and granulosa cell-types.

More recently, with the advent of organ and tissue culture, it has been possible to examine hormone production of the separate cell types under different hormonal conditions. Using these techniques Moor (1977) established that in the ovine ovary

i) intact follicles produced oestrogen, testosterone and some progesterone. FSH enhanced the production of these steriods and was accompanied by morphological luteinization.

ii) isolated theca cells produced only testosterone

iii) granulosa cells in monolayer did not produce either oestrogen or testosterone but

iv) with a combination of theca and granulosa cells the capacity to produce these hormones was restored.

These experiments confirmed that the cooperation of the two cell-types was mandatory for oestrogen biosynthesis and showed that the product of LH activity in cholesterol conversion was testosterone. Addition of testosterone to granulosa cells in culture caused the production of oestrogen and FSH enhanced the production (Dorrington et al.,1975; Moor, 1977).

The experimental evidence accumulated from such studies has given rise to the two-cell, two-gonadotrophin and two-steroid theory which is summarised in Fig 1.2.

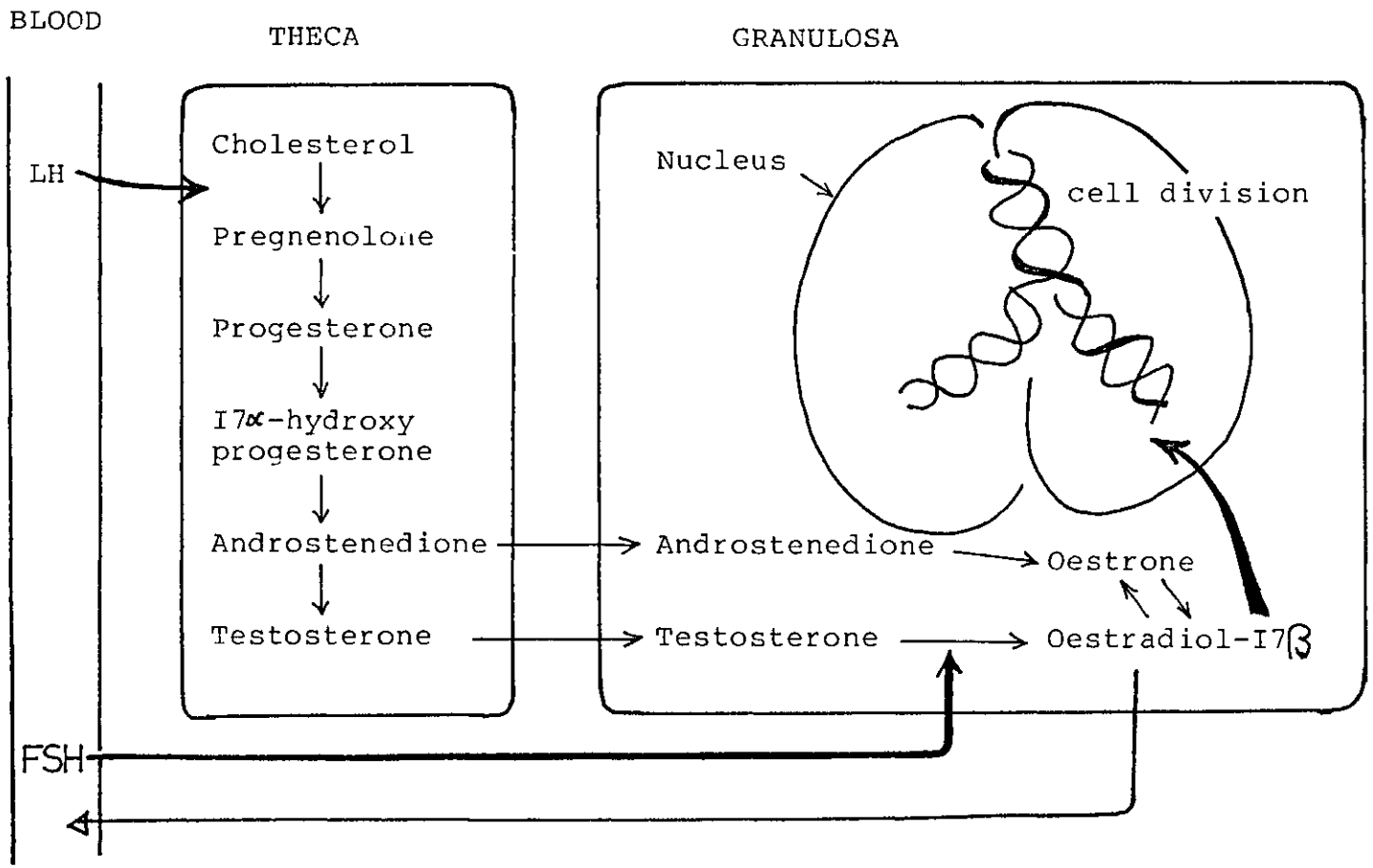


Fig.1.2. Gonadotrophin-Steroid interaction at the cellular level in the mammalian ovary.

Briefly LH acts on the theca cell where it stimulates the conversion of cholesterol to testosterone which then passes into the granulosa cell compartment probably by diffusion across the cell membranes. In the granulosa cell, testosterone is prevented from accumulating by FSH which stimulates the oestrogen synthetase to aromatize testosterone to oestradiol-17β. The oestrogen produced then passes into the general circulation or acts on the granulosa cell itself, stimulating the mitotic process leading to granulosa cell hyperplasia and the production of the follicular fluid (Richards, 1979).

1.4 Recruitment and Selection of Follicles for Ovulation

Any ovarian follicle destined to ovulate is derived from a cohort of growing follicles and various theories have been proposed to explain how the follicles are selected. One such theory is that the follicles are selected in response to the gonadotrophin surge immediately preceeding ovulation (Richards, 1979). In animals with a 4 to 5 day oestrous cycle a surge of FSH and LH occurs late in the afternoon of pro-oestrus with the FSH surge extending in to the morning of oestrus (Greenwald, 1980). Richards (1979, 1980) suggested that the follicles which are selected in response to the gonadotrophin surge of one cycle would actually have begun to grow at least three cycles earlier as part of a pool of growing follicles. Hodgen (1982) also reported a similar observation in

the monkey : cautery of the largest visible follicle at mid-cycle destroyed the follicle destined to ovulate and the accompanying expected surges of gonadotrophins and oestrogen secretion did not take place. These however occurred two weeks later and since the delay approximated to the length of the follicular phase, it indicated that apart from the ablated follicle there was no other follicle capable of ovulating. This suggested that the follicle destined to ovulate was selected probably before the cautery and that the next follicle to ovulate would have to come from a different cohort of follicles. The remaining follicles in a particular cohort are therefore destined to undergo atresia.

The phenomenon of compensatory ovulation by polytocous species such as rodents has been found to give an indication of when follicles may be set aside (Greenwald, 1980). This phenomenon is the mechanism whereby unilateral ovariectomy of the animals leads to the stimulation of the remaining ovary to produce twice as many ova to compensate for the absence of the other ovary. This way the ovulation rate characteristic of the species is maintained. The ability of the animal to compensate however depends on which day in the cycle that the ovariectomy is performed. By the method of unilateral ovariectomy, therefore, follicles can be seen to be committed to ovulate on day 3 to 4 (pro-oestrus) in the rat cycle

(Peppler & Greenwald, 1970), day 12 in the guinea-pig cycle (Hemreck & Greenwald, 1970) or day 3 in the hamster cycle (Bast & Greenwald, 1977). During follicular development, all follicles are equally exposed to the FSH and LH surges. It has been suggested that the FSH surge primarily serves to select a group of follicles for the final stages of growth and the LH surge, which appears later might terminate their growth allowing only a few follicles to ovulate (Richards, 1979). On the basis of this, it would appear that follicles are unequally responsive to the gonadotrophins and that the stage of differentiation of the follicular cells might determine which follicles will respond. Specific gonadotrophin receptors have been demonstrated in ovarian tissues. FSH receptors are reported to be restricted to only granulosa cells (Ireland & Richards 1978; Richards,1979, 1980) and LH receptors are present in the theca, interstitial, luteal cells (Channing & Kammerman, 1974; Richards et al., 1976; Richards, 1979, 1980) and in granulosa cells of large preovulatory follicles (Zeleznik et al., 1974; Richards et al., 1976; Richards, 1979, 1980). It has been postulated that alterations in follicular receptor content for these gonadotrophins might determine which follicles respond to the gonadotrophin surge. Since oestrogen modifies the ovarian response to the gonadotrophin FSH (Goldenberg et al., 1972) it has been presumed that it might have a direct effect

on the gondatrophin receptors. By measuring receptor content in granulosa cells harvested from hypophysectomised rats appropriately primed with oestradiol, FSH and LH or a combination of these hormones it has been shown that there are three types of receptor regulation mechanisms that operate during follicular development.

(a) Autoregulation. This is a process whereby a hormone alters its own receptor content, for example oestradiol was found to be capable of stimulating an increase in its own receptor (Richards, 1975).

(b) Coordinated regulation, a process whereby steroid and peptide hormones interact to alter the receptor content of the same or a different peptide hormone. For example, FSH acts on oestradiol-primed granulosa cells to increase LH and FSH receptors (Richards, 1975; Richards et al., 1976) and LH, in the absence of FSH, reduced LH and FSH binding sites.

(c) Heteroregulation. By this process, one hormone affects the receptor content of an entirely different hormone, for example LH acts on an oestradiol-FSH-primed granulosa cells to increase prolactin (PRL) receptor in addition to decreasing the receptors for FSH, LH and oestradiol (Richards & Williams, 1976; Richards et al., 1976). These results demonstrated that regulation of hormone receptors could participate in maturation and selection of follicles for ovulation. Thus although all granulosa cells possess FSH receptors, their cellular response to FSH can be drastically

modified by the presence of oestradiol which alters the FSH receptor content (Richards, 1976). As a follicle enters the final growth phase, the granulosa cells acquire LH receptors, presumably through the action of FSH, which permit it to respond to the LH surge to ovulate and luteinise (Richards, 1979). Rat granulosa cells from preovulatory follicles possess high oestrogen receptor content and luteinization of granulosa cells as well as atresia is said to be associated with a decrease in oestrogen receptor content (Richards, 1975). The development and selection of follicles destined to ovulate may therefore be related to their ability to produce and respond to oestradiol.

It has been demonstrated that oestradiol, of all the reproductive hormones, has by far the most profound effect on follicular development for it enhances the proliferation of granulosa cells and their response to basal levels of circulating gonadotrophins. At the preovulatory stage, elevated oestrogen production then triggers off the brain to produce the LH surge (Legan et al., 1975) by which time LH receptors appear on granulosa cells. Through the action of oestradiol therefore, follicular development and the LH surge are synchronized to effect ovulation.

1.5 Control of Ovulation

Ovulation is preceded by a gonadotrophin surge for

a species-specific number of hours. Prevention of the surge abolishes ovulation even though prepared follicles may be present in the ovary (Brown, 1968; Bingel & Schwartz, 1969; Schwartz, 1973). In a number of mammalian species, oestrogen constitutes the essential signal in a chain of events that trigger off the gonadotrophin surge (principally, LH). Ovulation may be spontaneous following a gonadotrophin surge in the absence of coitus as seen in the rat, mouse, man etc. In the rabbit however, ovulation occurs only after coitus which triggers off the gonadotrophin surge. The control of ovulation in the coitus-induced group of animal is proposed to result from the following sequence of events : elevated oestrogen production during follicular development acts as the afferent feedback signal from the ovary to the hypothalamic - pituitary axis. This induces mating behaviour and hypothalamic sensitivity to feedback from coital stimulation (Kawakami & Sawyer, 1967). The coital stimulation then triggers off the gonadotrophin surge followed by ovulation. In the spontaneous ovulating species, oestrogen is known to play a similar role to that in the coitus-induced ovulatory species e.g. as a feedback signal to the higher centres. In addition, oestrogen acts to determine what time of day or day of the cycle to induce the ovulatory surge of LH (Schwartz, 1973). As a result of the LH surge ovulation occurs accompanied by

(i) decline in LH receptors in the follicle

(ii) decrease in the production of thecal androgens

(iii) reduction in the aromatization of androgens

(iv) production of progesterone

(v) synthesis of pre-ovulatory enzymes

(vi) synthesis of proteoglycans

(vii) synthesis of prostaglandins

(viii) resumption of meiosis in oocytes.

Over 80% of these events involve the multifarious granulosa cells of the preovulatory follicle in ways which are discussed below. See also Fig.1.3.

1.5.1 Prostaglandins

The capacity of the ovary to produce prostaglandins is located predominantly in the granulosa cells (Clark et al., 1978; Erickson et al., 1977; Triebwasser et al., 1978) and increased prostaglandin synthesis can be stimulated in these cells with LH (Clark et al., 1978). In vivo increased prostaglandin production was found to be dependent on the preovulatory peak of circulating LH (Challis et al., 1974) In rabbits, there is a substantial increase in prostaglandin production within 4 - 5 hr after gonadotrophin stimulation (Le Maire et al., 1973). The preovulatory increase in prostaglandin is essential for ovulation for, in rats and many other laboratory animals, acute administration of indomethacin or antiserum to $PGF_{2\alpha}$ given systemically

or directly into the follicle inhibited ovum release. (Orczyk & Behrman, 1972; Tsafriri et al., 1972; Armstrong et al., 1974). Armstrong et al., (1974) observed that $PGF_{2\alpha}$ antiserum was more effective than PGE_2 antiserum in blocking follicular rupture. The fact that the peak of ovarian prostaglandin F production was coincidental with ovulation has led to the suggestion that it might participate in or be responsible for follicular rupture, by increasing ovarian contractility (Espey, 1978). Kuehl et al., (1970) showed the PGE_2 could mimic the effect of LH in isolated follicles and Lindner et al.,(1974) showed that in vivo indomethacin blocked ovulation without affecting LH-induced ovum maturation, steroidogenesis or the LH-surge. This indicated that LH and PGE_2 act independently probably through a common second messenger, cyclic AMP. The seperable effects of PGE and PGF was demonstrated by Le Maire et al. (1975) who found that follicles showing signs of ovulation had comparatively higher concentrations of PGE and PGF series in the follicular fluid than non-ovulating follicles and that after ovulation PGF fell sharply but PGE declined very slowly. Challis et al. (1974) had demonstrated that PGF was produced by rabbit granulosa cells in vitro. Lee & Novy (1978) have suggested that prostaglandin E may function solely as a mediator of ovarian hyperemia during the later stages of the ovulatory process. Diaz-Infante et al., (1974) showed that

$PGF_{2\alpha}$ induced ovarian contractility in subprimates and primates but PGE_2 inhibited it. $PGF_{2\alpha}$ but not PGE_2 has been found to increase follicular oestradiol production during superfusion of pig follicles and to stimulate the aromatase enzyme system in vitro (Dodson & Watson, 1980). PGE_2 rather caused an increase in progesterone production.

The rupture of the follicle is essential for completion of ovulation or expulsion of the ovum. Intrafollicular pressure has also been suggested as one cause of follicular rupture. Blandau & Rumery (1963) and Espey & Lipner (1963) have measured intra-follicular pressure of developing follicles in the rat and rabbit respectively, and found that follicles could not be ruptured by pressure alone. In the rabbit, post-ovulation pressure fell from 15mm to 5mm Hg rather than Zero mm Hg and some atretic follicles had pressures of about 50 mm Hg, far beyond that in the normal preovulatory follicle (17 mm Hg). Rondell (1970) has also suggested that the morphological changes in the area of the stigma indicates that the process of follicular rupture might also be under hormonal or enzymatic control.

1.5.2 Plasminogen Activator

Fibrinolytic activity has been observed to increase in the follicle during the hours preceeding ovulation and this activity is associated with production of

plasminogen activator (Beers et al, 1975; Strickland & Beers, 1976). This increase in plasminogen activator in the follicle may be related to the elevation of the urine plasminogen activator, urokinase, observed near the time of ovulation (Oster, 1978). Strickland & Beers (1976) have demonstrated that only granulosa cells from pre-ovulatory follicles possessed the ability to produce the plasminogen activator, molecular weight 75,000 in vitro. The substrate for this enzyme, plasminogen, is present in follicular fluid and the product of the reaction, plasmin,has been shown to weaken follicle wall strips in vitro (Beers, 1975). Cyclic AMP and prostaglandins E_1 and E_2 but not prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$ (Oster, 1978) FSH and Testosterone in combination or FSH alone stimulated granulosa cells to produce plasminogen activator in vitro (Liu et al., 1981). It has been proposed that the plasminogen activator might be responsible for disruption of the follicle at ovulation.

1.5.3 Proteoglycans

Jensen & Zachariah (1958) demonstrated the presence of substances, which recent workers have referred to as proteoglycans, in follicular fluid and suggested that they were produced by granulosa cells under oestrogen stimulation (Zachariah, 1959). These workers also identified 'hyaluronidase-like' activity which, by breaking down high-molecular weight proteoglycans

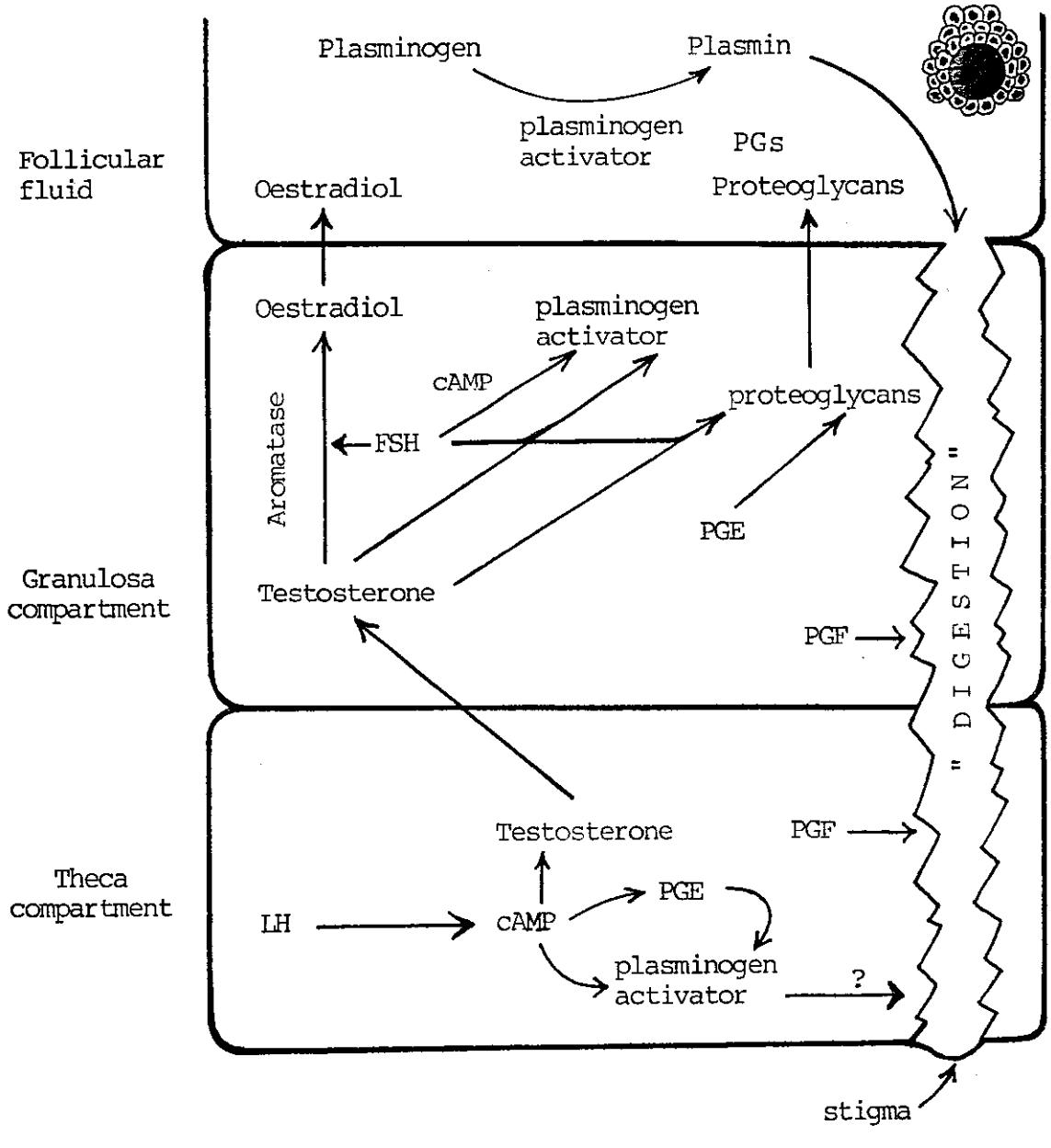


Fig.1.3. Factors involved in ovulation in the rat.

raised the colloid-osmotic pressure of follicular fluid from 20 to 60 cm H_2O (Zachariah, 1959). More recent work has confirmed that granulosa cells synthesise proteoglycans under the stimulation of FSH, LH,hCG, PGE_1,PGE_2 and testosterone (Mueller et al., 1978; Ax & Ryan, 1979; Yanagishita et al., 1981; Lenz et al., 1982). It has been suggested that proteoglycans might be involved in follicular rupture and probably fertilization because proteoglycans from bovine follicular fluid enhanced acrosome reaction in bovine spermatazoa (Lenz et al.,1982).

1.6 Reasons for this Study

FSH receptor levels do not appear to change during the period of ovarian maturation (Uilenbroek, 1982) but FSH responses are enhanced at later stages of follicular development through the action of oestrogen (Goldenberg et al., 1972; Richards et al., 1976; Richards, 1979, 1980). Development of the ovarian follicles and their selection for ovulation appears to depend predominantly on their ability to produce and respond to oestrogen. Ultimately ovulation depends on synchronisation by oestradiol of follicular growth with the gonadotrophin surge. The mechanism of action of oestrogen in the ovary is not well understood but its effect may be mediated via a receptor. Saiduddin & Zassenhaus (1977) have demonstrated the presence of an oestrogen receptor in the ovary and studies have been conducted on nuclear

(but not cytosol) oestrogen receptors in the granulosa cell (Richards et al., 1976).

Further studies are required in order to provide insights into how oestrogen could control ovarian follicular development via its binding proteins. It is appropriate to study the entire oestrogen receptor system (nuclear + cytosol) of the granulosa cell with respect to its ontogeny and its intracellular distribution during the postnatal development of the ovary.

1.7 Oestrogen Action via Intracellular Receptors

The studies which established that oestrogen stimulated ovarian growth also identified that the target for steroid action in the ovary was the granulosa cell which underwent hyperplasia. One of the properties of a steroid hormone target tissue is the ability to selectively bind and retain the hormone for a considerable period of time (King & Mainwaring, 1974). Experiments aimed at identifying the unique components of target cells which bound specific hormones were made possible in the late 1950's when a high specific activity tritiated oestradiol ([^{3}H]-oestradiol - 17β) became available. Jensen & Jacobsen,(1962) demonstrated the presence of unique steroid binding molecules in three tissues : the uterus, vagina and the anterior pituitary. These tissues were accepted as oestrogen target tissues because they retained the

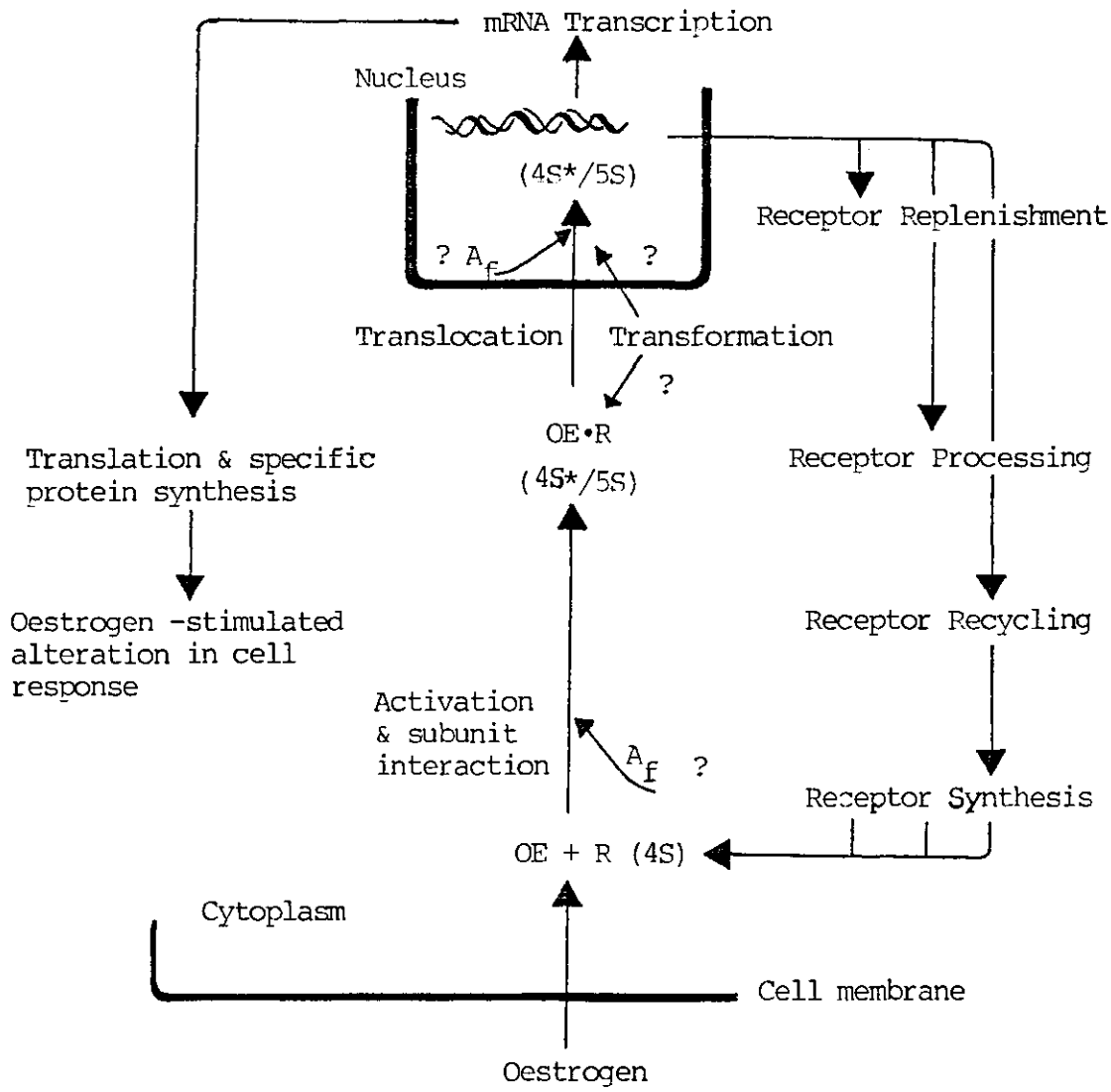
hormone against a concentration gradient in the circulating blood. Using dry-mount high resolution autoradiography,Stumpf (1969) showed that in addition to the uterus, vagina and anterior pituitary [^{3}H]-oestradiol injected into rats was retained in the nuclei of all cell types of the oviduct, the nuclei of cells located between the seminiferous tubules of the testis, cytoplasm of liver and adrenal cortex cells and in the ovary, especially the granulosa cell.

Stumpf (1969) also confirmed that the specific hormone binding molecules or 'receptors' could be present in two fractions of the cell; the high speed supernatant of tissue or cell homogenates called cytosol and in the nucleus. Similar experiments, both in vivo and in vitro,have identified receptors for other polypeptide and steroid hormones. Much of the present knowledge concerning the oestrogen receptor has been derived from studies in the rat uterus. Although not all the steps involved in the mechanism of hormone action at the cellular level are clearly understood, it is possible to construct a working hypothetical model. The steps involved are shown in Fig. 1.4.

1.7.1 Entry of Oestrogen into the Cell

The lipophilic nature of the cell membrane has been assumed to provide very little resistance to the diffusion of steroid into the cell. The diffusion

Fig.1.4.The Mechanism Of Oestrogen Receptor Action



4S Non-activated receptor } (M.W.60-80,000)
4S* Activated receptor }
5S Activated & transformed receptor, (M.W. I30-I40,000)
A_f Activating Factor
? Steps requiring further study.

process will be enhanced by the fact that 17β-oestradiol has high lipid solubility and a high lipid-water distribution coefficient (King & Mainwaring, 1974). The concept of free diffusion of 17β-oestradiol into a target cell was however challenged by Baulieu et al., (1972) who observed that [^{3}H]-oestradiol incorporation into uterine cells was inhibited by sulphydryl blocking agents. These workers therefore suggested that some membrane-bound proteins containing sulphydryl groups might be involved in the transport of the steroid into the target cell. As the sulphydryl-blocking agents may have entered the cell and inactivated the binding of [^{3}H]-oestradiol to the cytosol receptor itself, their suggestion has not attracted much attention. Cell labelling/affinity chromatographic techniques enabled Pietras & Szego (1977) to demonstrate that receptor components specific for 17β-oestradiol, but not its 17α-oestradiol isomer, were present on the external surfaces of oestrogen-responsive cells. At low temperatures (4°C) cell membrane-bound [^{3}H]-oestradiol was low matching a decline in the cells' accummulation of oestrogen and this was attributed to a reduction in cell surface receptor for the hormone probably due to re-orientation of binding sites on the cell membrane. These sites could be saturated with oestradiol probably by masking binding sites, but the inability of a technique like dry-mount autoradiography to identify these membrane-bound receptors may have

however caused investigators to overlook it as a potential mechanism of transporting hormone to the well-documented cytoplasmic receptor sites.

1.7.2 The Cytosol Oestrogen Receptor

1.7.2.1 Molecular Forms, sizes and their interconversions

The cytosol oestrogen receptor exists *in vitro* in a form which migrates on sucrose density gradients (SDG) in a discrete band with a sedimentation coefficient of 8-10S in hypotonic buffers (less than 0.IM). This macromolecule has been estimated to have a molecular weight of between 200,000 to 238,000; Stoke's radius, 67 Å; Frictional ratio (f/fo), 1.65; axial ratio (prolate/oblate), 8.3/0.09; isoelectric point (pH) 6.2 when electrofocussed (Erdos, 1968; Jensen *et al.*, 1968; Puca *et al.*,1971; Stancel *et al.*, 1973a; Notides & Nielsen, 1974). As the salt concentration of buffers are increased to 0.15M Na or KCl the complex migrated more slowly on SDG with a sedimentation coefficient of 3.8 - 7.5S. At 0.4M the complex sedimented at 3.8 - 4.5S, with an estimated molecular weight 60 - 80,000; Stoke radius 33-44 Å; frictional ratio (f/fo), 1.25 - 1.45; axial ratio (prolate/oblate) 3.4/0.31. When electrofocussed a heterogenous species was obtained with isoelectric points at 6.6 and 6.8 (Puca *et al.*, 1971; Notides & Nielsen, 1974). Salt concentration above 0.4M KCl however did not dissociate the receptor any further. Proteolytic

enzymes such as trypsin convert the 8-10S receptor to a 3.9S species with an isoelectric point of 6.2 (Puca et al., 1971). Removal of the salt by dialysis caused reaggregation of the 4S species back to the 8S (Baulieu et al., 1971)

Steggles & King (1970) reported the presence of both 8S and 4S forms of the receptor in the mature rat uterus even in the absence of salt. This had led to the suggestion that in vivo the oestrogen receptor (like other steroid receptors) existed as a 4S form rather than as an 8-10S form (Stancel et al., 1973a). This larger form could well be an artefact which is formed during in vitro fractionation process. The 8S form was suggested by Puca et al., (1971) to be a tetramer consisting of four of the 4S sub-units (the monomer) with only one of these possessing an oestrogen binding site.

1.7.2.2 Dissociation constants of receptor-oestrogen interactions

Various methods have been used to assess the equilibrium dissociation constants (Kd) for receptor-oestrogen interaction. These include sephadex gel filtration (Puca & Bresciani, 1969; Ginsburg et al., 1974) charcoal adsorption (Mester et al., 1970) hydroxylapatite adsorption (Erdos et al., 1970) equilibrium dialysis (Erdos et al., 1969) Polyacrylamide gel electrophoresis (Duffy & Duffy, 1977) and protamine sulphate precipitation (Steggles

& King, 1970). Values for the dissociation constant of the receptor-oestradiol interaction generally agree despite the differences in methodology and are in the range of 10^{-9} to 10^{-10} M (King & Mainwaring, 1974; O'Malley & Means, 1976). Binding studies on a variety of oestrogen target tissues, e.g. pituitary, hypothalamus and uterus reveal similar dissociation constants and this has lent weight to the assumption that all oestrogen receptors are identical. The measurement of the affinity of the receptor, however has been found to depend on the protein concentration of the cytosol. At high protein concentration (3 to 6 mg/ml) the Kd is proportional to the protein concentration while at protein concentrations below 1 mg/ml a biphasic scatchard plot is obtained; in the upper part the Kd is proportional to protein concentration and in the lower part the Kd increases at oestradiol concentrations below 10^{-10} M (Sanborn et al., 1971; King & Mainwaring, 1974). The Kd however, does not alter between 0 and 20°C. Only at higher temperatures was the Kd altered (King & Mainwaring, 1974).

1.7.2.3 Receptor Binding Capacity

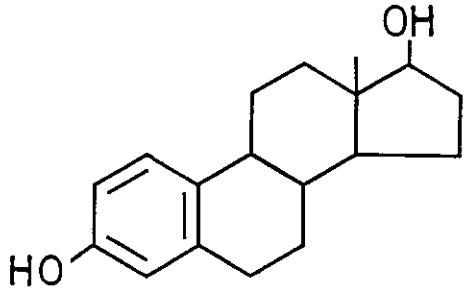
One of the criteria for establishing a true receptor-hormone binding interaction is a demonstration that the binding activity is saturable (Clark & Peck, 1979). Oestrogen-receptor binding is saturable and is of low capacity. There is a disagreement on the quantity of

cytosol receptor sites present in the immature rat uterine cell; Clark & Gorski (1970) reported 16-20,000 sites/cell while Jensen et al. (1968) measured at least 2.1 pmol/uterus which they estimated to be equivalent to more than 100,000 sites/cell (Jensen & DeSombre, 1972). Using affinity labelling Muldoon (1971) reported an estimated 4 pmol/uterus (about 290,000 sites/cell). These differences may reflect the instability of the receptor, the different methods of measurement or the different protein concentration in the cytosol used by various workers.

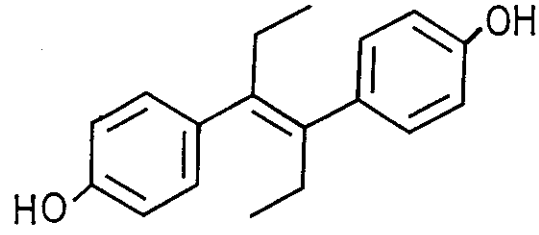
1.8 Specificity of the Oestrogen Receptor

The plurality of different receptor proteins for hormones that are structurally similar in the same target tissues makes the possibility of one steroid binding to different receptor proteins inevitable. It has been suggested that structural details of steroids might have direct and overriding influences on the level of cross-reactions in vivo. Duax et al., (1981) have compared by crystallography the structures of receptor-bound steroids and determined that probably the essential structural features required for binding to the oestrogen receptor are the A and D rings on steroids. They postulated that the A ring might be required for binding to the receptor, the energy for the binding being derived from the 3-hydroxyl group on oestradiol (Fig. 1.5) which may act as both hydrogen bond donor and acceptor.

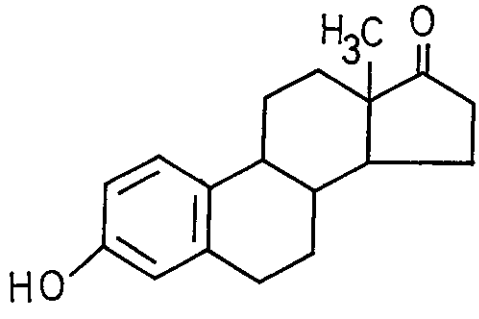
Fig.1.5. Structures of compounds that may or may not bind to the oestrogen receptor



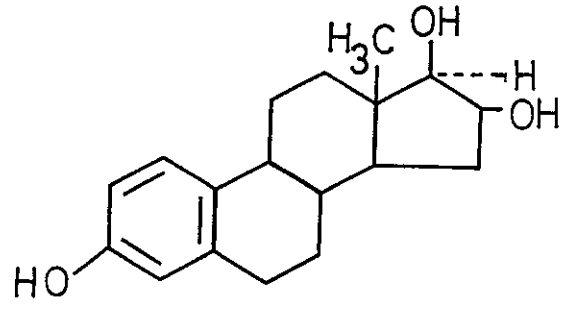
(a) 17β-oestradiol



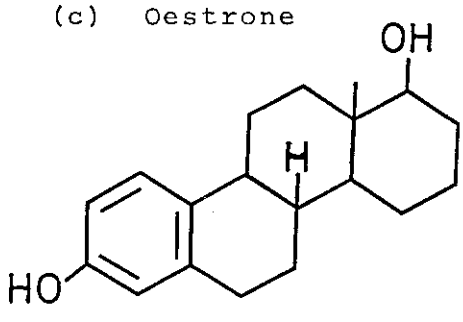
(b) Diethylstilboestrol



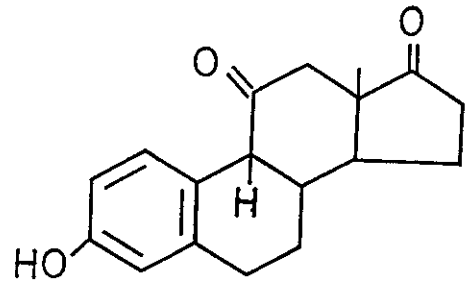
(c) Oestrone



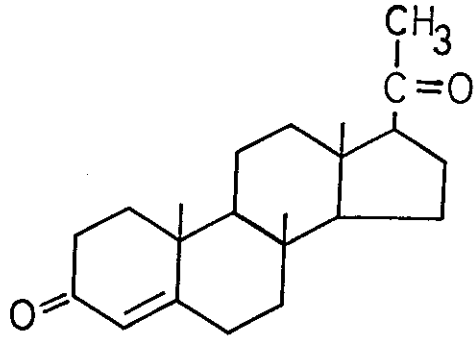
(d) Oestriol



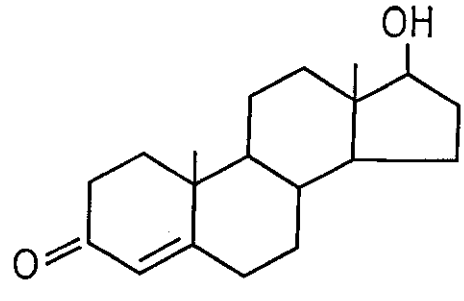
(e) 8α-D-homoestradiol



(f) 11-keto-9β-oestrone

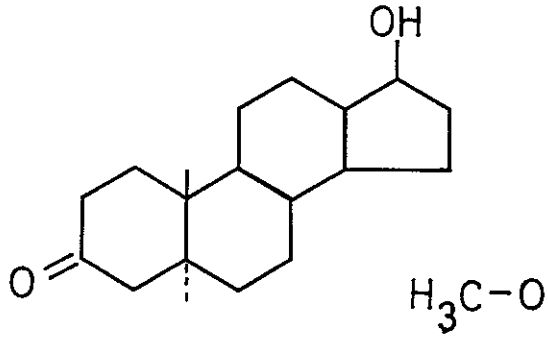


(g) Progesterone

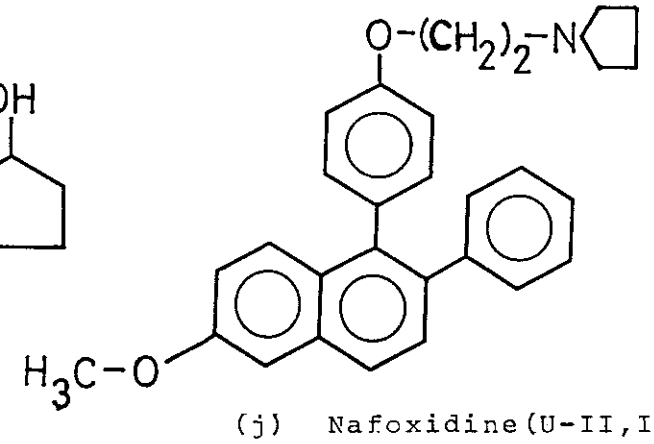


(h) Testosterone

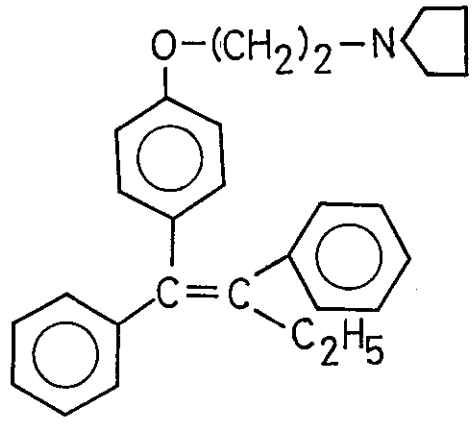
Fig.1.contd.



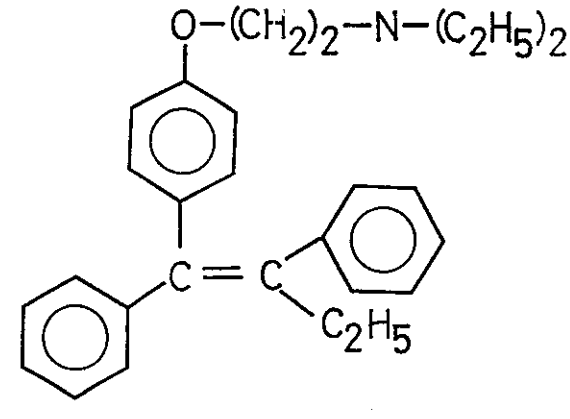
(i) 5α-dihydrotestosterone



(j) Nafoxidine(U-II,1OOA)



(k) CI-628(CN-55,945)



(l) Tamoxifen(ICI-46,474)

Conformational changes accompanying formation of the complex are then supposed to cause specific amino acids to be brought into contact with the D ring which might then control the activity of the complex depending on the functional groups on the steroids. Tseng & Gurpide (1976) have reported that only phenolic steroids possessing a 17β-hydroxyl group competed with oestradiol for nuclear binding. It appears that in the absence of certain functional groups, the complex might not be stabilised but released thus perhaps reducing its nuclear stay. Competition studies in vitro in a number of oestrogen target tissues show that the oestrogen receptor displays specificity for both biologically active natural oestrogens as well as the synthetic non-steroidal oestrogens. Oestradiol-17β, oestradiol-3-acetate, 17α - ethinyl - 19 nortestosterone, diethylstilboestrol, nafoxidine, clomiphene and tamoxifen are very good competitors. Oestradiol-17α, oestrone, oestriol, oestradiol-16α and dimethylstilboestrol compete mildly being 10-30% as effective as 17β-oestradiol. Corticosteroids, progesterone, 17α-testosterone, androstendione and 19-nortestosterone do not appear to compete for the oestrogen receptor at all (Baulieu et al., 1971; King & Mainwaring, 1974; Duax et al., 1981). Because DHT competed for the receptor at equilibrium conditions and testosterone could cause a slight decrease in the association rate of oestradiol-17β on its receptor, it was

suggested that *in vitro* inhibition of the oestradiol on its receptor was a stereospecific binding (Rochefort & Garcia, 1976). The high affinity stereospecific binding of oestrogen to the oestrogen receptor is characterised by a free energy change of about 55 kJ/mole which is thought to come from both hydrophobic and hydrogen bonds (King & Mainwaring, 1974; O'Malley & Means, 1976)

1.9 Stability of the Receptor

Cytosol receptor-oestradiol interactions can be reversibly dissociated by heating at 45°C for 5 min but higher temperatures destroy them (Baulieu *et al.*, 1971; Puca *et al.*, 1971; Myatt.,1979). 50% inactivation is observed in one week if stored at -20°C but at -57°C the cytosol receptor is stable for at least two weeks (Korenman, 1968). Lyophilization has no effect on steroid binding capacity of the receptor or its ability to be transformed (Janes *et al.*, 1982). The binding of oestradiol prolongs the stability of the receptor, stored at 4°C, from one week to one month (Puca *et al.*, 1971).

Oestrogen receptors tend to aggregate *in vitro* therefore to optimise receptor interaction with oestrogen, EDTA is required (King & Mainwaring, 1974). Blockage of sulphydryl-groups on steroid receptors in general usually minimises binding and so these groups are normally protected by sulphydryl reducing

agents such as dithiothreitol (DTT) and thioglycerol (Gorski et al., 1968; King & Mainwaring, 1974; Sando et al., 1979). Puca & Bresciani (1969) have suggested that these reagents also prevented receptor aggregation because sulphydryl-blocking agents such as iodoacetate and iodoacetamide caused aggregation of receptor thereby masking the steroid binding sites.

1.10 The Nuclear Oestrogen Receptor

The majority of nuclear receptor-oestrogen complexes are thought to be associated with components of chromatin and DNA (King et al., 1965; Shyamala & Gorski, 1967; Yamamoto & Alberts, 1974). Sucrose gradient analysis of 0.4M KCl extracts (pH 8.5) from nuclei or chromatin of rat uteri exposed to oestrogen in vivo or in vitro revealed predominantly 5S complexes with the occasional appearance of 4S complexes (Jensen et al., 1968; Puca & Bresciani, 1968; Siiteri et al., 1973). The formation of 5S nuclear receptor may depend on a number of parameters including

a) availability of oestrogen to the target cell in vivo or in vitro (Musliner et al., 1970)

b) presence of cytosol. No 5S complex was formed in vitro in the presence of oestrogen but in the absence of cytosol (Jensen et al., 1968)

c) an elevated temperature (25 - 37°C) (Jensen et al., 1968; Jensen & DeSombre, 1972)

1.10.1 Size and Physical Characteristics

The 5S receptor complexes extracted from nuclei of rats treated *in vivo* with oestrogen exhibit no significant differences from the *in vitro* temperature/ oestrogen-induced 5S cytosol receptor. The 5S receptor has been estimated to have a molecular weight of 110-140,000, approximately twice that of the 4S receptor, molecular Stoke's radius, 58.5 Å; frictional ratio (f/fo) 1.60; axial ratio (Prolate/ oblate), 3.4/0.31;isoelectric points 6.6, 6.8, 7.8 and Kd 2.9 -6.7 x 10^{-10} M (Jensen & De Sombre, 1972; King & Mainwaring, 1974; Notides & Nielsen,1974). On purification, the nuclear receptor sedimented as a 4.5S species with molecular weight around 61,000 (Puca *et al.*, 1971). This reduction in the size of the 5S molecule may be due to loss of a moiety that might be attached to the receptor protein in the native state. Because there is no reduction in oestrogen binding capacity as a result of the purification such a moiety may not be involved directly with oestrogen binding.

1.10.2 Nuclear/Chromatin binding sites

The binding capacity of rat uterine nuclei has been estimated to be around 1 pmol/uterus (Markaverich & Clark, 1981). This however, may be an overestimation since under exchange conditions, other types of nuclear receptor may be measured (Eriksson *et al.*, 1978). The quantity of receptor measured also depends on the

oestrogen status of the animal. For example, in the uterus of the cycling rat, 930 sites/cell are found at metoestrus, 3300 at dioestrus, 4700 at pro-oestrus and 1000 sites/cell at oestrus (Clark et al., 1972) There is some dispute as to whether receptor binding to nuclear sites is a non-saturable process (Chamness et al., 1974; Hemminki , 1976) or is a saturable process with a finite number of binding sites (Kon & Spelsberg, 1982). Although the precise nature of the interaction between steroid receptors and nuclear sites is still not very clear, results from many studies indicate that DNA and chromosonal proteins might be of prime importance (Yamamoto & Alberts, 1974; André et al.,1976; Kallos & Hollander, 1978; Thanki et al., 1978).

1.10.3 Receptor - DNA interaction

Receptor-DNA interaction has been reported to be characterised by a Kd of 2-4 x 10^{-10} M at 4°C with about 3500 acceptor sites/nucleus (Clark & Peck, 1976). Yamamoto & Alberts (1974) in sharp contrast,measured, by sedimentation partition chromatography, as many as 30,000 acceptor sites/cell (Kd,10^{-4} M). These workers postulated that in spite of this large number of sites, biological response was stimulated only when the receptor bound to a few specific sites which were normally masked by low affinity acceptor sites. In an attempt to identify receptor acceptor sites on DNA, Kallos & Hollander (1978) observed that receptor

bound preferentially to A.T-rich segments of native double-stranded DNA rather than denatured DNA and Kumar et al (1980) showed that receptor bound to poly (dA-dT) rather than to other synthetic polynucleotides. The intercalating agents actinomycin D, ethidium bromide and 9-hydroxyellipticine inhibit the binding of oestrogen receptor to DNA-cellulose and native DNA. As actinomycin D intercalates in the minor grooves of the DNA molecule with preference for guanine (Sobell & Jain, 1972; Kallos & Hollander, 1978) and ethidium bromide and 9-hydroxy ellipticine, at low concentrations modify the 3-dimensional structure of DNA intercalating between base pairs (André et al, 1976) it has been suggested that probably specific nucleotides in the DNA helix might be directly involved with receptor binding. Steroid receptor binding to DNA however has been designated as a weak non-specific interaction because of the failure to identify specific binding sequences within the preferred regions of the polynucleotide. Using oligo (dT) - cellulose to determine the conditions of binding of oestradiol receptors of mouse kidney and uteri, Thanki et al (1978) found that the greater part of the non-specific binding was associated with salt-sensitive interactions with oligodeoxypyrimidines, the simplest structural components of DNA. Limited proteolysis of oestrogen receptor complexes has been shown to destroy only the DNA binding site without any effect on oestrogen binding capacity (Sala-Trepat & Vallet-Strouvé, 1974).

Kumar et al.(1979) used the sulphonated polyaromatic dye Cibacron Blue F3GA to demonstrate direct competitive inhibition of the polynucleotide binding site (termed the "dinucleotide fold" of the receptor protein) without any effect on the oestradiol binding site. From these results it was deduced that the receptor possessed distinct binding sites for oestradiol and DNA. Thanki et al. (1978) found that oligo(dT)- and oligo (dC)-cellulose had greater binding capacities for the mouse kidney and uterine cytosols than oligo (dA)-cellulose indicating that in addition to the electrostatic interactions between cationic amino acids side chains of the receptor protein and the backbone phosphate groups of the polynucleotides, there was a recognition locus which could discriminate among the nucleotide bases. Kumar et al.(1980) reported that a distinct order of specificity among oligomers existed such that mouse uterine cytosol receptor bound in the following decreasing order of magnitude: oligo (dG), oligo d(T), oligo (dC) and oligo d(A).

1.10.4 Receptor - Histone Protein interaction

In vivo, virtually all the DNA in the eukaryotic nucleus is enveloped by histones, the structural proteins around which genes are packaged (Kornberg, 1977). Kallos et al.(1981) have demonstrated that the oestrogen receptor from the rabbit uterus binds to histone proteins: strongly to histones 2A and 2B, moderately to H3 and H4 and rather weakly to H1.

Using mild trypsin digestion these workers could distinguish the stable histone and oestrogen binding sites from the proteolysis-sensitive DNA binding sites on the receptor. Thus the oestrogen receptor may possess distinct binding sites for oestrogen, DNA and histones. Kallos and his co-workers (1981) observed further that histones, especially H2B, stabilised the binding of oestrogen receptor to DNA. They have therefore suggested that binding of receptor to histone protein promoted a conformational change which enhanced the receptors affinity for DNA. This way the histone could serve as an 'escort protein' directing or guiding receptors to specific high affinity acceptor sites.

1.10.5 Receptor - Non-histone Protein Interaction

It has been suggested that specific non-histone proteins (NHP) might also act as nuclear acceptor sites. Their removal reduced progesterone receptor binding to chick oviduct chromatin to the levels seen in non-target tissue chromatin (Spelsberg et al., 1972). The experiments of Webster et al.(1976) also showed that Guanidine Hydrochloride (GuHCl) extraction of hen oviduct chromatin to remove acidic proteins AP_1 and AP_2 caused an increased acceptor activity. However, removal of all histone species failed to increase binding (Spelsberg et al.,1976a) suggesting that perhaps in addition to themselves being acceptor sites, acidic proteins could also be responsible for

masking or repressing other acceptor sites. Removal of the remaining non-histone protein (AP_3) led to complete loss of binding sites. By reconstituting all these proteins back on to the DNA, the masking role and the high affinity binding capacity were restored (Spelsberg et al.,1976b). These workers further showed that removal of AP_1 and AP_2 from non-target chromatin resulted in a binding equivalent to that found in target tissue chromatin depleted of AP_1 and AP_2. It was therefore postulated that non-target cells also possessed acceptor sites but were completely masked (100%) compared to 70% masking in target tissues. These results suggested that, at least for the progesterone receptor system, acidic proteins rather than histones might determine the high affinity nuclear binding for steroids. Progesterone and oestrogen antagonise each other in biological responses and Kon & Spelsberg (1982) have shown that, in the avian oviduct, the antagonism is at the level of transcription, essentially on the stimulation of polymerase activity. Using in vivo and in vitro competitive binding techniques, these workers demonstrated that progesterone and oestradiol receptors bound to their distinct specific acceptor sites in the nucleus. Antagonism did not occur at the level of nuclear binding. The glucocorticoid acceptor has also been found to be distinct from the oestrogen acceptor site (Higgins et al., 1973). These results suggest that the steroid acceptor loci

are distinct and do discriminate among receptors in a very specific manner.

1.10.6 Nuclear Occupancy and Response to oestrogenic Stimulation

To stimulate true uterine growth in the immature rat, the oestrogen receptor appears to have to be retained for 4 - 6hr in the nucleus. This long-term nuclear oestrogen receptor binding may trigger off the oestrogen-associated biochemical events (Hardin et al, 1979). Only a limited number of nuclear binding sites were however, found to be involved in producing the maximum uterotrophic response (Anderson et al, 1973). These workers found in the rat uterus that the higher the dose of oestrogen, the greater the quantity of receptor that accumulated in the nucleus during the first hour after injection. Over the next 5 hours there was a decline in nuclear receptor levels, the rate of which was also proportional to the dose administered. The amount of receptor retained in the nucleus for longer than 6 hr was, however, independent of the dose. A prerequisite for uterine growth appeared to be the long term retention of these sites which amounted to 2-3,000 sites/cell (Anderson et al., 1975). This concentration was lower than the peak levels capable of being translocated by exogenous oestrogen. The period of nuclear retention of oestrogen-receptor complexes appears to be related to the biological potency of a particular oestrogen and its apparent affinity for the oestrogen

receptor (Clark & Peck, 1977). Thus oestriol, when administered as a single injection and at comparable doses, was found to elicit a shorter nuclear stay of receptor than did oestradiol. Two forms of the nuclear receptor have been identified (Clark & Peck, 1976; Baudendistel & Ruh, 1976; Ruh & Baudendistel, 1977): Firstly, a form extractable *in vitro* with 0.4M KCl (or less) which exhibits a shorter term stay in the nucleus (1 to 3 hr) and a ten-fold lower affinity for oestradiol (Kd, about 3×10^{-9}M) than does a second form resistant to KCl extraction. This latter form of receptor could not be extracted with 0.4M KCl, was retained in the nucleus for a longer period after oestrogen administration (4 to 12hr) and had a Kd for oestradiol of about 3×10^{-10}M. It has been postulated that nuclear receptor binding involved at least two nuclear sites: the specific acceptor constituting a small proportion of the available sites and corresponding to the long term and KCl-resistant sites. The second was the loosely bound high capacity KCl-extractable long term receptor sites which have been presumed to act as a storage facility optimising binding to the KCl-resistant sites.

Barrack *et al*. (1977) using 0.6M KCl, have confirmed the existence of KCl-resistant nuclear binding sites and suggested that the nuclear matrix, devoid of nuclear DNA and representing 5 to 10% of total nuclear

proteins, might be involved in the binding of the salt resistant form. This nuclear matrix was said to consist of 3 acidic polypeptide fractions with molecular weights 60-70,000 and exhibited a saturable high affinity oestradiol binding with an equilibrium dissociation constant in the 10^{-10}M range.

The results of the work of Juliano & Stancel (1976) were however, in disagreement with this concept of two nuclear acceptor sites for oestrogen receptor, based on long term stay and resistance to 0.4M KCl extraction. These workers found that equilibrium dissociation constants of nuclear receptor, determined at 1, 3 and 6 hr after an oestradiol injection (0.1 µg) were identical (3.12, 2.56 and 2.46nM, respectively), even though the total amount of the receptor extracted decreased between 1 and 6 hr. Moreover, these workers observed that the same proportion of salt extractable to salt resistant receptor was present at 1, 3 and 6 hr after oestradiol injection. The extracted receptor at all the three time points was found to be in the 5S form.

1.10.7 "Unoccupied" Nuclear oestrogen Receptors

Increased concentrations of nuclear oestrogen receptor accompany rat uterine biochemical and morphological differentiation induced by exogenous oestrogen (Hardin et al., 1979) or by endogenous oestrogen stimulation as seen at the pro-oestrous phase of the oestrous

cycle (Clark et al., 1973); Thrower et al., 1981) White et al.(1978) made a similar observation in the hypothalamus. A fraction of nuclear receptor populations are reported to remain "unoccupied" even after maximum nuclear receptor accumulation under oestrogen stimulation in diverse target tissues including human mammary tumours (Zava & McGuire, 1977; Panko & McCleod, 1978) rat hypothalamus (White & Lim, 1978, 1980; Thrower et al., 1981) rat uterus (Thrower et al., 1981) rat brain and pituitary (Clark et al., 1982). Unoccupied nuclear sites have been estimated on the basis of their ability to bind [^{3}H]-oestradiol at low temperatures (0-4°C). Total nuclear receptor content is customarily estimated at elevated temperatures (25-37°C) when the bound endogenous steroid is exchanged with [^{3}H]-oestradiol (Anderson et al., 1972) Filled or occupied sites are reckoned to be the difference between these two populations of binding sites. Unoccupied and occupied oestrogen receptors have been found to have similar physicochemical characteristics in terms of their sedimentation velocities, equilibrium dissociation constants and ligand specificities (Clark et al., 1982) but the fact that the 'unoccupied' sites are measurable at low temperatures suggest that either these are normally occupied by low affinity ligands which are exchanged with [^{3}H]-oestradiol at 4°C or they are truly unoccupied. Substantial amounts of unoccupied receptors are reported to be present in most tissues.

In the brain and the pituitary gland of ovariectomised rats, these could constitute as much as 75% of the total nuclear receptor population (Clark et al., 1982). Following injection of rats with oestradiol, Clark et al. (1982) observed a 20% increase in unoccupied nuclear sites in the hypothalamus and the pituitary at a time when nuclear binding sites were at a maximum (1 hr). White & Lim (1980) made a similar observation in the rat hypothalamus but by 6 hr post-injection, unoccupied sites constituted 71% of total receptor sites. Based on the assumption that long term nuclear receptors (present at 6 hr post-injection) were responsible for mediating the physiological responses of oestrogen (Clark et al., 1973; Clark & Peck, 1976; Anderson et al., 1975), White & Lim (1980) have suggested that the unoccupied sites represented the physiologically active component of the nuclear binding sites. The observation by Thrower et al.(1981) that the population of the unoccupied sites fluctuated during the oestrous cycle with a peak at pro-oestrus, suggested that in vivo their concentration may be under hormonal control and reinforces the concept of a physiological role. The role, however, still remains unresolved.

1.11 Translocation

Intracellular movement of [^{3}H]-oestradiol receptor complexes from the cytoplasm into the nucleus has been demonstrated both in vivo and in vitro (Jensen

et al., 1968; Giannopoulos & Gorski, 1971; Williams & Gorksi, 1972). [^{3}H]-oestradiol labelled cytosol receptor has also been shown to be taken up by nuclei in a cell-free system (Haselbacher & Eisenfeld, 1976; Buchi & Villee, 1976). The process of receptor translocation appears to occur by virtue of the receptor acquiring an increased affinity for nuclear acceptor sites, a phenomenon referred to as activation (Buchi & Villee, 1976). Early workers (Jensen et al., 1968) found that receptor extracted from the nuclei appeared to be predominantly 5S and so the translocation step was assumed to be accompanied by a transformation of the receptor from a 4S to 5S form. Conversion of cytoplasmic 4S receptor to a 5S form has been demonstrated in vitro using either treatment with 0.4M KCl or incubation at any temperature between 10° and 37°C (Notides, 1978). This cytoplasmic 5S receptor was found to be indistinguishable from the 5S receptor isolated from the uterine nuclei of oestrogen-treated animals. Some authors use the alteration in receptor sedimentation coefficient as an indication of activation having taken place (Weichman & Notides, 1977). The terms activation and transformation are therefore very often used interchangeably. Buchi & Villee (1976) demonstrated that activation preceeded transformation and that during prolonged incubation at temperatures above 25°C, the oestrogen receptor was first activated followed by inactivation (as assessed by receptor uptake by nuclei). However,

transformation became increasingly more prominent (as determined by sucrose density gradient centrifugation) with time. Thus activation was shown to be distinct from transformation.

Siiteri et al.(1973) and Linkie (1977) showed that both 4S and 5S receptor complexes could be isolated by salt extraction from nuclei of immature rat uterus, pituitary and hypothalamus after oestrogen stimulation in vivo. Therefore it appeared that activated receptor could be translocated without undergoing transformation and thus transformation possibly occured in the nucleus.

1.11.1 Measurement of Activation

Activation of receptor in vitro has been measured by assessing its ability to bind to nuclei (Buchi & Villee, 1976; Haselbacher & Eisenfeld, 1976) and artificial matrices such as oligo (dT)-cellulose and DNA cellulose (Thrower et al.,1976; Myatt et al., 1982). Activation may also be assessed by the elution pattern on DEAE cellulose chromatography (Chong & Lippman, 1982) and by analysis of receptor-ligand dissociation kinetics (Weichman & Notides, 1977; Mauck et al.,1982; Myatt et al., 1982) where the activated form of receptor has a slower dissociation rate than the non-activated form (see section 1.16).

1.11.2 Assessment of Activation/Transformation

1.11.2.1 Nuclear Uptake/Receptor binding to oligo (dT) -cellulose

Thrower et al.(1976) showed that the rat uterine oestradiol receptor-complex that bound oligo (dT)-cellulose constituted only 30 to 40% of the original amount of receptor in the cytosol. Sucrose gradient analysis of the remaining receptor that did not bind to oligo (dT)-cellulose showed that no 5S receptor was present. That proportion of receptor which bound to oligo (dT)-cellulose, however, was transformed to a form with an apparent molecular weight of 100 to 120,000 on sephadex G-100 corresponding to the 5S nuclear receptor. These workers suggested that an activating factor with a concentration about 30 to 35% of that of the cytosol receptor, was present in the cytosol and could interact with the receptor to produce the 5S form. This activating factor was presumably identical to the sub-unit described by Yamamoto (1974) which was reported to participate in the in vitro interconversion of the 4S to 5S receptor. Yamamoto (1974) found that the 5S receptor complex bound to DNA fifteen times more tightly than did the 4S receptor indicating that the receptor was probably transformed before binding to DNA. This bound sub-unit may direct the 5S receptor complex to specific genomic sites. Haselbacher & Eisenfeld (1976) found that more [^{3}H]-oestradiol was bound when nuclei were incubated with native prelabelled cytosol

than when the cytosol was partially purified with 30% ammonium sulphate. As the binding could be restored upon addition of untreated cytosol to the purified preparation, these workers concluded that the limited nuclear binding might depend on a macromolecule present in the cytosol which could not be precipitated with 30% ammonium sulphate. Myatt et al. (1982) however observed an increased oligo (dT)-cellulose binding upon 50% ammonium sulphate purification of the cytosol. They postulated that the increase could have been due to both the 4S receptor and the activating factor being precipitated out at 50% saturation leaving oligo (dT)-cellulose to facilitate formation of the 5S.

Dilution and dialysis have also been found to activate the oestrogen receptor (Myatt et al., 1982) confirming the results of Sato et al. (1978,1979). The observed increase in oligo (dT)-cellulose binding after these manipulations could possibly have been due to the removal by dilution or dialysis of a low molecular weight inhibitor of activation or transformation.

Notides (1978) has proposed that non-activated uterine oestrogen receptor contained an inhibitor of the activation reaction from which it must dissociate before being converted to the activated form. Thus high salt treatments were presumed to promote dissociation of the 4S receptor from the inhibitor and

rapid desalting or dilution facilitated binding of the 4S to the activating factor to form 5S. Myatt et al. (1982) found that the fraction of the mature rat uterine cytosol oestrogen receptor that bound to oligo (dT)-cellulose showed a single slow dissociating component (activated form) when analysed by dissociation kinetics. Since only 5S receptor bound to oligo (dT)-cellulose (Thrower et al., 1976), this confirmed that activation and transformation were intimately connected. Activation obtained by ammonium sulphate treatment, dilution and dialysis may also represent the removal of the inhibitor from the DNA binding site of receptor. Alternatively it may come from a site remote from it but which allosterically affects the DNA binding site.

1.11.2.2. The Dimeric Structure of the 5S Receptor

The increase in sedimentation coefficient of the oestrogen receptor from 4S to 5S has not been shown to be due to a conformational change but is proposed to be an increase in the size of the macromolecule (Notides, 1978). The frictional ratio and Molecular Stoke's radius show only 10 and 30% change from 4S to 5S respectively. The molecular weight accompanying the conversion however, changes by 85 to 100% (King & Mainwaring, 1974; Notides, 1978). The general consensus is that the 5S receptor is a dimer. There is disagreement however, as to whether the 5S receptor is a heterodimer (involving a 4S receptor

monomer and a second dissimilar molecule of identical size) or a homodimer (involving two identical 4S receptor monomers).

1.11.2.3 Heterodimer

Thrower et al.(1976) and Myatt et al.(1979) suggested that dimerisation to 5S involved the 4S receptor and the putative activating factor. The activating factor could interact with receptor in the absence of oestradiol but oestradiol facilitated the binding (Thrower et al., 1976). Myatt (1979) suggested that it could further be distinguished from the receptor by its sensitivity to heat denaturation and that the oestradiol-activating factor interaction was less stable than the receptor-ligand interaction. The activating factor (Mol. wt.60,000) interacted with the 4S receptor in vitro to give a 120,000 molecular weight species which was indistinguishable from the 5S nuclear receptor found in vivo (Thrower et al., 1976). On the basis of these findings it was suggested that the 5S was a heterodimer.

1.11.2.4 Homodimer

Notides et al.(1981) have proposed that the 5S dimer is rather a homodimer, consisting of two identical 4S receptor macromolecules. This postulate was based on the observation that the formation of the activated 5S receptor was a second order co-operative process. Cooperative binding decreased upon dilution of

cytosol and this was taken to be indicative of dissociation of binding sites. Notides et al.(1981) inferred from these observations that for the 5S to be a heterodimer, 4S receptor would have to possess two binding sites and that would then make ligand site to site interaction independent of receptor concentration. In suggesting that the 5S was a homodimer, Notides and his co-workers were thus proposing that the 5S had two binding sites and the 4S one binding site.

This view however is contradictory to the results of Giannopoulos & Gorski (1971) and Williams & Gorski (1971) who found that both 5S and 4S possessed only one ligand binding site each.

1.11.3 Assessment of Activation/Transformation by Dissociation Kinetics

The studies of Notides & Nielsen (1974) established that the dissociation of [^{3}H]-oestradiol from calf uterine cytosol receptor at 25°C followed biphasic reaction kinetics. The first component showed rapid dissociation and was thought to represent a lower affinity or inactive state of the receptor (4S; Kd, 10^{-9}M; rate constant K_{-1}, about $0.12 min^{-1}$) and the second slow dissociating component represented the higher affinity and activated state (5S; Kd, 3×10^{-10}M; rate constant K_{-2}, about $4 \times 10^{-3} min^{-1}$). Weichmann & Notides (1977) demonstrated that pre-incubation of the receptor complex under conditions which

activated the receptor (e.g. 30 min at 25-30°C) caused the disappearance of the fast dissociating component. The slow dissociating form was found to be a 5S species on sucrose density gradients. Myatt et al.(1982) showed that dialysis and dilution of cytosol increased oligo (dT)-cellulose binding of rat uterine oestrogen receptor and there was a concomitant increase in the magnitude of the slow dissociating form of receptor.

1.11.4 Effects of Sodium Molybdate on Activation/ Transformation

Sodium molybdate affects several molecular properties of the receptor that appear to be related to the receptor's activation/transformation process. Under conditions which normally promote activation but in the presence of 10mM sodium molybdate, the magnitude of the fast dissociating component of the calf and rat uterine cytosol receptor increased (Mauck et al., 1982; Myatt et al., 1982). The proportion of the receptor that exhibited slow dissociation kinetics was roughly equivalent to the amount (10%) that bound to oligo (dT)-cellulose (Myatt et al., 1982). Therefore molybdate appears to inhibit receptor activiation. When molybdate was added after receptor had been activated then it was incapable of reversing the activation (Mauck et al., 1982). There appears to be a certain level of tissue specificity associated with the action of molybdate, for it could not prevent MCF-7 cell cytosol oestrogen receptor binding to DNA cellulose (Chong & Lippman, 1982). Sodium

molybdate inhibits dialysis-induced receptor activation of calf uterine cytosol but receptor can be reactivated upon removal of the molybdate by dialysis (Mauck et al., 1982). The process is however, irreversible in the mouse uterine oestrogen receptor (Shyamala & Leonard, 1980).

The mechanism by which sodium molybdate prevents activation of receptor-complexes is still unknown. However it is possible to postulate that the mechanism might involve interference with some of the following mechanisms associated with receptor activation.

a. Phosphorylation/Dephosphorylation

The nuclear phosphatase enzyme which inactivates the oestrogen receptor appears to be highly sensitive to sodium molybdate inhibition for other phosphatase inhibitors such as sodium fluoride and levamisole are inactive towards it (Chong & Lippman, 1982). If molybdate is acting in cytosol by reducing phosphatase activity (Auricchio et al., 1980a) then inhibition of receptor activation by molybdate implies that activation may be a dephosphorylation process involving phosphatase enzyme. However the enhancement of MCF-7 cell cytosol oestrogen receptor binding to DNA cellulose by ATP (Chong & Lippman, 1982) suggests that phosphorylation might produce activation of the receptor. This receptor activation, produced by other ribonucleotide triphosphates as well, could be blocked

by sodium molybdate and thus suggests that molybdate might also interfere with a phosphorylation process. There is no conclusive evidence that activation is a phosphorylation or a dephosphorylation step. Only the implied effects of molybdate action suggests this.

b. Prevention of dissociation of low-molecular weight inhibitors or direct interaction with Receptor

Molybdate may also inhibit activation by preventing dissociation of low molecular weight inhibitors from the macromolecular receptor complex (Sato et al., 1978, 1979; Notides, 1978; Noma et al., 1980; Myatt et al., 1982). Since molybdate is also capable of forming complexes with phosphate and thiol groups (Sando et al., 1979) and the thiol group is adjacent to the steroid binding site on the receptor (Ikeda, 1982) direct interaction of molybdate with these groups could affect both steroid binding and activation of receptor.

1.12 Mechanisms of Regulation of Oestrogen Receptor Concentration in a target cell

Following translocation of the cytosol receptor-complex into the nucleus, there is a progressive loss of the nuclear receptor a situation termed "processing". This can be detected 30 min after injection of oestradiol

and continues for 3 to 5 hr. The rate at which filled nuclear receptors are lost depends on the dose and the type of hormone used (Anderson et al., 1973; Clark et al., 1973a; Katzenellenbogen & Ferguson, 1975; Horwitz & McGuire, 1978). The reappearance of the receptor in the cytosol (replenishment) is thought to be required to ensure propagation of hormonal responses (Cidlowski & Muldoon, 1974; Horwitz & McGuire, 1978). Using castration and adrenalectomy to control the level of circulating oestrogen, Cidlowski & Muldoon (1978) showed that the extent of replenishment depended on the oestrogen status of rats and only a fraction of the replenished receptor was attributable to de novo protein synthesis, the rest being presumably due to oestrogen induction of receptor recycling. Nuclear loss of receptor without reappearance in the cytosol has been attributed to inactivation of receptor (Auricchio et al., 1982).

1.12.1 Dephosphorylation as a mechanism of Nuclear inactivation of Receptors

The experiments of Auricchio and his co-workers have suggested a probable mechanism by which the nuclear receptor be inactivated, as part of a process that might control the intracellular level of active receptor. Auricchio et al.(1980a) demonstrated that nuclei of mouse uteri and mammary gland were endowed with a phosphatase enzyme capable of inactivating oestrogen receptor binding in vitro. When partially purified this enzyme was found to have a very high

affinity for both the oestrogen-receptor complex and the unbound receptor (Km, 0.8 - 1.5 x 10^{-9}mol of 17β-oestradiol binding sites/L). Its binding activity was enhanced by the sulphydryl stablising reagent dithiothreitol (DTT) and since the thiol group on the receptor resides at the oestrogen binding sites (Ikeda, 1982) it would appear that the phosphatase enzyme attacked the oestrogen binding site. The binding was inhibited by phosphatase inhibitors including sodium molybdate. Because the nuclear receptor inactivation led to loss of hormone binding ability, direct evidence of the presence of an inactivated receptor is absent. The process however was suggested to be due to dephosphorylation because the inactivation of receptor could be reversed by an ATP - dependent cytosol enzyme.

1.12.2 Dephosphorylation of anti-oestrogen - oestrogen receptor complex

The delayed nuclear loss observed when oestrogen receptor translocation was induced by anti-oestrogens may in part be due to the inability of the phosphatase enzyme to dephosphorylate the anti-oestrogen - oestrogen receptor complex (Auricchio et al., 1981a). The result of this is an impaired receptor processing step, as discussed elsewhere in this thesis.

1.12.3 Progesterone antagonism of oestrogen action through Dephosphorylation.

Progesterone antagonism of oestrogen action may be

due, in addition to other factors, to its ability to diminish nuclear retention of the oestrogen receptor (Buller & O'Malley, 1976; Okulicz et al., 1981) or by a progesterone induced inactivation of the nuclear oestrogen receptor by a factor termed the oestrogen receptor - regulatory factor (Evans et al., 1980). Recent reports suggest that an acid phosphatase in the nuclei mediates the action of this factor (Leavitt, 1982) supporting the view that progesterone antagonism of oestrogen action might be through the process of dephosphorylation (MacDonald et al., 1982).

This inactivation of receptor has been observed in the glucocorticoid receptor system as well but, unlike the oestrogen receptor system, phosphatase inactivation appears to be localised in the cytoplasm and the nuclei are not involved (Nielsen et al., 1977; Sando et al., 1979; Housley et al., 1982). Moreover the phosphatase enzymes appear to be highly specific for each receptor system. For, in a mixed receptor system containing both oestrogen and glucocorticoid receptors,alkaline phosphatase inactivated only the glucocorticoid receptor (Nielsen et al., 1977).

1.12.4 Phosphorylation or Reactivation of Inactivated Receptor

Auricchio et al.(1981b) have purified a cytosol ATP-dependent enzyme from calf uterus, which exhibited a high affinity for the inactive oestrogen receptor (Km, 0.3 x 10^{-9}mol of 17β- oestradiol binding sites/

L). Its activity was enhanced by magnesium and calcium and evidence has been produced to suggest that this kinase was responsible for phosphorylation of inactivated dephosphorylated nuclear receptor (Migliaccio et al., 1982). To examine post-translocational events, Auricchio et al.(1982) incubated receptor- depleted mouse uterine cytosol with ATP and found that for a limited period,the apparently 'lost' nuclear receptor could be detected and recovered in the cytosol. These workers showed that this restoration of receptor binding in the cytosol could not be due to de novo synthesis as cycloheximide had no effect on the recovery. The detection of receptor in cytosol following translocation and nuclear loss was time dependent as the receptor's ability to be phosphorylated was gradually lost, probably through degradation. The cells' constitutive level of oestrogen receptor however, is restored through receptor resynthesis about 20 hours later. A hypothetical model of how a dephosphorylation - phosporylation mechanism might control the level of active receptor in an oestrogen target cell is shown in Fig. 1.6.

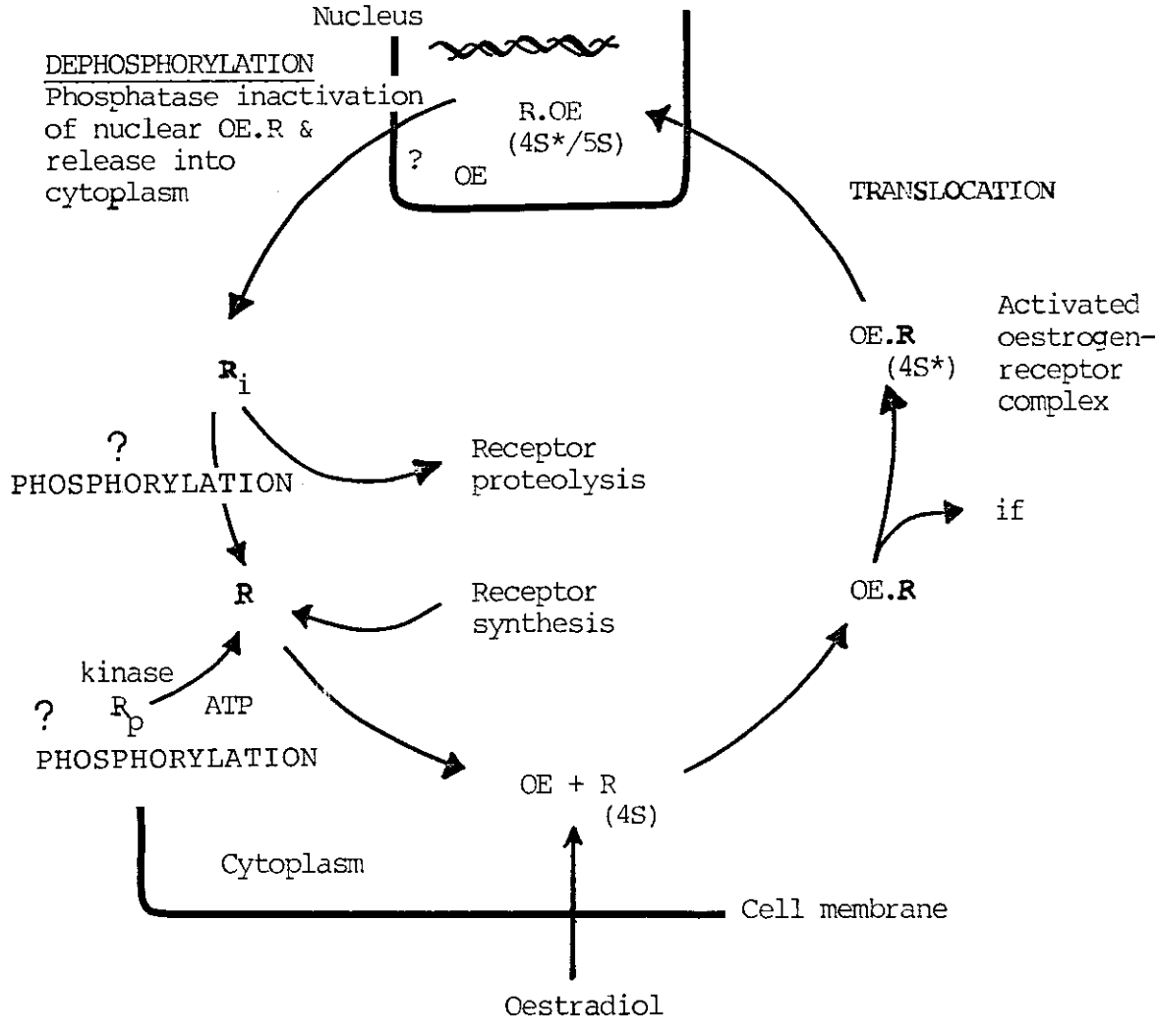
1.13 Effect of steroidal/non-steroidal hormones on Oestrogenic Stimulation

1.13.1 Oestrogens

Oestriol is as potent as oestradiol in stimulating early uterotrophic responses but is ineffective in

Fig.1.6.

Postulated intracellular control of oestrogen receptor mechanisms by Phosphorylation/Dephosphorylation



4S	Non-activated receptor (M.W. 60–80,000)
4S*	Activated receptor
5S	Activated & transformed receptor, (M.W. I30–I40,000)
R_i	Inactive receptor
R^1	Active receptor
R_p	Receptor precursor
if	inhibitory factor
?	steps requiring further study

stimulating uterine weight gain (Hisan, 1959; Hamilton 1963; Gorski & Raker , 1973). Injection of equal amounts of both oestradiol and oestriol produced equivalent nuclear oestrogen receptor accumulation at 3 hr but by 6 hr the oestriol-induced nuclear receptor concentration decreased to control (non-injected) levels while that induced by oestradiol remained well above control levels (Anderson et al.,1975). The inability of oestriol to stimulate the later uterotrophic response has been connected with its inability to promote long-term nuclear occupancy (Katzenellenbogen & Gorski, 1975). This explanation forms the basis of classifying oestrogens into long-acting and short-term nuclear retention respectively. Oestrogens like dimethylstilboestrol (DMS), oestriol, mesestrol and 16α -oestradiol are short-acting whilst 17β-oestradiol and diethylstilboestrol are the best recognised long-acting oestrogens. Kassis & Gorski (1981) demonstrated that receptor replenishment in cytosol following injection of either 17β -oestradiol or diethylstilboestrol was very slow taking 12 - 16hr but in contrast, a short-acting oestrogen such as 16α -oestradiol produced a very fast replenishment which was completed within 4 hr and furthermore the disappearance of the nuclear receptor closely corresponded to the reappearance of receptor in the cytoplasm. Even after multiple injections, no overall receptor loss was observed. It was established, using cycloheximide, that this replenishment

process was not dependent on protein synthesis indicating that it occurred principally through recycling. This is in contrast to 17β-oestradiol-induced replenishment which has been proposed to occur through recycling and resynthesis of receptor (Cidlowski & Muldoon, 1978; Mester & Baulieu, 1975; Kassis & Gorski, 1981).

1.13.2 Anti-oestrogens

Non-steroidal anti-oestrogens such as tamoxifen, CI628,nafoxidine and clomiphene are structurally related compounds (triphenylethylene derivatives, Fig 1.7 ijk) which prevent oestrogens from expressing their full effect in oestrogen target tissues. Generally, antagonists of a hormome action may interfere with either the metabolism of the hormone or the synthesis of any of the proteins that might be essential for eliciting that hormones' response or else by competing directly for the hormone binding site on the receptor (King & Mainwaring, 1974; Clark & Peck, 1979). It is generally thought that anti-oestrogens antagonise oestrogen action by inhibiting oestrogen binding to its receptor and this concept has led to the use of anti-oestrogens to treat advanced oestrogen-dependent mammary tumours (Korenman, 1970; Ruh & Ruh, 1974; Katzenellenbogen & Fergusson, 1975; Mouridsen et al., 1978; Evans et al, 1982). Distinct anti-oestrogen binding sites have also been purportedly demonstrated in a number of tissues

(Sutherland et al., 1980; Sudo et al., 1983) but how these sites could be involved with the recognised oestrogen antagonism is still not clear.

1.13.2.1 Anti-oestrogen competition for oestrogen binding sites

Anti-oestrogens have affinities for the oestrogen receptor relative to 17β-oestradiol (100%) ranging from about 10% for clomiphene to about 1100% for H-1285 (Ruh et al., 1979). The actual mechanism by which anti-oestrogens might inhibit oestrogen action could take place at any of the stages of oestrogen interaction with the receptor mechanism. Ruh et al. (1979) observed that pretreatment of immature rat uteri with anti-oestrogens inhibited expression of full uterotrophic responses expected from subsequent oestradiol injection. The association rate of anti-oestrogens with the oestrogen receptor is identical to that of oestradiol and oestrone (Katzenellenbogen et al., 1978) suggesting that anti-oestrogen - oestrogen receptor interaction at this level does not exhibit any impairment. De Boer et al.(1981) however found that the dissociation of CI628 from the anti-oestrogen - oestrogen receptor complex was very rapid and followed monophasic kinetics, indicating that an anti-oestrogen - oestrogen receptor complex may have an impaired activation step. These workers found that CI628 was ineffective in activation of the oestrogen receptor and incapable of inducing 5S receptor

formation. The fact that anti-oestrogens induce translocation implied the anti-oestrogen - oestrogen receptor complex was perhaps only partially activated to a state where it could be translocated (Baudendistel & Ruh, 1976; Ruh & Bandendistel, 1978). Anti-oestrogens caused cytosol oestrogen receptor depletion and the anti-oestrogen - oestrogen receptor complex was retained in the nucleus for a much longer period than oestrogen-receptor complexes but the whole complex was completely extractable with 0.3MKCl and spermine (Baudendistel & Ruh, 1976). Translocation of oestrogen receptor induced by anti-oestrogens not only produces long-term nuclear stay but also displays an aberrant replenishment process (Cidlowski & Muldoon, 1976; Horwitz & McGuire, 1978). The inefficiency of the replenishment process is now believed to be the key to the action of anti-oestrogens and Ruh et al.(1979) have suggested that this may be a reflection of partially activated anti-oestrogen - oestrogen receptor complexes which are structurally different from the oestrogen-receptor complex proper. These complexes may then be bound to different acceptor sites on the chromatin from the true oestrogen-receptor complex. Evans et al.(1982) have found that the oestrogen receptor binds in vitro to non-specific double stranded DNA with a lower affinity when it has been activated by an anti-oestrogen than when activated by 17β-oestradiol. Ruh et al.(1979)

also observed that anti-oestrogens were capable of exchanging with oestradiol in the nucleus as they do in the cytosol. When anti-oestrogens were administered subsequent to oestradiol injection, oestrogen receptor replenishment was halted, prolonging nuclear stay and eliminating the KCl-resistant fraction of the oestrogen-receptor complex to control levels (Ruh & Baudendistel, 1978). Actinomycin D, which interacts in the minor groove of DNA intercalating between GC base pairs, was found to extract a greater proportion of the anti-oestrogen - receptor complex than the oestrogen-receptor complex. However, extraction with ethidium bromide, which intercalates in the major groove without any base specificity, showed that a greater proportion of the anti-oestrogen receptor complex was resistant to extraction than oestrogen-receptor complex (Baudendistel & Ruh, 1976; Ruh et al., 1979). It was therefore postulated that the differential extraction of the complexes might be due to their being bound to different loci in the nucleus, which might result in different rates of recycling or synthesis of receptor for receptor replenishment. Ruh et al.(1979) however demonstrated that anti-oestrogens presumably stimulated normal oestrogen receptor synthesis but it was a block in the receptor recycling process which inhibited replenishment (Fig 1.3).

1.13.2.2. Distinct Anti-oestrogen binding sites

Distinct anti-oestrogen high affinity binding sites

have been reported in a number of target tissues (Sutherland et al., 1980; Sudo et al., 1983) including the rat uterus, ovary, kidney, human endometrium, chick oviduct and mammary carcinoma. By comparing the concentration of saturable binding sites for both oestradiol and anti-oestrogens in the same cytosol preparation and by competition experiments with [^{3}H]-anti-oestrogens, Sutherland et al. found that anti-oestrogen binding sites distinct from oestrogen receptor were present in most tissues. In particular, only 5 to 7% of [^{3}H]-tamoxifen bound to oestrogen receptor in mammary carcinoma. Faye et al.(1980) reported that the concentration of these distinct anti-oestrogen binding sites in the uterus fluctuated during the rat oestrous cycle implying that they were under hormonal control.

Sudo et al.(1983) have reported that in the uterus, the anti-oestrogen specific binding sites fractionated with the microsomal fraction and only small amounts were present in the high speed cytosol. These workers also demonstrated that anti-oestrogen - specific binding sites and oestrogen receptor sites differed in their thermal stability, pH stability and their pattern of depletion from the 'microsomal fraction supernatant'. These workers have suggested that these sites may not be directly involved with oestrogen antagonism but might influence the action of anti-oestrogens in vivo in a manner which is still not clear.

1.13.3 Progesterone

Studies in the rat uterus and in the human breast cancer cell line, MCF-7, have helped to elucidate the probable mechanism by which progesterone could antagonise an oestrogen response. Progesterone's action is generally considered to involve:

1. Interference with the oestrogen receptor mechanism e.g. impairment of receptor replenishment, control of the amount of cytoplasmic oestrogen receptor or reduction of the nuclear stay of the receptor.

2. Control of oestrogen availability to a target tissue through enhancement of 17β-hydroxysteroid dehydrogenase activity.

3. Enhancement of nuclear receptor dephosphorylation.

1.13.3.1 Interference with the Oestrogen Receptor System

There is no evidence of progesterone interfering with the initial binding of oestrogen to its receptor neither does it impair the translocation process (Toft & Gorski, 1966; Anderson et al., 1972; Clark et al., 1977). When oestrogen-primed rat uteri were treated with oestradiol alone or oestradiol plus progesterone, Hsueh et al.(1976) observed similar patterns of translocation in both groups up to 8 hr after the treatments. Thereafter there were significant differences in the replenishment process. Cytosol oestrogen receptor continued to increase up to 24 hr in the oestradiol-treated group but plateaued in the

oestradiol plus progesterone treatment group. Coulson & Pavlik (1977) showed that the progesterone inhibitory effect could not be due to an alteration of protein synthesis. Further experiments involving sequential administration of progesterone to oestradiol primed rats showed that between 8 and 24 hr, progesterone imposed a time dependent inhibition on oestrogen receptor replenishment: the longer the delay in administration the greater the quantity of cytosol receptor measured. It has been suggested that the receptor replenishment took place in two phases.

(i) the progesterone insensitive recycling process which takes place 4 - 8 hr after injection. The quantity of replenished cytosol receptor measured was equivalent to that depleted.

(ii) the progesterone sensitive phase occuring 8 - 24 hr after injection which involves receptor synthesis (Clark & Peck, 1979). The reduced ability of the uterus to respond to oestradiol after progesterone exposure was thus postulated to be due to decreased cytoplasmic oestrogen receptor as a result of an impaired receptor synthesis.

1.13.3.2 Control of oestradiol level by 17β-hydroxysteroid dehydrogenase

Progesterone has been shown to enhance the activity of 17β-hydroxysteroid dehydrogenase, an enzyme which catalyses the conversion of oestradiol to oestrone. As oestrone has only about 10% of the affinity of oestradiol for the receptor, it has been suggested

that the presence of this enzyme could reduce the oestrogenic response (Gurpide et al.,1976; Tseng et al., 1977) and could constitute a major mechanism by which progesterone might antagonise oestrogen action. Indeed Abul-Hajj (1979) reported that higher levels of oestrone were observed in oestrogen receptor negative tumours in which significantly higher levels of 17β-hydroxysteroid dehydrogenase activity were measured. It was speculated that perhaps the enzyme was responsible for controlling the amount of active oestrogen which might affect the growth and development of hormone dependent tumours.

1.13.3.3 Nuclear Oestrogen Receptor Inactivation via Dephosphorylation

Apart from interfering with receptor synthesis progesterone might also antagonise oestrogen action by reducing nuclear retention of the oestrogen receptor (Buller & O'Malley, 1976) a mechanism which is selective for only occupied oestrogen-receptor (Okulicz, et al., 1981). Because the process of early nuclear receptor loss could be blocked by inhibitors of protein and RNA synthesis, it was suggested that the nuclear receptor inactivation process was mediated by a factor which depended on protein synthesis and was induced by progesterone. Okulicz et al.(1981) extracted such a factor termed the oestrogen receptor-regulating factor which promoted inactivation of the nuclear oestrogen receptor in vitro. Progesterone treatment increased nuclear phosphatase

activity (Auricchio et al., 1981a) and MacDonald et al. (1982) showed that acid phosphatase activity could mediate the progesterone induced inactivation of the nuclear receptor. From these studies it was suggested that progesterone controlled oestrogen action in the uterine nuclei possibly by promoting inactiviation of the oestrogen receptor by dephosphorylation.

1.13.4 Androgens

In vitro pre-incubation of immature rat uteri with the androgens testosterone and 5α-dihydrotestosterone (DHT) inhibited the specific nuclear uptake of [^{3}H]-oestradiol indicating that in vivo, they might compete for the oestrogen receptor (Ruh et al., 1975). In low doses androgens produced anti-uterotrophic responses which could not be blocked by antiandrogens but in very high doses, they are known to stimulate uterine growth (Lerner et al., 1966; Gonzales-Diddi et al., 1972). Rochefort & Garcia (1976) showed that in vitro, DHT and to a lesser extent testosterone were competitive inhibitors of oestrogen receptor which they translocated. Administered in high doses androgens induced a specific uterine protein (IP) indistinguishable from that induced by oestradiol treatment (Garcia & Rochefort, 1977). The physiological significance of this in the normal uterus is questionable as the doses administered in order to produce uterotrophic responses were very high (5 mg or more daily for 10 days). These concentrations

are unlikely to be present *in vivo*. It may also be argued that the androgens were probably converted to oestrogen prior to binding to the oestrogen receptor. Dubé *et al.*(1978) have found that 5α -androstane-3β, 17β-diol, a major metabolite of DHT, bound to dog prostate cytosol oestrogen receptor with high affinity. Van Doorn *et al.*(1982) have also shown that the androgen 5-androstene - 3β, 17β-diol (Adiol) bound to the oestrogen receptor of the immature rat uterine cytosol *in vitro* and *in vivo* administration of 1mg Adiol caused oestrogen receptor translocation.

1.14 Oestrogen mediation of Gene Expression

1.14.1 RNA Polymerase Activity

RNA polymerase enzymes catalyze the synthesis of all types of RNA for which DNA codes are available (Weiss, 1960). Their activities in the rat uterus have been observed to increase in response to oestrogen stimulation (Gorski, 1964). Recent studies have shown that when solubilized and chromatographed on diethyl-aminoethyl (DEAE) Sephadex, four distinct forms of the three classes of uterine RNA polymerase are observed: Polymerase I, II, IIIA and IIIB (Roeder & Rutter, 1970; Stancel *et al.*,1979). In mammalian systems, RNA polymerase I is thought to catalyse the synthesis of ribosomal RNA, polymerase II the synthesis of heterogenous RNA (mainly high molecular weight species that appear to contain mRNA) and

the class III polymerases are thought to catalyse transfer (t) RNA synthesis (Chambon, 1970). These enzymes could also be distinguished on the basis of their sensitivity to α-amanitin, an octapeptide isolated from mushroom. Weil et al.(1977) and Stancel et al.(1979) found that RNA polymerase I was insensitive to high concentration of α-amanitin, RNA polymerase II was sensitive to low concentration (50% inhibition at 6ng/ml)and RNA polymerase IIIA and IIIB were sensitive to very high concentrations of α-amanitin (50% inhibition at 18 μg/ml). The effect of oestrogens on rat uterine nuclear RNA polymerase activity was examined by Glasser et al.(1972). A biphasic activity of polymerase II was observed within 15 min after injection of 17β-oestradiol. The first peak was observed at about 30 min which then fell to control levels by 1 to 2 hr. This was followed by a second increase above the control levels from 2 to 12 hr. It has been suggested that the first increase in polymerase II activity might be required for production of mRNA (Borthwick & Smellie, 1975). Oestradiol probably acts by increasing chromatin template activity or stimulating changes in chromatin template composition (initiation sites?) thus enhancing RNA transcription and ultimately protein synthesis. This may explain the observed oestrogen induced synthesis of nuclear RNA in vivo in the uteri of immature rats (Means & Hamilton,1966), the early RNA synthesis observed by De Angelo & Gorski (1970), the synthesis of higher molecular weight RNA (>45S)

observed by Knowler & Smellie (1971) and ultimately the synthesis of induced protein (IP) as observed in ovariectomised or immature rats between 30 to 45 min after injection of 17β-oestradiol (Notides & Gorski, 1966; Wira & Baulieu, 1971). Both long-acting oestrogens (such as 17β-oestradiol and nafoxidine) and short-acting ones (such as oestriol) have been found to cause an early stimulation of RNA polymerase II followed by an increase in RNA polymerase I activity (Hardin et al., 1979). These investigators found however, that only 17β-oestradiol and nafoxidine could sustain RNA polymerase I activity and cause a secondary rise in RNA polymerase II. Clark et al.(1973) had earlier shown that oestradiol and nafoxidine caused a long-term nuclear retention of the oestrogen receptor and produced true uterine growth. The inability of oestriol to cause long-term retention of the oestrogen receptor was therefore reflected in its inability to stimulate RNA polymerase activities to the same extent as oestradiol. These results also indicate that the prolonged stimulation of RNA polymerase II activity was a prerequisite for stimulation of true uterine growth. On the basis of stimulation of RNA polymerase activities, oestradiol and nafoxidine (an anti-oestrogen with aberrant receptor processing machinery, Horwitz & McGuire, 1978) therefore are classified together as long-acting oestrogens.

1.15 Oestrogen Receptor mediation of transcription

The induction of specific translatable messenger RNA

(mRNA) by oestrogen has been studied in both avian and mammalian nuclei. In the chick, oestrogen induced growth and differentiation and the synthesis of many egg-white proteins including ovalbumin (O'Malley & McGuire, 1968; Kohler et al, 1969).O'Malley et al.(1972) demonstrated that oestrogen treatment led to the appearance of new mRNA populations from the oviduct chromatin template which correlated with ovalbumin synthesis. The rate of induction of the specific mRNA activity was moreover dependent on the state of oestrogen stimulation so that withdrawal of the oestrogen led to disappearance of the RNA. Early studies designed to demonstrate oestrogen receptor mediation of the transcription process employed crude receptor preparations of rat and calf uteri. Arnaud et al.(1971) and Mohla et al.(1972) demonstrated that increased RNA synthesis required the presence of activated or nuclear bound receptor complexes. Concurrent with the transcription, the RNA polymerase pool was observed to decline implying that the RNA synthesis was catalysed by the polymerase molecules. More recent studies by Taylor & Smith (1982) using purified receptor added to isolated oviduct nuclei showed that RNA polymerase II activity was stimulated in a receptor concentration-dependent manner. Using saturating concentrations of purified oestrogen receptors, endogenous RNA polymerase activity could be increased four-fold leading to increased ovalbumin gene expression in vitro. The

longer the period of oestrogen withdrawal, the lesser the quantity of ovalbumin mRNA synthesised even in the presence of receptor. Hence it was postulated that oestrogen receptors might increase RNA synthesis by increasing the activity of the polymerase II for its DNA template. Such a hypothesis would tend to support the observations of Tsai et al.(1975) that oestrogen withdrawal from DES-stimulated chicks, essentially decreased the number of chromatin initiation sites available to RNA polymerase for RNA synthesis. Oestrogen withdrawal presumably decreased the level of receptor complexes at the acceptor sites and so it appeared that in order to ensure maximum gene expression, the oestrogen receptor complex had to remain bound to the nuclear chromatin. Palmiter et al (1981) have shown that the decrease of oviduct nuclear receptor sites (from 4500 sites/cell to about 200 sites/cell) of oestrogen stimulated chicks upon oestrogen withdrawal, was accompanied by a decline in both conalbumin and ovalbumin gene transcription. Upon oestrogen restimulation, transcription rates recovered and mRNA levels increased. From these studies it was found that nuclear receptor levels were directly proportional to conalbumin gene transcription at all times during stimulation and withdrawal but ovalbumin gene transcription showed an exponential relationship to receptor concentration.

In addition to mRNA both rRNA and tRNA have been shown to be stimulated by oestrogen (Luck, 1979) and there is considerable support for the view that growth rates of mammalian cells depend on the growth rate of ribosomes, a feature which is regulated by oestrogen and other growth-promoting hormones.

1.16 Kinetic Studies of Oestrogen-Receptor Interaction

The interaction between oestrogens and the oestrogen receptor involves a noncovalent reversible bonding such that intact oestrogen molecules are recoverable (Jensen, 1962; Maurer & Chalkley, 1967).

The dissociation of the [^{3}H]-oestradiol-receptor complex may be rendered irreversible with respect to [^{3}H]-oestradiol by addition of a large excess of unlabelled ligand or by dilution of the reaction volume (Sanborn et al., 1971). The dissociation of the unstable receptor complex then depends on the establishment of a new temperature-dependent equilibrium which follow biphasic first-order reaction kinetics independent of protein concentration up to about 3mg/ml for temperatures below 37°C (Sanborn et al., 1971; Notides & Nielsen, 1974; Notides et al., 1975; Weichman & Notides, 1977, 1979).

The biphasic reaction shows slow and fast dissociation rates of [^{3}H]-oestradiol from the receptor. The rate constant for the slow component (K_{-2}) was

determined from the second phase of the biphasic reaction curve and its entire reaction when subtracted from the first phase, produced the rate constant for the faster component, K_{-1} (Frost & Pearson, 1971). The two-component dissociation reaction suggested that the receptor consisted of either two interdependent binding sites with similar affinities or two independent species with different affinities (Sanborn et al, 1971). Activation of the receptor has been accepted as a prerequisite to translocation and is thought to be accompanied by a transformation of the receptor from 4S to 5S form (Jensen & De Sombre, 1972). Notides et al.(1975) showed, using kinetic analysis in association with Sucrose gradient centrifugation, that the 4S to 5S transformation was a bimolecular reaction involving two forms of the receptor which possibly existed at two different affinity states. The 4S monomer was considered to be the inactive form of the receptor from which [^{3}H]-oestradiol dissociation produced a fast component with rate constant (K_{-1}) 0.12 min^{-1} and the slower dissociating form was the activated 5S dimer (the biologically active form) with a rate constant (K_{-2}) 4 x 10^{-3} min^{-1} (Weichman & Notides, 1977)

1.16.1 Kinetic studies for assessment of Biological Activity of Ligand

In vivo studies have correlated the biological activity of different oestrogens with their capacities to translocate the oestrogen receptor and their

ability to be subsequently retained in the nucleus (Anderson et al., 1972; Ruh et al., 1973; Horwitz & McGuire, 1978). Using oestrogens of differing potencies (with regard to their abilities to stimulate uterotrophic response after a single injection) Weichman & Notides (1980) suggested that the kinetics of dissociation of these oestrogens from the receptor could also be used as an dex of their biological activity.

Association rate constants (K_{+1}) and equilibrium association constants (K_a) for the receptor were similar for oestradiol, oestriol and oestrone. The dissociation rate constant was the only parameter which could distinguish between the three oestrogens. The dissociation rate constants for the fast component K_{-1} (non-activated receptor) at 25°C were 0.12 min^{-1}, 0.16 min^{-1} and 0.57 min^{-1} for oestradiol, oestriol and oestrone respectively. The dissociation rate constants for the activated state (K_{-2}) were 4 x 10^{-3} min^{-1}, 1.7 x 10^{-2} min^{-1} and 0.24 min^{-1} for oestradiol, oestriol and oestrone respectively. From these studies it was suggested that the slower a ligand dissociated from the activated state of the receptor, the longer the period that the receptor is likely to remain activated. This would cause a long period of nuclear stay and ultimately the more biologically potent the ligand is likely to be. De Boer et al.(1981) showed that the anti-oestrogen, [^{3}H]-CI628, dissociated from

the oestrogen receptor 250-fold faster than did oestradiol at 0°C.

1.16.2 Kinetic studies for the assessment of ligand-receptor interaction

There is disagreement as to whether the binding of oestrogen to its receptor is by a negative or a positive cooperative process. Most reports have based their conclusions on the shapes of Scatchard plots in which deviations from linearity has been taken to be indicative of either of these processes. A positive cooperative binding process (where increasing levels of ligand enhance the binding capacity of the receptor) was said to produce scatchard plots with a characteristic convex curve at receptor concentrations of 1-10nM. The monomer (4S) - dimer (5S) equilibrum has been found to be a positive cooperative binding process (Notides et al., 1981). At low receptor concentrations the scatchard plot approached linearity but still retained the positive cooperative binding. Only upon trypsin treatment to abolish the formation of the 5S, was the positive cooperative binding abolished. Sanborn et al. (1971) had also observed that at low receptor concentrations but at increasing oestrogen concentrations, the ratio of bound to free steroid increased suggesting that binding of oestrogen to one site promoted additional binding to other sites.

Rosner et al.(1979) using dissociation kinetics

however have suggested that the interaction of oestrogen with its receptor was a negative cooperative binding process: that in the presence of additional labelled or unlabelled oestrogen, the dissociation rate of the receptor-ligand complex was enhanced, presumably due to more oestrogen binding sites being exposed. These dissociation experiments were carried out at low receptor occupancy (2-15%, compared to levels of about 80% in other scatchard plot experiments). Another difference was that the charcoal adsorption method used by Notides and his co-workers to separate bond steroid from the free would introduce errors as at low protein concentrations, the receptor adsorbs to charcoal (Powell et al., 1982). Rosner and his co-workers however estimated the bound ligand by the hydroxylapatite method which has no effect on the receptor even at low concentrations. The difference in interaction of oestradiol with its receptor therefore may depend on how the determinations are carried out. The biological significance of a negative cooperative binding process would seem to outweigh the possibility that in vivo the binding is a positive cooperative process. It has been suggested that negative cooperative binding would ensure that the target cell maintained a high sensitivity to low concentration of hormone but would protect it from acute levels. Negative cooperative binding has already been described for the insulin receptor (De Myets et al., 1973) the binding of thyroid

stimulating hormone (Tate et al., 1975) and thyrotrophic hormone (Grant et al., 1972).

1.17 Alpha-fetoprotein as determinant of oestrogen action in the Ovary

Steroid hormones are normally transported to their sites of action in association with plasma proteins (King & Mainwaring, 1974). It has been estimated that only 1-3% of biologically active oestrogens remain unbound in the general circulation (Burke & Anderson, 1972). Despite the low concentration, it is this free oestrogen rather than the bound oestrogen which gives rise to oestrogenic responses (Raynaud, 1973).

By comparing the ability of two oestrogens to induce uterine growth in immature and adult rats, Raynaud (1973) observed that the natural oestrogen 17β-oestradiol, which binds to plasma proteins,could induce uterine growth only in the adult whereas the synthetic oestrogen R2528, which does not bind to plasma proteins, produced uterine growth irrespective of the age of the rat. It was suggested that by binding to the plasma proteins, the oestrogen was rendered inactive. The major plasma oestrogen binding component in the adult is albumin which transports about 60% of circulating oestrogen (King & Mainwaring, 1974) With a Kd between 10^{-5} and $^{-6}$M, rapid dissociation and interchange of oestrogen in target tissue is facilitated. In the immature rat, the foetal plasma

protein alpha-fetoprotein (AFP) has been shown to be the predominant oestrogen binding species. In the light of present knowledge, AFP has been presumed to have a regulatory role in the control of postnatal ovarian development in the rat.

Serum concentrations of AFP have been estimated to be as high as 2-4 mg/ml at birth (Sell et al., 1976) approximately 700 µg/ml at 21-25 days old and by day 35 only traces (20-30 µg/ml) could be measured (Messeyeff et al., 1974) a level which is maintained into adulthood. Germain et al.(1978) confirmed that there was an exponential decline in the AFP with age and also demonstrated that [^{3}H]-oestradiol uptake by the rat uterus was inversely proportional to the age of the animal. This implied that by binding oestrogen, AFP might control its availability to target tissues.

Both mouse and rat AFP are known to bind to oestrogens with high affinity. Studies by Versée & Barel (1978) have shown that rat AFP binds 17β-oestradiol and oestrone but not 17α-oestradiol with an association constant of 5-9 x 10^{-7}M at pH 8.5, 25°C. Of all the androgens tested only those with non-aromatic A rings of the steroid nucleus could displace 17β-oestradiol from the AFP-oestrogen binding site. Using oestrogen-sensitive and insensitive tumour cells, it has been shown that AFP might control cell multiplication.

Sonnenschein & Soto (1980) and Soto & Sonneschein (1980) observed that when cells from cloned pituitary tumour cell line $C_2$9RAP, which exhibited oestrogen dependent multiplication, were injected into newborn rats, cell multiplication was hampered. However when F_4C_1 cells, cloned pituitary tumour cells which are oestrogen insensitive were used, there was no observable alteration of growth rate. Culturing these different cell lines in media supplemented with sera from rats of different ages confirmed the 'in-animal' observation which suggested that AFP decreased cell multiplication in oestrogen responsive cells. Direct evidence of the localisation of AFP in the ovary was provided by Castelli et al.(1982) who observed that AFP was present only in the zona pellucida of secondary follicles and in the liquor folliculi of ovaries of rats aged 5-26 days old. No AFP was observed in primordial or primary follicles nor was it present in any part of the ovary after day 30. These results implicate AFP in the regulation of ovarian growth but failure to identify AFP in the granulosa cells suggest that regulation of the growth or multiplication of the oestrogen responsive granulosa cells might be performed by a different mechanism hitherto not identified. Since oestrogens are synthesised in the granulosa cell, any regulatory species might be intracellular if it is to have any effect.

1.17.1 Relationship of AFP to the Cytosol Oestrogen Receptor

Because of their relatively high serum concentrations in immature rats, alpha-fetoproteins have been considered as potential contaminants of the immature rat uterine cytosol. Sucrose gradient analysis of immature rat uterine cytosol in hypotonic buffers reveals the oestrogen receptor as an 8S species and in high salt, as a 4S species. Initial results obtained when such cytosols were treated with specific immunoadsorbents to alpha-fetoprotein and by competitive assays (Uriel et al., 1976) indicated that alpha-fetoproteins might account for almost the entire high specific oestrogen binding properties. These workers postulated that in low salt, the alpha-fetoprotein was present partly as a free protein and partly bound to some intracellular components to form an 8S sedimenting complex. This apparently led to a temporary loss of antigenic reactivity to alpha-fetoprotein antibodies. La Barbera & Linkie (1978) have supported this view but suggested that the AFP moiety of the cytosol receptor migrated with a sedimentation velocity of 4S even in low salt gradients. Uriel et al.(1976) however reported that only in 0.4M KCl gradients did the receptor and AFP sediment as 4S and under this condition, by some unknown mechanism, the antigenic and binding capacities of the native alpha-fetoproteins were restored. It is difficult to envisage how the cytosol receptor could be an alpha-fetoprotein-receptor

aggregate as their specifities remain unaltered both in the immature and mature rats (King & Mainwaring, 1974). The fact that AFP may be detected in the plasma of adult individuals with various pathological conditions (Uriel, 1969; Abelev, 1971) when oestrogen receptor was thought to be involved has obvious implications on our current knowledge of the oestrogen receptor molecule. In an attempt to confirm or deny these proposals, Toft & Tomasi Jr. (1978) made an immunological comparison between mouse alpha-fetoprotein and the oestrogen receptor and noted that while on sucrose density gradient in 0.4MKCl, alpha-fetoproteins cosedimented with the receptor, charcoal treatment revealed a clear distinction between the alpha-fetoprotein and the receptor. The alpha-fetoprotein-[^{3}H]-oestradiol interaction was highly unstable in the presence of activated charcoal presumably because of the difference between its equilibrium dissociation constant (Kd,25nM) and that of the receptor's (Kd, 0.5 x 10^{-10}M). AFP selectively bound, and the receptor nonspecifically, to affinity resins containing anti-AFP antibodies. Richard-Foy (1977) also showed that even on high salt sucrose density gradient the AFP and receptor peaks could be resolved. AFP migrated as 4.5S and the receptor 5.5S. The half-time dissociation of AFP-[^{3}H]-oestradiol was 100 to 200 times more rapid than that of the receptor complex. Furthermore AFP had a very weak affinity for diethylstilboestrol to which the receptor bound with a very high affinity. In the

light of available evidence then, AFP is not considered a component of the oestrogen receptor. Instead its role could be related to its ability to control levels of circulating oestrogen (Germain et al, 1978) Inability to demonstrate AFP binding to DNA-cellulose has led to the suggestion that it had no direct effect on genomic activity (Fox, 1975).

1.18 Ontogeny of Oestrogen Receptors

Studies in most mammalian oestrogen target tissues suggest that receptor synthesis is a constitutive property of target cells. Oestrogen receptor is present in the rat uterus from birth and probably earlier (Clark & Gorski, 1970; Peleg et al., 1979; Medlock et al., 1981). The oestrogen receptor system of the rat brain, though detectable in 17-day foetuses (Vito et al., 1979) and perhaps earlier, increases rapidly after birth up to day 6 (Plapinger & McEwen, 1973; Maclusky et al., 1979a,b; McEwen, 1980). The oestrogen receptor of the hypothalamus is also present from birth(Kato et al., 1971; Plapinger, 1973) but prior to 2 weeks of age, no oestrogen receptor activity was demonstrable in mouse mammary tissue (Muldoon, 1979). The late appearance of the receptor in the mouse mammary tissue is thought to be of physiological relevance as the mammary glands begin to grow only after 25 days of age, and the rapid rise of oestrogen receptor in the brain has been observed to coincide with the onset of the critical

period for the defeminizing aspects of rat brain sexual differentiation (McEwen, 1980). The neonatal uterine cytosol receptor has been found to have similar physiochemical characteristics (dissociation constants and sedimentation velocities) as the adult receptor (Medlock et al., 1981). The receptor binding capacities however do alter with age, increasing from about 5000 sites/cell on day 1 of age to about 20,000 sites/cell on day 10 (Clark & Gorski, 1970). Thereafter the level dropped to about 16,000 sites/cell on day 20 and remained unaltered until ageing and sensecence set in and caused a further 45% reduction (Clark & Peck, 1979; Gesell & Roth, 1981). A progressive increase in cytosol oestrogen receptor concentration has been observed in the rat hypothalamus from 6 days of age to day 33 (Kato et al., 1971; Plapinger, 1973). Receptor content per cell, particularly in the cytoplasm, is characterized by fluctuations which seem to correlate with circulating endogenous oestrogen. In the rat, ovarian oestrogen synthesis starts on day 8-10 (Falck, 1953; Presl et al., 1965; Dohler & Wuttke, 1975) and this presumably may be the cause of cytosol receptor synthesis in the hypothalamus. However there is a decrease in cytosol oestrogen receptor in the uterus after day 10 presumably due to redistribution because during this time, the uterus grows about two to three times faster than between day 1 and 10 after birth (Price, 1947). The fact that most target

tissues possess the receptor prior to the onset of endogenous oestrogen production suggests that synethesis of the receptor could also be an autonomous property of the target tissue. As a result of the increase in endogenous oestrogen, uterine nuclear receptor increased more than 8-fold on day 12 compared with the day 3 levels and remained unaltered up to day 28 (Medlock et al., 1981). In the rat brain however the dramatic increase in nuclear receptor occurred later than in the uterus, namely between day 20 and 26 (McEwen et al., 1974). It has been suggested that the increase in nuclear receptor binding observed may be the result of translocation (Clark et al., 1973) which would suggest that different target tissues have their own threshold levels of sensitivity to the same oestrogen stimulus. In the rat brain, [^{3}H]-oestradiol uptake studies in the regions known to bind and retain oestradiol namely hypothalamus-preoptic area-amygdala-midbrain (HPAMB) show that the presumptive oestrogen receptor is initially low at birth both in the cytosol and nuclei but nuclear receptor was dramatically increased between day 21 and 25 without a corresponding decrease in the cytosol (Plapinger & McEwen, 1973). This shows that both receptor synthesis and translocation may be taking place at about the same time.

Attempts have been made to correlate biological response with receptor content in cells. Anderson et al.(1973) found that oestrogen-induced uterotrophic

responses in the rat could be correlated with nuclear receptor content but not the cytoplasmic receptor as this was always present in excess of that required to produce maximal response. In general growth responses were proportional to the quantity of receptor retained in the nucleus for about 6 hr. In an earlier study,Flux (1954) observed that mouse mammary tissue did not respond to oestrogen or progesterone between day 10 to 15. Induced protein (IP) could not be induced in uteri of 5 day old rats (Somjén et al., 1973). Uteri of 6 to 10 day old rats did not show a late growth response, such as increased DNA synthesis and cell division, in response to oestradiol (Peleg et al., 1979). Peleg and his co-workers demonstrated that the inability to respond could be due neither to lack of oestrogen receptor replenishment nor a curtailed nuclear oestrogen receptor retention. The fact that competence was acquired after the appearance of endogenous levels of oestrogens has led to the speculation that oestrogen might have prompted maturity of the receptor system or conferred competence in some unknown way. The inability to respond may also be due to the level of circulating alpha-fetoprotein which is present in substantial amounts in the very young (Dohler & Wuttke, 1975, Germain et al., 1978). In adult cycling female rats, in the absence of AFP, physiological variations of circulating oestrogen are reflected in the fluctuations of nuclear receptor content (Clark et al., 1972).

Even though the nuclear receptor content fluctuates, the total receptor level/cell remains relatively constant inspite of cell hypertrophy. It has therefore been speculated that receptor synthesis may be related to cell number but not directly to oestrogen induction (Clark & Peck, 1979). This may be particularly so in the uterus where receptor sites appear to be distributed to re-establish a finite receptor concentration per cell. For tissues which respond to oestrogen without hyperplasia e.g. the brain, receptor ontogeny and maturation and maintenance of cell receptor level might involve some other mechanism not known at present.

1.19 Heterogeneity of Oestrogen Binding Species

1.19.1 Type II Cytosol Oestrogen Receptor

In addition to the classical oestrogen receptor, now termed type 1, Clark et al., (1978) identified the presence of a secondary rat uterine cytosol oestrogen binding species, termed the type II oestrogen receptor. This secondary species has received little attention possibly because it has been considered to be a serum albumin or alpha-fetoprotein (Kimmel & Harman, 1980) or because the amount of [^{3}H]-oestradiol normally used for quantitative binding studies have been adequate to saturate only the classical receptor (type I) binding sites. Saturation analysis over a wide range of [^{3}H]-oestradiol concentration (0.5 to

80nM) revealed two distinct specific binding sites which were resolved by scatchard analysis using the methods of Rosenthal (1967) and Feldman (1972) for correction of non-linear Scatchard plots. One of these sites was the type I and the other the type II which had a lower affinity for oestradiol (Kd, 30nM) but a higher binding capacity (about 1-4 pmol/uterus, 30 to 35 mg, wet weight) which was 4-8 fold greater than the concentration of type I sites (Clark et al., 1978; Eriksson et al., 1978). It was also observed that the type II cytosol receptor could not be translocated out of the cytosol by oestrogen administration and was present in both adult and immature rat uterine cytosol (Clark et al., 1978). Specificity studies revealed that oestrone, oestradiol,oestriol and diethylstilboestrol all competed well for the type II sites but testosterone and progesterone could not inhibit [^{3}H]-oestradiol binding. Markaverich & Clark (1981) showed that the type II could only be measured in buffers not containing sulphydryl stabilising agents and under this condition, sucrose density gradient runs caused extensive dissociation. Thus the type II receptor was either underestimated or not measured at all. By postlabelling the fractionated gradients, the type II receptor was found to migrate with a sedimentation coefficient of 4S in both low and high salt gradients. It is not clear whether the type II is similar to the 4S receptor species found in mature uterine cytosol by Steggles & King

(1970). The possibility that it could be AFP is dispelled by the fact that Clark et al (1978) demonstrated its existence in both mature and immature rat uterine cytosol. The type II receptor does not seem to be unique to the rat uterus. It has been identified in the human uterus (Smith et al., 1979; Gibbons et al., 1979) rabbit corpus luteum (Yuh & Keyes, 1979) and in the chick oviduct (Smith et al., 1979). This latter group made their measurements even in the presence of the sulphydryl stabilising reagent thioglycerol. It is not know whether the type II sites are intra or extracellular but it is postulated that by virtue of having a high binding capacity they might be involved in retention of oestrogen in the target tissue so as to maximise binding to type I sites. Alternatively it has been suggested that they might be precursors for the type I sites (Eriksson et al., 1980).

1.19.2 Type II Nuclear Oestrogen Receptor

Eriksson et al., (1978) also identified a type II nuclear oestrogen receptor which displayed a sigmoidal saturation curve and which yielded a hyperbolic scatchard plot. Consequently the concentration of these sites could not be accurately determined (Markaverich et al., 1979). However the Kd as determined from the concentration of [^{3}H]-oestradiol required to half saturate type II sites has been estimated to be in the range of 15-20 nM. Oestradiol treatment of mature ovariectomized rats causes an

increase in both type I and type II sites in the nucleus with the type II being located predominantly in the uterine myometrium (Markaverich et al., 1981). The increase in type I sites represents the high. affinity and low capacity receptor translocated from the cytosol but the increase in type II does not appear to be due to translocation from the cytosol (Markaverich & Clark, 1979; Eriksson et al., 1980; Markaverich et al., 1980) and does not appear to be related to the type II cytosol oestrogen receptor (Eriksson et al., 1980). Hormone specificity of type II sites are similar to those of the nuclear type I sites and therefore introduce error into estimations of type I sites. To quantitate types I and II separately in a mixed system, Markaverich & Clark (1981) made use of the fact that type II sites do not bind [^{3}H]-oestradiol in the presence of sulphydryl reducing agents and at 4°C occupied type I sites are exchanged very slowly. Type II sites were therefore measured using a single saturating concentration of [^{3}H]-oestradiol (40 - 80nM + 300 fold excess DES) and the nuclei incubated at 4°C for 60 min. Under these conditions, these authors claimed that unoccupied type I sites (White & Lim, 1978, 1980; Thrower et al., 1981) were not measured.

1.19.2.1 Type II Nuclear Oestrogen Receptor and Uterotrophic Responses

Prior to oestradiol injection, rat uterine nuclei contain extremely low levels of both types I and II

nuclear receptor. The pattern of nuclear retention of type II receptor following administration of 10μg oestradiol to mature ovariectomized rats were similar to that observed for type I sites. Maximal levels were reached by 1 hr after injection when 4 - 30pmol/ uterus can be measured. Since type II sites were not translocated from the cytosol, it has been suggested that they represent de novo synthesis of a new class of binding species or oestrogen activation of a pre-existing inactive species or a combination of these possibilities (Clark & Markaverich, 1981; Markaverich et al., 1979). Once they appear, the type II sites are sustained in the nucleus for 24 to 48 hr at levels 2- to 3-fold above controls. This is in contrast to the concentration of type I sites which peak at one hour and by six hour had declined to control levels (Anderson et al., 1975). Markaverich & Clark (1979) found that a single oestradiol injection induced a rapid increase in type I but not type II nuclear sites and no uterine growth was observed. It has therefore been postulated that oestrogen stimulation of true uterine growth may require long term nuclear retention of type I sites and a sustained elevation of type II. This postulate was supported by the observation that oestriol could induce uterine growth when delivered as a paraffin implant which sustained elevated levels of types I and II receptor sites. Thus there is a better correlation between type II sites and uterine growth than for type I sites.

The significance of the multiplicity of the oestrogen receptor has not been established for any target tissue. It has been speculated that they could be responsible for different oestrogenic actions: e.g. participating in the negative and positive feedback actions of oestrogen on the tonic and cyclic centres in the hypothalamus (Smith et al., 1979). Whatever their functions may be, it is now well documented that other oestrogen binding species exist which may not necessarily be involved with expression of genomic responses.

1.19.3 Other Oestrogen Binding Proteins

1.19.3.1 Z-binder

In addition to type I and II, Taylor & Smith (1982) have reported that the existence of another oestrogen binding species in the chick oviduct cytosol. These workers have demonstrated that when oestrogen-withdrawn chicks were treated with oestrogen, the two previously described oestrogen receptors (types I & II) are translocated leaving an oestrogen binding protein termed Z with Kd, 30nM. The fact that the cytosol type II receptor in chick oviduct was translocated indicated that it was different from that described for the rat and human uterus. Rather the Z oestrogen binding species appeared to be similar to the rat uterine cytosol type II receptor. Being found at a consistently higher concentration

than the other oestrogen binding proteins, Z levels were also found to be enhanced in chicks receiving exogenous oestrogen, in a manner which could be likened to the rat uterine nuclear type II receptor. Competition studies revealed that a variety of androgens and progestogens inhibited [^{3}H]-oestradiol binding to Z as effectively as oestradiol but DES was ineffective. In low ionic strength buffers, Z sedimented predominantly as 8S complex and shifted to 4S in the presence of 0.4M KCl.

1.19.3.2 Hepatic Steroid Binding Protein

The most unusual oestrogen binding species with regard to its high binding capacity and steroid binding specificity has been termed the 'Hepatic Steroid Binding Protein' and was observed in the crude liver cytosol of the chick (Lazier, 1979). HSBP has a low affinity for oestradiol (Kd,0.1 to 1μM in crude cytosol but a Kd of 30 to 60nM when purified to 95% homogeneity). The protein was found to constitute about 5 x 10^6 binding sites per cell in the liver of egg laying hens but was reduced in the non-laying hens or roosters and was about ten-fold lower still (5 x 10^5 sites/cell) in the cockerel. The protein displayed a ten-fold higher affinity for oestrone than for oestradiol or dihydrotestosterone and bound pregnenolone and progesterone. Oestradiol-17α, oestriol and corticosterone did not bind to it. On sucrose gradients,it migrated with sedimentation coefficient of 2.7S and a molecular weight of about

30,000 and could not be translocated into the nucleus.

The physiological significance of these oestrogen binding proteins has not been established but it has been speculated that the Z oestrogen binder in the chick oviduct could be an intracellular protein which might regulate oestrogen concentration to which the translocatable cytosol receptors are exposed. This would seem probable as the chick, unlike most mammals,has no sex hormone binding globulin which is presumed to serve such a purpose (Taylor & Smith, 1982). With the Hepatic Steroid binding protein having characteristics clearly inconsistent with the criteria ascribed to a receptor, it could only be speculated that it might act as a reservoir for holding the hormones until required, and its low Kd would facilitate a rapid interchange.

CHAPTER TWO

MATERIALS & METHODS

2.1 Materials

Sigma Chemical Company Ltd., Poole, Dorset, UK supplied the following:

17β-oestradiol [3, 17β-Dihydroxy-1, 3, 5 (10)-oestratriene]

5α-dihydrotesterone [5α-Androstan-17β-ol-3-one]

Testosterone [17β-Hydroxy-3-oxo-4-androstene]

Progesterone [4 - Pregnene - 3,20 - dione]

Hydrocortisone [11β, 17 , 21-Trihydroxypregn-4-ene -3, 20-dione]

Diethylstilboestrol (DES)

Deoxyribonuclease (DNase)

Ribonuclease (RNase)

Trypsin

Calf thymus deoxyribonucleic acid (DNA) Type 1

Dithiothreitol (DTT) or Cleland's Reagent

Trizma [Tris; 2-Amino-2 (hydroxymethyl)-1,3-propandiol]

Bacitracin

Phenylmethylsulphonylfluoride (PMSF)

Ethylenediaminetetra acetic acid (EDTA)

Pharmacia (G.B.) Ltd., Milton Keynes, U.K. supplied:

Sephadex LH-20

Pre-swollen Concanavalin-A Sepharose

Pre-swollen Sephacryl S-200, S-300 superfine

High and low molecular weight Gel Filtration Calibration

Kits.

Chromatographic Columns (30 x 15mm; 60 x 9 mm)

Dextran T70

British Drug Houses (BDH) Ltd., Poole, Dorset, U.K. supplied all the general purpose Analytic Reagent (A.R.) Grade chemicals including:

Butyl-PBD [5-(4-biphenylyl)-2-)4-t-butylphenyl)-1-oxa-3,4-diazole]

Activated charcoal, Norit A

Toluene

Spheroidal hyroxylapatite gel

Luckhams Ltd., Burgess Hill, Sussex U.K. supplied LP3 and LP5 polystyrene tubes.

Pasteur pipettes (H99/3) were supplied by Jencons (Scientific) Ltd., Leighton Buzzard, U.K.

[2,4,6,7n-^{3}H]-oestradiol (85-106 Ci/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K.

Liquid Scintillant Unisolve I was purchased from Koch-Light, Colnbrook, Bucks, U.K.

Bovine Serum Albumin (BSA) fraction IV was supplied by Miles Laboratories Ltd., Slough, U.K.

MSE Polycarbonate tubes (4.2 ml) was purchased from

Fisons, Manor Royal, Crawley, Sussex, U.K.

Medium 199 was purchased from Wellcome Research Laboratories, Beckenham, Kent, England.

Pergonal was purchased from Serono Laboratories (U.K.) Ltd.

Gonadtrophon F.S.H. was purchased from Paines & Byrnes Ltd., Greenford, Middlesex, England.

Nafoxidine was supplied by Drug Development Branch, National Cancer Institute, Bethesda, U.S.A.

Tamoxifen was purchased from Imperial Chemical Industries Ltd., Cheshire, U.K.

Clomiphene Citrate was supplied by UpJohn, U.K. Ltd.

R1881 (17 -hydroxy-17 -methyl-4, 9, 11-estratrien-3-one) was a gift from Mr R Ghanadian, R.P.M.S., London.

2.2 Apparatus

Dounce's all-glass tissue grinder (7 ml), Jencon's (Scientific) Ltd., Leighton Buzzard, U.K.

Centrifugation procedures were performed on the following MSE centrifuges (Fisons, Manor Royal, Crawley, U.K.):

MSE 'Coolspin' and Prepsin 65.

Spectrophotometric determinations were made using a Gilford 252 spectrophotometer (Gilford Instruments Ltd., Middlx. U.K.)

Sucrose density gradients were fractionated with Density Gradient Fractionator model 640 (Instrumentation Specialities Company, Nebraska, U.S.A)

Chromatographic columns were run in a laboratory refrigerator (Combi Cold Rac, LKB Instruments Ltd., Croydon, Surrey, U.K.) and fractions collected using an automatic fraction collector, LKB model 2070 ultrorac II.

Radioactivity was determined using either an Inter-technique SL 3000 Liquid Scintillation Counter (Inter-technique Ltd., Uxbridge, Middx. U.K) or a Packard Liquid Scintillation Counter (Packard Instruments Ltd., Reading, U.K)

2.3 Measurement of Radioactivity

Radioactivity extracted into 2 ml absolute ethanol, was counted in 10 ml Toluene containing butyl-PBD (4.5g/L) and radioactivity in aqueous solution was counted in Unisolve I (usually 1.0-1.2 ml aqueous phase per 4 ml scintillant). Radioactivity measurements made using the Intertechnique SL 3000 were expressed

in disintegrations per minute (d.p.m.) using external standard ratio counting coupled to quench correction curves, and counting at an efficiency of 45%. Measurements made on the Packard counter were expressed in counts per minute (c.p.m.).

2.4 Animals

Immature female Wistar rats, aged 8-28 days purchased from Charles River (U.K) were either used immediately or housed until required in the Animal Unit of the Royal Postgraduate Medical School, London.

2.5 Removal of Granulosa Cells

Rats were sacrificed between 8.30 and 10.00 a.m. on the day of the experiment. Animals aged 8 to 15 days old were sacrificed by decapitation and older ones by cervical dislocation. Usually 15 to 20 rats were used for each experiment. Ovaries were quickly removed and denuded of all extra-ovarian tissues, washed and placed in chilled (0-4°C) TED buffer (10mM Tris/HCl, 1mM Dithiothreitol, 1.5mM EDTA pH 7.6) in embryo dissecting dishes, kept on an ice bucket. With the aid of a dissecting microscope, as many ovarian follicles as possible were randomly punctured with a dentists 30 gauge needle causing granulosa cells to exude out of the follicle. To maximise granulosa cell recovery, the punctured ovaries were than placed in a Dounce all-glass tissue grinder containing TED buffer and gentle pressure exerted

on them with pestle B whereupon more granulosa cells, from especially small follicles, were expressed out. The granulosa cell-depleted ovaries were either discarded or washed thoroughly with TED buffer and processed for oestrogen binding assays. The granulosa cell suspensions were pooled and the cells pelleted out by centrifugation at 800 x g for 10 min at 4°C and subsequently washed three times with TED buffer.

2.6 Estimation of Cell Number

In a preliminary study, the quantity of granulosa cells harvested per ovary was estimated by counting cells in a Neubauer haemocytometer and then by determining the DNA content of a known number of cells using the method of Burton (1956). In this manner the DNA content of a granulosa cell could be estimated based on the assumption that all granulosa cells were uninucleate with a haploid genome. Subsequent estimates of cell number in granulosa cell nuclear suspensions were made by determining the DNA content and computing the approximate cell number.

2.7 Determination of Cellular DNA Content

A known number of granulosa cells was aliquoted in duplicate (from 5 x 10^5 to 10^7 cells/tube) and pelleted down by centrifugation at 1200 x g for 10 min. The cells were then washed twice with 0.2M PCA at 4°C, resuspended in 0.5 ml of 0.5M PCA and incubated at

70°C for 60 min. The reaction was stopped by placing the tubes on ice and the hydrolysate recovered following centrifugation at 2000 x g for 5 min. In routine measurements, the granulosa cell nuclear fraction was treated similarly and the hydrolysate prepared in the same volume of 0.5M PCA as the reference DNA standard solution.

2.7.1 Reagents

(i) A solution of 1.5% diphenylamine (w/v) and 1.5% sulphuric acid (v/v) was prepared in glacial acetic acid and kept in the dark.

(ii) Aqueous acetaldehyde was prepared to a final concentration of 16.2 mg/ml.

(iii) Prior to use, these two reagents were mixed in the proportion 0.1 ml of the aqueous aceltaldehyde to 20 ml of the diphenylamine solution.

2.7.2 Reference DNA Standard Solution

Calf thymus DNA stock solution (400µg/ml) was prepared in 5mM NaOH and kept at 4°C. Prior to use equal volumes of this solution and 1.0M PCA were mixed and heated at 70°C for 15 min. From this stock, a range of standard solutions (0 to 200 µg/ml) were prepared in 0.5M PCA.

2.7.3 DNA Assay

One volume of the hydrolysate or the standard DNA Solution (usually 0.5 ml) was mixed with two volumes

of diphenylamine solution and incubated at 30°C for 18 hr in a shaking water bath. The absorbance was then measured at a wavelength of 600 nm and a standard curve constructed for the DNA standards, from which the DNA content of the intact granulosa cells or nuclear fractions was estimated.

2.8 Protein Estimation

The total protein concentration of the cytosol was measured using the technique of Lowry et al.(1951).

2.8.1 Reagents

(i) A copper tartrate carbonate solution was prepared containing 2% sodium carbonate in 0.1M NaOH: 4 ml of 1% sodium tartrate and 0.4 ml 5% copper sulphate was added to 200 ml of this solution.

(ii) Folin's Reagent was diluted 1 in 3 in distilled water prior to use.

2.8.2 Reference Protein Solution

A solution of bovine serum albumin (1.4 mg/ml) was prepared in distilled water and kept at -20°C. When required it was diluted to give a range of standard solutions from 0 to 140 µg/ml.

2.8.3 Protein Assay

Copper tartrate carbonate solution (2.5 ml) was added to 0.5 ml of the cytosol or standard BSA solution and the mixture allowed to stand on the

bench for 15 min at ambient temperature. Then 0.25 ml of dilute Folin's Reagent was added, the solution mixed and left to stand for 30 min before measuring the absorbance at 720 nm. A standard curve was prepared for the known protein solution and from this the protein concentration of the cytosol estimated. Generally the cytosol was diluted 2 to 3 fold prior to estimation of its protein content.

2.9 Histology

Granulosa cell smears were prepared from exudates of punctured follicles to ascertain the homogeneity and purity of the cell harvest.

Sections of whole ovaries from animals of various ages and granulosa cell-depleted ovaries with 'empty' follicles were also made to ascertain the efficiency of granulosa cell removal. Each specimen was first fixed in 10% phosphate buffered formal saline for at least 24 hr. The tissues were then impregnated with wax on an Elliot Tissue processor and embedded in paraffin wax. The tissues were then cut to a thickness of approximately 5 μm, placed on clean sides, dewaxed in xylene and stained in Ehrlich's Haematoxylin for 15 min (as were the cell smear slides). The slides were then differentiated with 70% ethanol containing 1% hydrochloric acid, followed by treatment with Scott's tap water substitute. The slides were then stained for 3 min in 1% aqueous eosin Y, washed under

tap water, dehydrated in alcohol and cleared in xylene. Finally, the sections were mounted under coverslips with DePex resin mountant.

2.10 Whole Cell [^{3}H]-oestradiol Uptake In Vitro

Granulosa cells were harvested from 24 to 28 day old rats, collected with chilled Medium 199 and placed on ice. The cells were sedimented by centrifugation at 800 x g for 10 min at 4°C and resuspended in a known volume of Medium 199. The cell number was estimated by counting in a Neubauer haemocytometer and their viability assessed using the trypan blue dye exclusion test. Approximately 10^6 viable cells/ml of Medium 199 were aliquoted into LP3 tubes in duplicates and incubated with 5, 10 or 20 nM [^{3}H]-oestradiol in the presence or absence of a 100 fold excess of diethylstilboestrol (DES) or testosterone at either 4, 25 or 37°C for 1 hr. After incubation, the tubes were placed on ice for 5 min to terminate the reaction and the cells suspensions centrifuged at 800 x g for 10 min at 4°C. The cells were then washed with 3 x 1 ml 1% bovine serum albumin (BSA) in TED buffer and finally with 1 ml TED buffer. The cells were extracted with 2 ml ethanol and radioactivity counted 10 ml butyl PBD/Toluene scintillant.

2.11 Homogenisation of Granulosa Cells

All procedures were conducted at 0 to 4°C. TED buffer was added to the granulosa cell pellet in the

Dounce all-glass tissue grinder in the ratio of 1 ml of buffer to the pool of cells from 4 ovaries. The cells were then homogenised with 6 gentle strokes using pestle A in the Dounce homogeniser and the homogenate fractionated as shown in Fig 2.1. The granulosa cell-depleted ovaries, termed the Residual Ovarian Tissue (ROT), were homogenised after blotting the ovaries dry with filter paper and chopping them finely with scissors.

The ratio of ROT to TED buffer was the same as used for the granulosa cell homogenisation. A similar procedure was adopted for homogenisation of whole ovaries.

2.12 Subcellular Fractionation

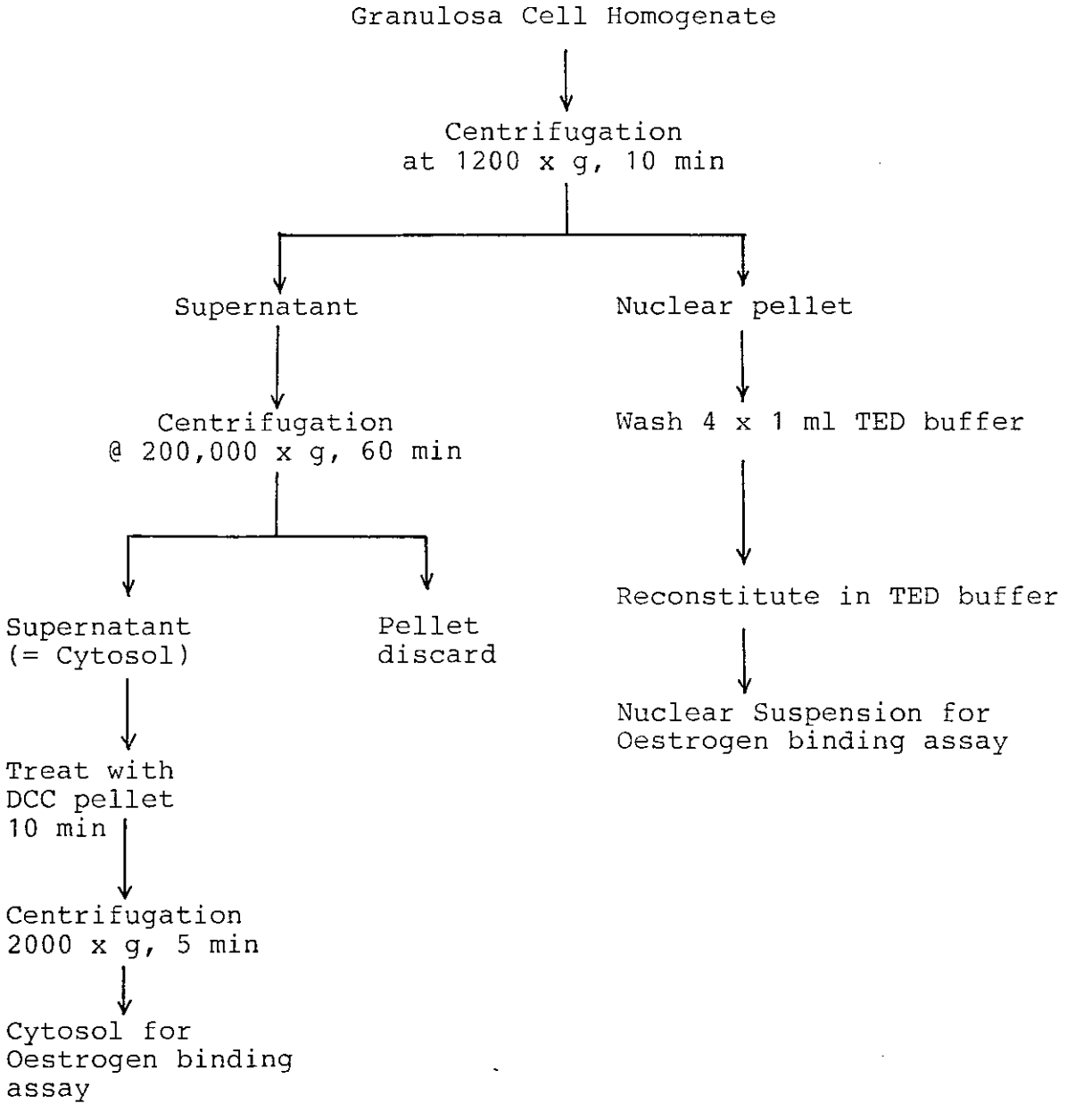
The granulosa cell (or ROT/whole ovarian) homogenate was centrifuged in a MSE Coolspin at 1200 x g for 10 min to yield crude nuclear fraction and a cellular supernatant.

2.12.1 Preparation of Cytosol Fraction

The cellular supernatant was further centrifuged in an MSE Prepsin 65 ultracentrifuge at 200,000 x g for 60 min to yield a clear supernatant termed the cytosol. The endogenous steroids in the cytosol were adsorbed with freshly prepared pellets of Dextran Coated Charcoal (0.5% activated charcoal, 0.05% Dextran T70, DCC).

Fig 2.1

Subcellular Fractionation of Granulosa Cells



To prepare the DCC pellet, aliquots of the charcoal suspension (0.5 ml for each ml of cytosol) were centrifuged at 2000 x g for 5 min, the supernatant removed and the pellet washed once with the original volume of TED buffer. The cytosol was added to this pellet and left standing on ice for 10 min with intermittent mixing. The stripped cytosol was obtained by centrifugation at 2000 x g for 5 min and kept on ice until required for oestrogen binding assay.

2.12.2 Preparation of the Nuclear Fraction

The nuclear pellet obtained from the initial centrifugation of the homogenate was washed four times with 1 ml TED buffer, reconstituted in the original homogenate volume and kept on ice until ready for oestrogen binding assay.

2.13 Assay of Cytosol Oestrogen Binding Species

Duplicate 0.2 ml aliquote of cytosol were incubated for 18 hr at 0-4°C with a range of [^{3}H]-oestradiol concentrations (0.2 to 36nM) with or without a 100 fold excess of oestradiol-17β (OE_2) or diethylstilboestrol (DES) in LP3 tubes to determine the equilibrium dissociation constants for the cytosol oestrogen binding species - oestradiol interaction.

For routine determination of levels of cytosol oestrogen binding species, 10nM [^{3}H]-oestradiol was used with or without 1 μM DES or OE_2 as competitor.

The macromolecular bound [^{3}H]-oestradiol was separated from free steroid on spheroidal hydroxylapatite columns or more routinely by sephadex LH-20 chromotography.

2.13.1 Sephadex LH-20 Exclusion Chromotography

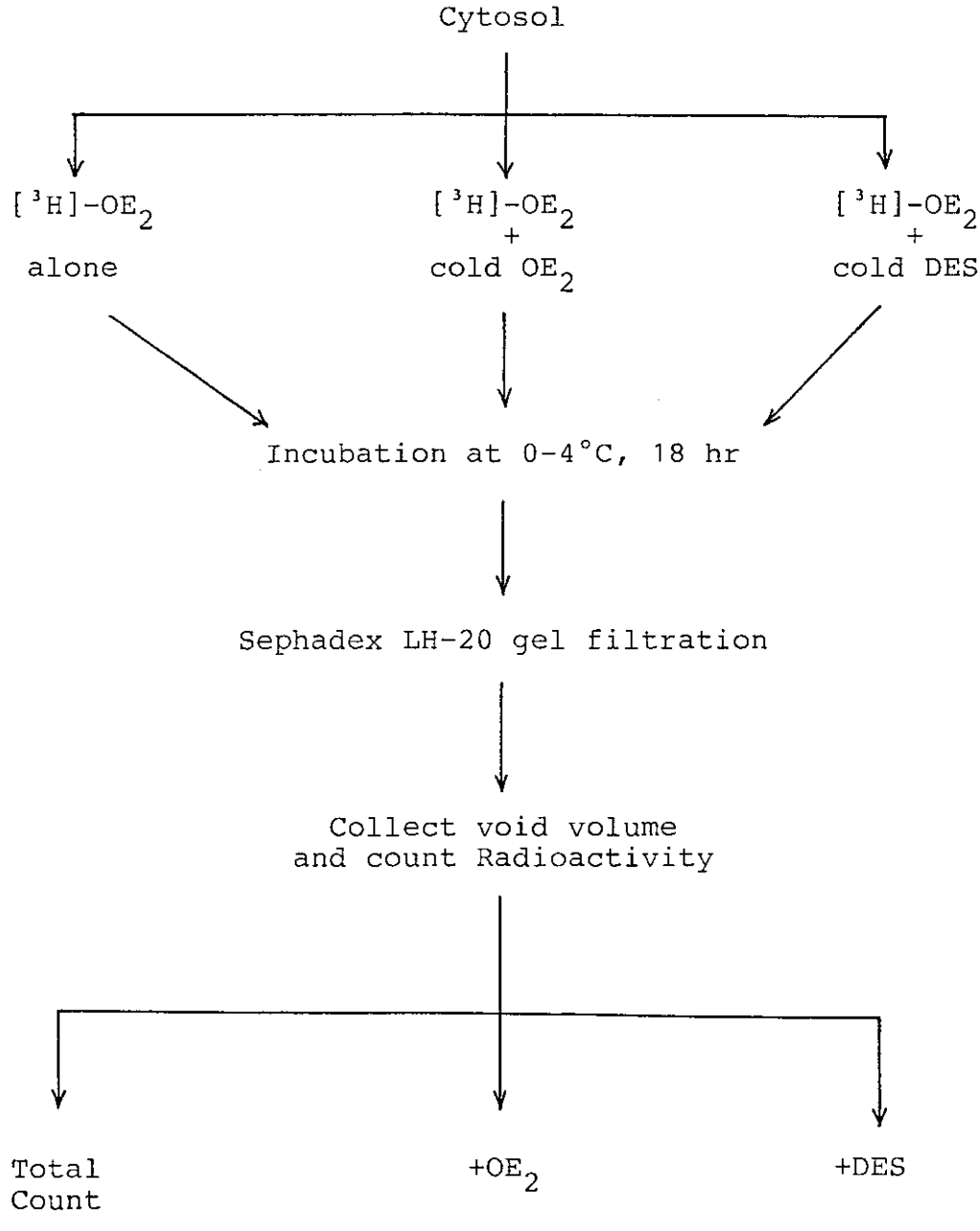
The technique originally described by Ginsburg et al (1974) was used. Sephadex LH-20 was swollen with 8 to 10 volumes (w/v) of TED buffer at 4°C for 18 to 24 hr prior to use. The slurry (3.4 ml) was poured into borosilicate glass pasteur pipettes (H99/3) whose necks had been plugged with glass beads. These columns were then inserted into purpose-built perspex tanks and surrounded by ice. These columns were then equilibrated with TED buffer or, where necessary, the appropriate buffer with required salt concentration (Fig 2.2). An aliquot (0.2 ml) of cytosol, prelabelled with [^{3}H]-oestradiol was applied to the top of the column and allowed to enter the gel bed. Then 0.5 ml TED buffer was added to the top of the gel to initiate separation. Macromolecular bound [^{3}H]-oestradiol was excluded from the gel bed with 1.2 ml TED buffer and was collected as the void volume, directly into scintillation vials. Prior to counting for radioactivity, 4 ml of Unisolve I scintillant was added and mixed thoroughly.

2.13.2 Spheroidal Hydroxylapatite Chromatography

The technique of Thrower and Lim (1980) was used.

Fig 2.2

Assay of Oestrogen Binding Species



2.13.2.1 Preparation of Gel Columns

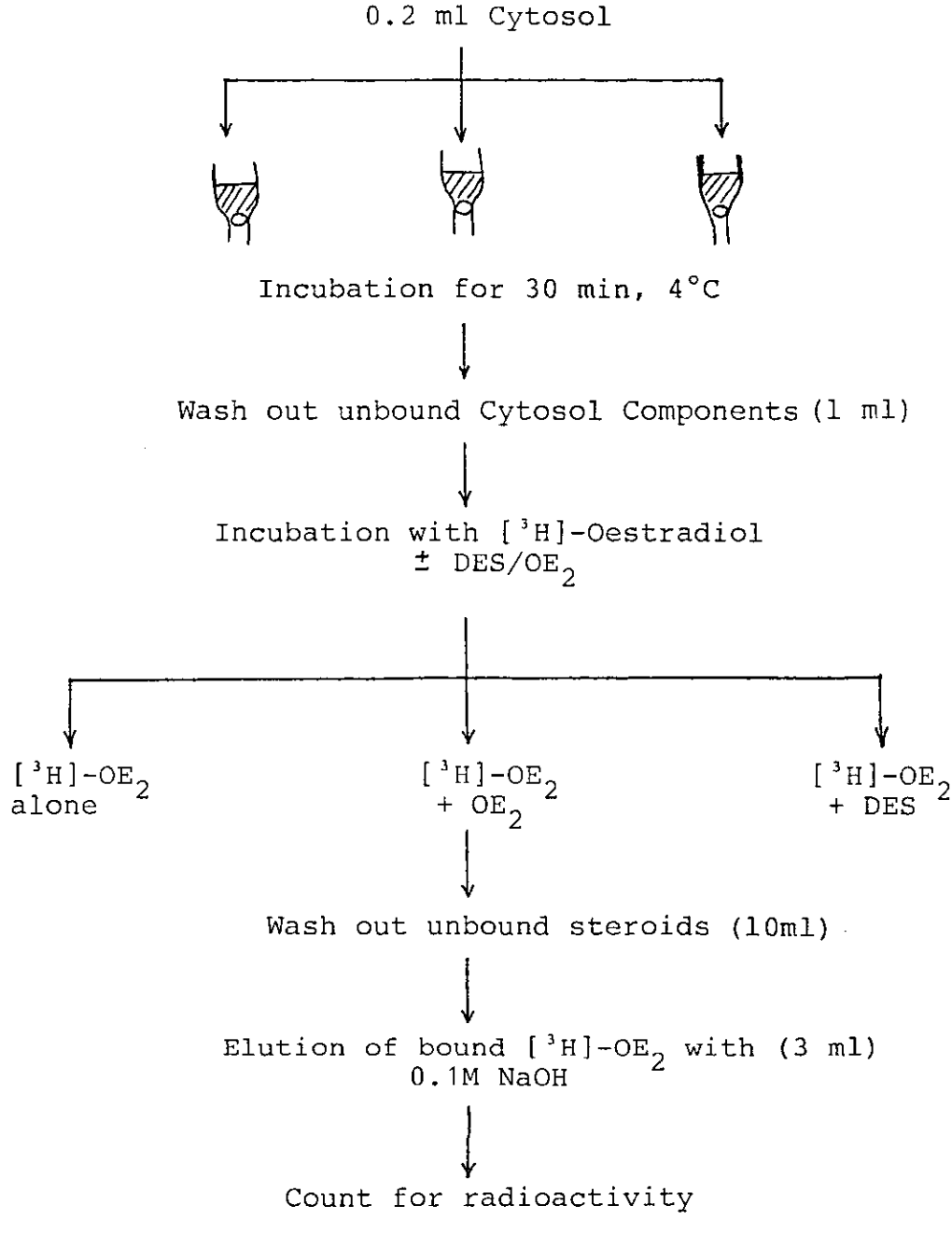
A 33% hydroxylapatite gel suspension (w/v) was prepared in 0.2M NaOH with continuous stirring for 10 min. The sodium hydroxide solution was poured off the settled gel and the suspension reconstituted to 10 to 12% (w/v) with distilled water to thoroughly wash the gels. After three more washings, the gel was made up in TED buffer and kept at 0-4°C until required.

2.13.2.2 Assay of Oestrogen Binding Species

The spheroidal hydroxylapatite gels were poured into borosilicate-glass pasteur pipettes, plugged with glass beads to a height of about 1.5 cm above the glass bead. The tubes were then inserted into ice-filled purpose-built perspex tanks and the gels washed with 2 ml TED buffer. Binding studies were conducted as shown in Fig 2.3. Cytosol(0.2 ml) was added directly to the top of the gels and allowed to incubate for 30 min after which the gels were washed with 1 ml TED buffer to remove unbound proteins. TED buffer (0.6 ml) containing 10nM [^{3}H]-oestradiol in the presence or absence of 1 μM DES or OE_2 was then added to the gels and allowed to incubate for 18 hr at 4°C. The free label in the gel columns was eluted with 3 ml TED and collected directly into scintillation vials for counting. After this the gels were washed 5 times with 1 ml TED buffer at 15 min intervals. Next 3 ml TED buffer was run in and the eluates collected for counting. Macromolecular

Fig 2.3

Assay of Oestrogen binding Species by Spheroidal hydroxlapatite Chromatography



bound [^{3}H]-oestradiol was eluted with 3 x 1 ml 0.1 M NaOH which was collected directly into scintillation vials for counting.

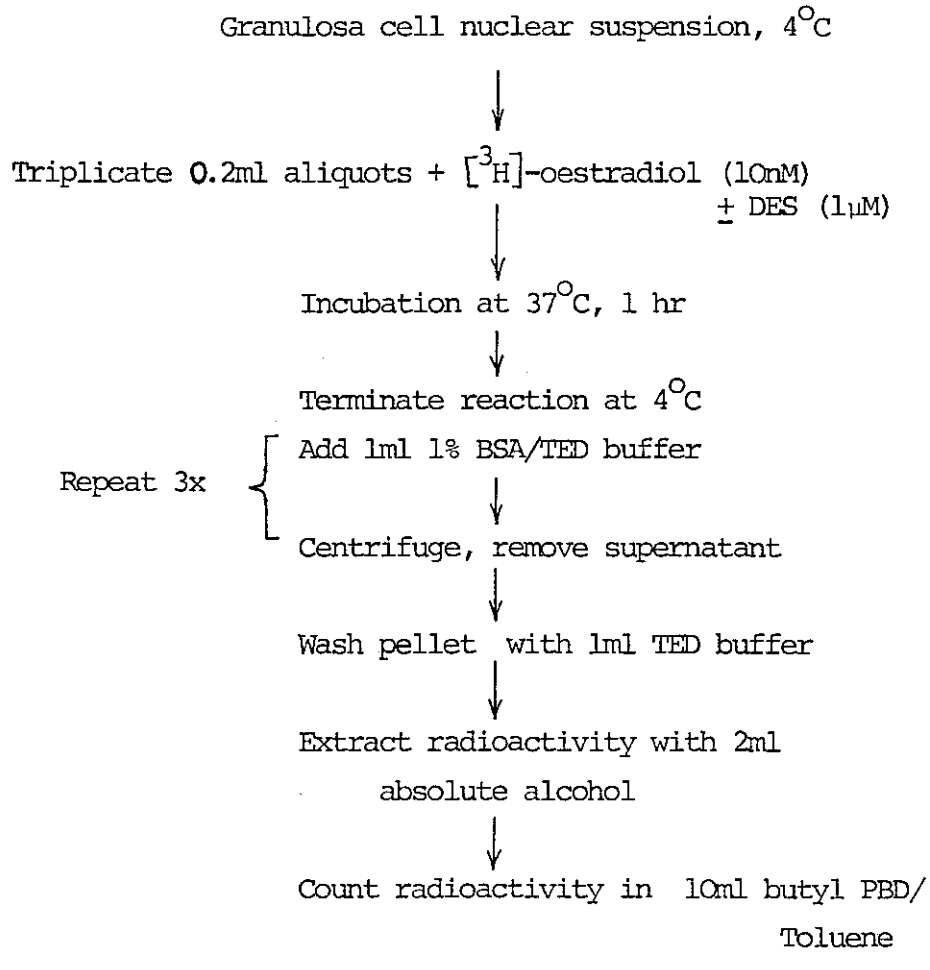
In a preliminary study, it was determined that after incubation of the cytosol with 10 nM [^{3}H]-oestradiol, washing the gels with 10 ml TED buffer was sufficient to remove all free [^{3}H]-oestradiol. Therefore for routine assays only the three NaOH eluates were collected and counted.

In all these oestrogen binding assays, the difference between [^{3}H]-oestradiol binding in the absence and presence of DES was designated as DES-specific binding and the difference between the presence and absence of OE_2 = OE_2-Specific binding.

2.14 Nuclear Oestrogen Receptor Assay

Triplicate 0.2 ml-aliquots of nuclear suspensions were incubated with a range of [^{3}H]-oestradiol concentrations (0.3 - 15nM), with or without a 100 fold excess of OE_2 or DES as competitors, to determine the equilibrium dissociation constants for oestrogen-receptor interaction. For routine measurements 10nM [^{3}H]-oestradiol with or without 1μM DES was used. Binding studies were performed at 37°C for 1 hr which gave a measure of the total nuclear oestrogen receptor sites (i.e. occupied and unoccupied) or for 1 hr at 4°C to estimate only the putative unoccupied sites (Fig 2.4).

Fig.2.4. Assay of Nuclear Oestrogen Receptor



The reaction was terminated by addition of 1 ml ice-cold 1% BSA in TED buffer and the tubes placed on ice. After 10 min the nuclear suspension was centrifuged at 2000 x g for 2.5 min at 0-4°C and the supernatant decanted. The nuclear pellet was washed twice more with 1 ml 1% BSA in TED buffer and then with 1 ml TED buffer. Radioactivity in the nuclear pellet was extracted with 2 x 1 ml absolute ethanol for at least 15 min and counted in 10 ml of butyl-PBD/Toluene scintillant.

2.15 Nuclear Receptor Degradation During Assay Procedures

To examine the extent of thermal degradation at elevated temperatures, nuclear oestrogen receptor binding assays were performed on nuclear suspensions prepared from granulosa cells of 28 day old rat ovaries.

Triplicate 0.2 ml-aliquots of nuclear suspensions, prepared as described earlier, were incubated with 10nM [^{3}H]-oestradiol with or without 1μM DES at 25 and 37°C for 1 hr and at 4°C for 1 and 18 hr. After washing out free unbound [^{3}H]-oestradiol from the nuclei, as described previously, specific [^{3}H]-oestradiol binding was estimated by measuring radio-activity in the nuclei in 10 ml of butyl-PBD/Toluene scintillant. Receptor binding obtained after 1 hr incubations at 4, 25 and 37°C was compared with that obtained after 18 hr incubation at 4°C.

2.16 KCl Extraction of Nuclear Oestrogen Binding Sites

Granulosa cells were harvested from 28 day old rat ovaries, homogenised in TED buffer and the nuclear pellet obtained after the initial centrifugation washed 4 times with TED buffer. A nuclear suspension was prepared in the required volume of TED buffer and divided into 2 portions. One portion was used for estimation of nuclear oestrogen receptor in incubations at 4 and 37°C for 1 hr as described earlier. The other portion was centrifuged at 1200 x g for 10 min and the pellet resuspended in 90% of the original volume with TED buffer. The suspension was brought to 0.4M KCl by addition of 10% of the volume of 4M KCl/TED buffer. The suspension was kept at 0 to 4°C with intermittent shaking for 1 hr after which it was centrifuged at 2000 x g for 5 min. The supernatant containing the extracted nuclear oestrogen receptor was removed and labelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2 for 1 hr at 37°C or 4°C. The macromolecular bound [^{3}H]-oestradiol was separated by sephadex LH-20 gel filtration using 0.4M KCl/TED solution and radioactivity measured in 4 ml Unisolve I scintillant. The residual nuclear pellet from the extraction was washed twice with TED buffer and the oestrogen receptor remaining in the nuclei estimated at 4 and 37°C in a 1 hr incubation as described previously. Because earlier studies showed that both OE_2 and DES were equally effective in displacing [^{3}H]-oestradiol from

nuclear receptor sites, only DES was used routinely for nuclear binding assays.

2.17 Ligand-Competition Studies on Oestrogen Binding Sites

Granulosa cells were harvested from 26-28 days old rats. Duplicate 0.2 ml aliquots of cytosol and triplicate 0.2 ml aliquots of nuclear suspensions were incubated for 18 hr with 10nM [^{3}H]-oestradiol with or without 1μM of OE_2 DES, Oestrone, Oestriol, testosterone, 5α-dihydrotestosterone (DHT), Progesterone, cortisol, tamoxifen, clomiphene citrate, nafoxidine or R1881. Where two competitors were added to the same tube, each was maintained at a 100 fold excess with respect to the radiolabelled ligand. Macromolecular bound [^{3}H]-oestradiol was separated from free steroids by Sephadex LH-20 chromatography. The experiment was also performed, incubating the cytosol with 10nM [^{3}H]-oestradiol in the presence or absence of OE, DES, DHT, DHT + DES or DHT + OE_2 for 18 hr at 4°C on spheroidal hydroxylapatite, as described previously. Radioactivity was counted in Unisolve I scintillant.

Specific nuclear binding of [^{3}H]-oestradiol was determined by extracting radioactivity in the nuclear pellet with 2 x 1 ml absolute ethanol and counting in 10 ml of butyl-PBD/Toluene scintillant.

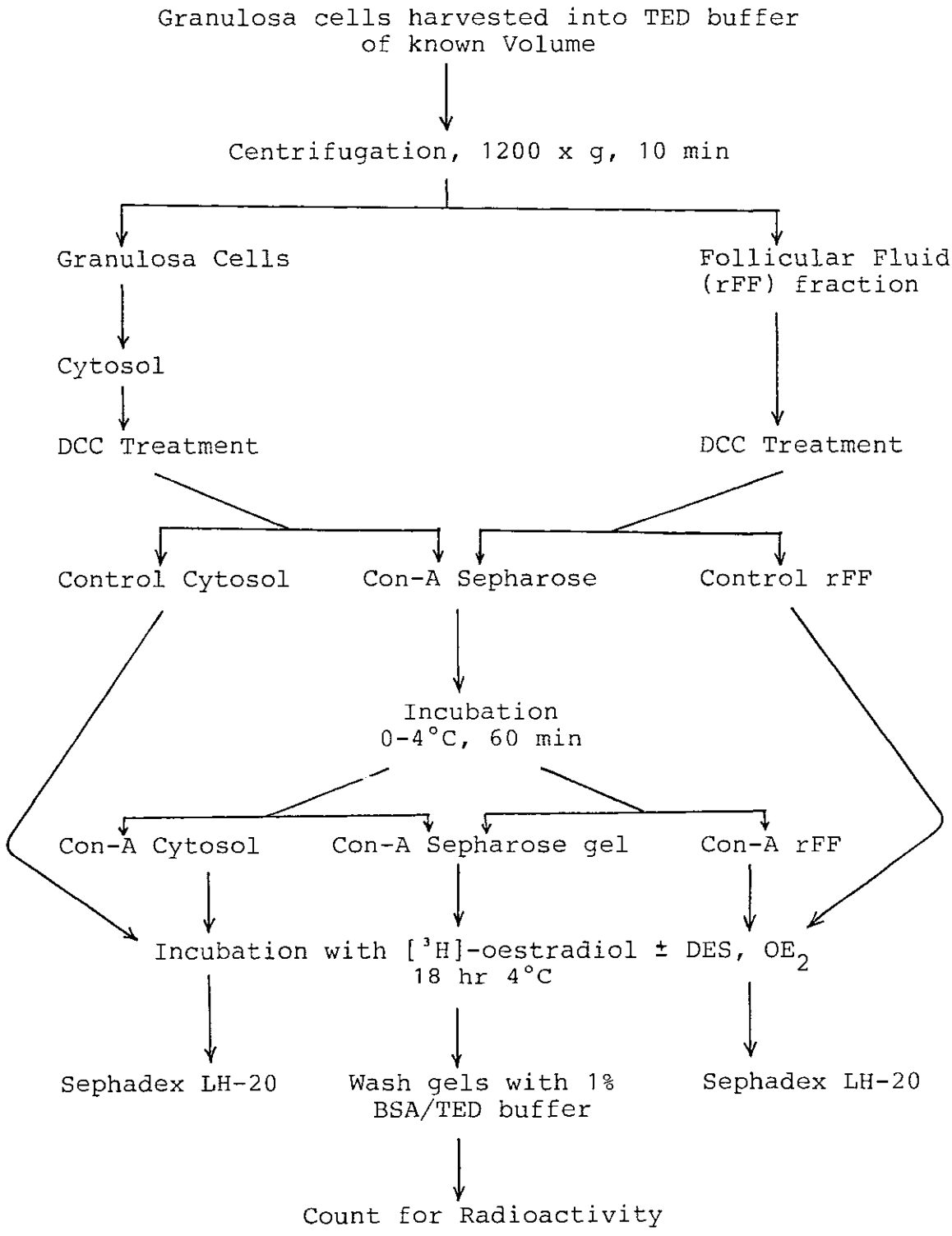
2.18 Concanavalin - A Sepharose Binding Studies

Granulosa cells were harvested from 28 day old rats into a known volume of TED buffer corresponding to that volume of TED buffer to be used to homogenise the granulosa cells. The supernatant obtained after the initial centrifugation of the granulosa cell suspension was treated with DCC at 4°C, as described earlier, and termed the rat follicular fluid (rFF) fraction (Fig 2.5). The granulosa cell cytosol was prepared as described earlier and the concanavalin A - binding assay performed using a modification of the method of Nisula et al. (1978).

A Concanavalin (Con)-A Sepharose gel sediment was washed in TED buffer and 50% (v/v) gel slurries prepared in TED buffer. Eight ml of the gel slurries were placed on ice and after sedimentation, the TED buffer supernatant was removed with a pasteur pipette. Cytosol or rFF fraction (4 ml) was added to the gel sediments to obtain 50% (v/v) gel slurries and incubated for 60 min at 0-4°C with mixing at 15 min intervals. After the incubation, the cytosol and the rFF fraction were removed with a pasteur pipette, the gels washed with 2 ml TED buffer/ml gel and resuspended in TED buffer corresponding to the volume of the cytosol or rFF fraction added (4 ml). Duplicate 0.2 ml aliquots of treated cytosol or rFF fraction and triplicate 0.2 ml aliquots of their corresponding Con A - gel slurry were incubated with

Fig 2.5

Concanavalin-A Sepharose Binding Assay



10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2. Macromolecular bound [^{3}H]-oestradiol in the cytosol and the rFF fraction was separated from free steroids by Sephadex LH-20 gel filtration. Unbound [^{3}H]-oestradiol was washed off the Con-A Sepharose gels with 3 x 1 ml 1% BSA/TED buffer and finally 1 ml TED buffer. The gels were emptied directly into scintillation vials with 1.2 ml TED buffer and counted for radioactivity in 4 ml Unisolve I.

In a separate experiment cytosol prepared from immature rat uterine homogenate (5%, w/v) was treated with Con-A Sepharose in the manner described above, to ascertain binding of uterine cytosol oestrogen receptor to the gel.

2.19 Sucrose Density Gradient Centrifugation (SDG)

2.19.1 Preparation of Sucrose Density Gradients

A 30% sucrose solution (w/v) was initially prepared in TED buffer and then the following concentrations prepared by serial dilution in TED buffer: 27.5, 25.0, 22.5, 20.0, 17.5, 15.0, 12.5, 10, 7.5 and 5%. Aliquots (0.36 ml) of each of these sucrose solutions were carefully layered in turn into an MSE polycarbonate 4.2 ml tube so that a gradient, 30% (bottom) to 5% (top) was prepared. The gradient tubes were carefully kept in a fridge at 4°C for at least 2 hr before centrifugation.

2.19.2 Preparation of Samples and Centrifugation

Cytosol to be used for sucrose density centrifugation was prepared at a protein concentration 5 times greater than usual and incubated with 10nM [^{3}H]-oestradiol with or without 1μM DES or OE_2 for 18 hr at 4°C. Free steroids were separated from bound [^{3}H]-oestradiol by Sephadex LH-20 gel filtration and 0.2 ml of the void volume containing macromolecular bound [^{3}H]-oestradiol layered on the gradients. An aliquot (0.2 ml) of a solution of BSA or catalase (both 2 mg/ml) were added to separate gradients to serve as molecular weight markers. The gradients were centrifuged for 16.5 hr at 2°C in an MSE 6 x 4.2 Swing-out rotor at 300,000 x g. After centrifugation each gradient was fractionated by upward displacement using 50% sucrose solution (containing 1 mg/ml blue dextran dye) on an ISCO Gradient fractionator model 640). Fractions of 0.1 ml were routinely collected and radioactivity counted in 3 ml Unisolve I. The sedimentation profiles of the protein standards were recorded using an absorbance/fluorescence monitor, ISCO model UA-5 to measure absorbance at 280nm during fractionation of the gradients. The ratio R was then determined where

$$R = \frac{\text{distance travelled by Unknown } (d_1)}{\text{distance travelled by Standard } (d_2)}$$

OR

$$R = \frac{\text{sedimentation contant of Unknown } (s_1)}{\text{sedimentation constant of standard } (s_2)}$$

and Molecular weight of the Unknown (MW_1) estimated using the relationship

$$R = \frac{S_1}{S_2} = \frac{d_1}{d_2} = \left[\frac{MW_1}{MW_2}\right]^{2/3}$$ where MW_2 is the molecular weight of the standard protein (Martin & Ames, 1966).

2.19.3 Postlabelling of Oestrogen Binding Species from SDG

Oestrogen receptor-depleted cytosol (the preparation of which is described later) was prelabelled with 20nM [^{3}H]-oestradiol in the presence or absence of 2μM OE_2 for 18 hr at 4°C after which macromolecular bound [^{3}H]-oestradiol was separated from free steroids on Sephadex LH-20. Aliquots (0.2 ml) of this treated cytosol were fractionated on sucrose gradients, as described above, the gradients then analysed and 0.2 ml fractions collected. These were relabelled with 20nM [^{3}H]-oestradiol in the presence or absence of 2μM OE_2 for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol in each fraction was then separated from free steroids on sephadex LH-20 and the radioactivity counted as described earlier.

2.20 Sephacryl Gel Exclusion Chromatography

2.20.1 Gel Preparation procedure (a) Sephacryl S-200 superfine

Preswollen settled gel (70 ml, equivalent to 125%

of the required packed volume for a 30 x 1.5 cm column) was added to 180 ml TED buffer and left overnight. The TED buffer was then decanted and replaced with 30 ml of fresh TED buffer to produce a 70% slurry which was thoroughly degassed for 30 min before use. The slurry was packed into the chromatographic column under gravity and 100 ml of degassed TED buffer (equivalent to 2 bed volumes) allowed to flow through under 40 cm of water pressure before chromatography was commenced. All procedures were carried out in a laboratory refrigerator maintained at 4°C.

(b) Sephacryl S-300 superfine

Preswollen settled gel (50 ml) was processed as described above and packed into a 60 x 0.9 cm column under 200 cm water pressure. Two bed volumes of degassed buffer was allowed to run through under 180 cm water pressure before chromatography was commenced.

2.20.2 Calibration of Sephacryl Gel Columns

Standard proteins, ferritin, thyroglobulin, catalase, aldolase, BSA, ovalbumin, chymotrypsin and ribonuclease were prepared at concentrations of 1 to 5 mg/ml. Samples (0.5 to 1.2 ml) of these standard proteins were applied directly onto the top of the sephacryl gel columns and chromatographed with thoroughly degassed TED buffer. Forty to fifty 22-drop fractions

(each equivalent to about 1 ml) were collected directly into scintillation vials. The absorbance of the effluents was monitored at 280nm either simultaneously using an absorbance/fluorescence monitor, ISCO model UA-5, or the absorbance of the fractions was measured on a spectrophotometer at 280nm. In order to construct a standard curve, Kav was determined for the various molecular weight markers using the relationship

$$Kav = \frac{V_e - V_o}{V_e - V_T}$$

Where V_0 = the column void volume which is the elution volume of molecules which are larger than the largest pores in the gel. These are therefore excluded first. Blue dextran 2000 with molecular weight 2×10^6 was used for the determination of this parameter.

V_e = elution volume of a particular protein
V_T = Total bed volume of the column

Using semilogarithmic graph, Kav values for each protein was plotted on the linear scale against its corresponding molecular weight on the logarithmic scale. Molecular sizes of the samples were also determined from plots of $-\log (Kav)^{1/2}$ against the Stokes' radii of the proteins.

2.20.3 Elution of Samples of Unknown Molecular weight/size

Cytosol prepared from 28 day old rat ovaries were incubated with 10 or 20nM concentrations of [^{3}H]-oestradiol in the presence or absence of a 100 fold excess of OE_2 or DES for 18 hr at 4°C. Samples of this cytosol (1.0 to 1.2 ml) which had been previously chromatographed on Sephadex LH-20 to remove free steroid was applied to the top of the Sephacryl column and allowed to run under gravity. Next 0.2 ml 10% sucrose solution in TED buffer was layered and after that had run in, elution was commenced by mounting a reservoir containing degassed TED buffer. The samples were eluted at 40 or 180 cm water pressure for the 30 x 1.5 cm and 60 x 0.9 cm columns respectively, and 50 or 40 22-drop fractions collected, respectively. Radioactivity in the fractions was measured in 4 ml Unisolve I scintillant. Kav was determined and the apparent molecular weight/size estimated from standard curves. Samples prepared in salt concentrations (0.4M KCl) were eluted with TED buffers containing the appropriate concentration of salt and buffers were always thoroughly degassed before use.

2.21 Postlabelling of Cytosol Chromatographed on Sephacryl

Granulosa cell cytosol was prepared in TED buffer containing 5nM cold OE_2. Cytosol (1 ml) was then chromatographed on Sephacryl S-200 gels and 50 22-drop

fractions collected. The fractions which were known from earlier studies to contain oestrogen binding activities were saved and the other fractions discarded. Each fraction was then stripped of steroids with DCC as described previously and 0.2 ml aliquots incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM OE_2 or DES for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated from free steroids by Sephadex LH-20 gel filtration. Radioactivity was counted in 4 ml Unisolve I scintillant.

2.22 Thermal Stability of Cytosol Oestradiol Binding species

A cytosol was prepared from granulosa cells removed from 28 day old rats and duplicate 0.2 ml aliquots incubated with 10nM [^{3}H]-oestradiol with or without 1μM DES or OE_2 for 18 hr at 4°C. To determine the effect of temperature on the [^{3}H]-oestradiol binding species, some of the samples were further incubated at 37°C, 55°C and 65°C for 30 min before being brought back to 4°C. Macromolecular bound ^{3}H-oestradiol was separated from free steroids by Sephadex LH-20 chromagraphy and radioactivity counted as described previously.

2.23 Ammonium Sulphate fractionation of Cytosol Oestrogen Binding Species

A cytosol was prepared from granulosa cells from 28 day old rats and divided into 4 portions and placed on ice. Sufficient solid ammonium sulphate

was then added slowly with intermittent stirring to 3 of these cytosol preparations to obtain 30, 50 and 70% saturation respectively. After 45 min the cytosols were centrifuged at 20,000 x g for 30 min at 4°C, the supernatant discarded and the precipitate redissolved in TED buffer corresponding to the original volume of cytosol. This partially purified cytosol was then incubated with 10nM [^{3}H]-oestradiol with or without 1μM of either OE_2, DES, DHT, DHT + OE_2 and DHT + DES for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated from free steroid by Sephadex LH-20 and Spheroidal hydroxlapatite chromatography and radioactivity counted as described earlier.

The effect of cytosol protein concentration on the yield of oestrogen binding species during this procedure was determined by adding BSA to obtain a protein concentration of 2 mg/ml in cytosol before treatment with ammonium sulphate as described above.

2.24 Interaction of Oestradiol with the Cytosol Oestrogen Binding Species

2.24.1 Association Studies

A cytosol preparation from granulosa cells of 28 day old rats was divided into three portions for incubation with [^{3}H]-oestradiol at a final concentration of 10nM alone or with [^{3}H]-oestradiol plus either OE_2 or DES at a final concentration of 1μM respectively at either

4°C or 25°C. At 15 min intervals duplicate 0.2 ml aliquots were taken and applied directly on to Sephadex LH-20 columns for separation of macromolecular bound [^{3}H]-oestradiol. The difference in radioactivity between incubations containing [^{3}H]-oestradiol alone and each competitor (OE_2 or DES) was calculated. The rate of association of the radiolabelled oestradiol with cytosol binding species was established from the relationship

$$\left[\frac{1}{S - B_{max}}\right] \log_e \left[\frac{S - B_T}{B_{max} - B_T}\right] \quad \text{against Time}$$

where S = [^{3}H]-oestradiol concentration used, = 10nM

B_{max} = The maximum amount of Bound oestrogen binding species.

B_T = Bound oestrogen binding species at a given time, T.

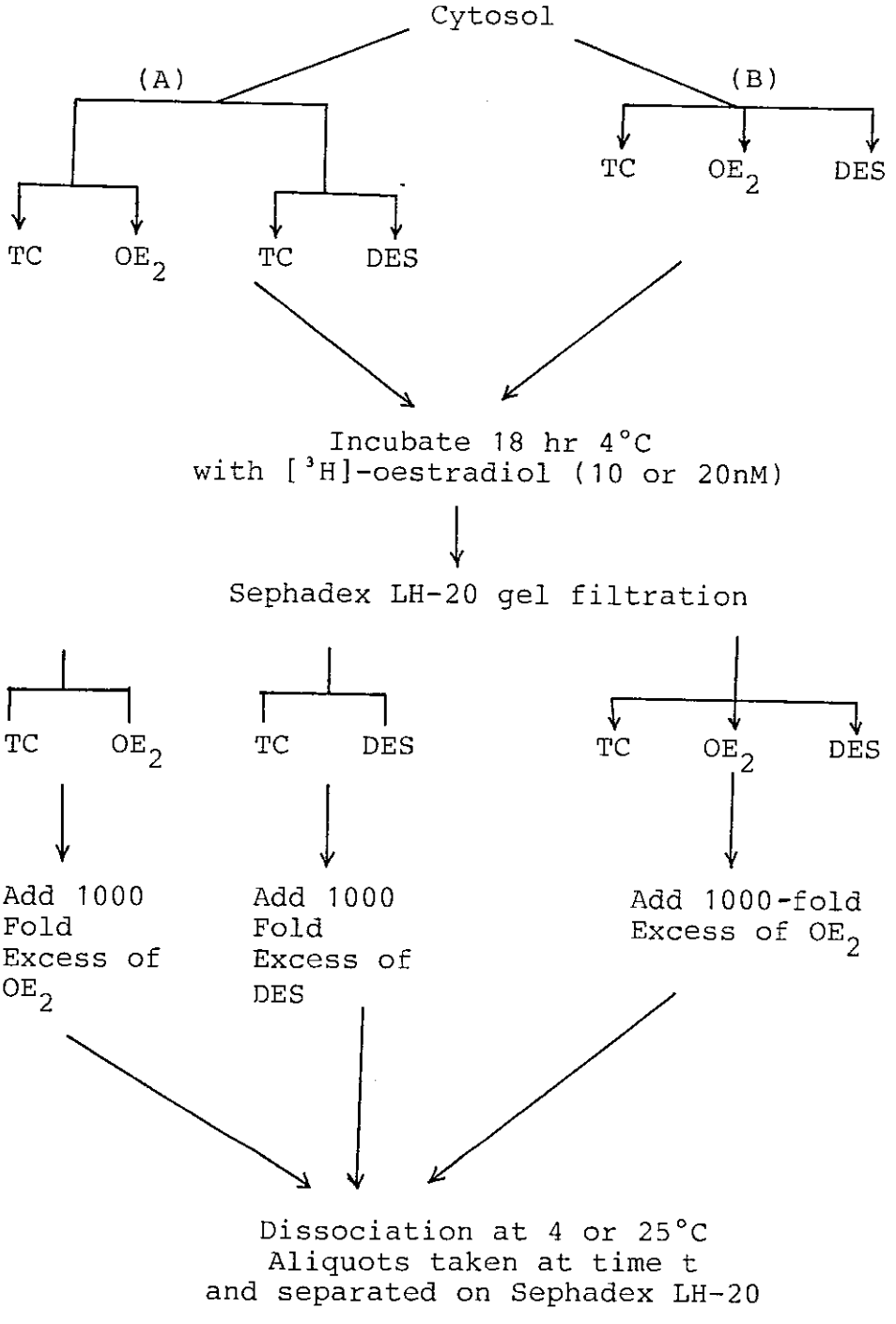
The rate of association between [^{3}H]-oestradiol and the oestrogen binding species was determined from the slope of the plots.

2.24.2 Dissociation Studies

Cytosol was prepared from granulosa cells from 28 day old rats and divided into four portions : two were incubated with [^{3}H]-oestradiol at a final concentration of 10 or 20nM without any competitor and the other two were incubated with [^{3}H]-oestradiol in the presence

of a 100-fold excess of DES or OE_2 respectively. To avoid excess dilution of the cytosol and to maintain protein concentration, minimum volumes of steroid stock solutions were used to achieve the required concentrations. After incubation at 4°C for 18 hr, macromolecular bound [^{3}H]-oestradiol was separated by Sephadex LH-20 gel filtration, which was slightly modified to avoid dilution of the cytosol. After equilibration of the Sephadex gel column with TED buffer, 0.1 ml of blue dextran dye solution (2 mg/ml) was applied to the top of the gel. Next 0.1 ml of TED buffer was added followed by the cytosol reaction volume (usually 5 to 6 ml). Cytosol collection commenced after the marker dye had passed through the column and was stopped after the volume of the eluate had reached the original volume applied to the gel. The stripped cytosol was then placed on ice until required. Dissociation of [^{3}H]-oestradiol from cytosol oestrogen binding species was initiated with a 1000 fold excess of either OE_2 or DES with respect to the radioligand and performed at 4 and 25°C. The amount of bound [^{3}H]-oestradiol determined at the start of the experiment after addition of the competitors to initiate dissociation was designated as 100%. Duplicate 0.2 ml aliquots of cytosol were removed from the incubates at various times for up to 2 hr and these were applied directly onto Sephadex LH-20 gels for filtration and determination of macromolecular bound radioactivity at each time point. The difference in radioactivity between incubations

Fig 2.6 Determination of Dissociation Kinetics of Oestrogen Binding Species in Granulosa Cells



containing [^{3}H]-oestradiol alone and those in the presence of competitors at each time point (specific binding) was calculated as a percentage of the amount of the commencement of dissociation (time 0). These concentrations were plotted on a logarithmic scale against their corresponding time points plotted on the linear scale of a semilogarithmic graph. Dissociation rate constants and half-lives of the [^{3}H]-oestradiol bound species were determined as described by Weichman & Notides (1977). Experiment were also performed by incubating cytosol with radiolabelled ligands for 18 hr at 4°C and for 30 min at 25°C (to promote temperature-induced activation of receptor) prior to gel filtration on Sephadex LH-20. Dissociation of [^{3}H]-oestradiol from oestrogen binding sites was determined as described above.

An alternative procedure to the one described earlier was also used (Fig 2.6B). Three portions of cytosol were prepared. One portion was incubated with 10nM [^{3}H]-oestradiol alone and the other two incubated with 10nM [^{3}H]-oestradiol plus 1μM OE_2 and DES respectively for 18 hr at 4°C. The cytosols were stripped on Sephadex LH-20 gels as described above. Dissociation of [^{3}H]-oestradiol from both OE_2-suppressed and DES-suppressed sites was assessed using a 1000 fold excess of cold OE_2. This is unlike the procedure described earlier where DES-suppressed sites were dissociated with DES. Dissociation rate constants

and half-lives of these oestrogen binding species were determined as described previously.

Immature rat uterine cytosol, prepared from 5% homogenates (w/v), was also incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES. After gel filtration on Sephadex LH-20 as described above, dissociation of [^{3}H]-oestradiol from the oestradiol receptor was performed at 4 and 25°C using 10μM cold oestradiol. Dissociation rate constants and half-lives were determined as described previously.

2.25 Oligo (dT) - Cellulose Binding

Oligo (dT) - Cellulose (100 mg) was placed in a pasteur pipette plugged with a glass bead and the pipette then inserted into an ice-filled perspex tank. The oligo (dT) - cellulose was washed with 5 ml TED buffer and then 0.2 ml cytosol, previously incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2 for 18 hr at 4°C, was applied to the oligo (dT) - cellulose in the column. The cytosol was allowed to incubate for 45 min at 4°C and then the column was washed with 15 ml TED buffer followed by 4 x 1 ml 1.2M KCl/TED. The eluates were collected directly into scintillation vials and the radioactivity counted in Unisolve I scintillant. The difference in total radioactivity in the presence or absence of OE_2 or DES was denoted as OE_2-specific binding and DES specific binding respectively.

2.26 Translocation of Cytosol Oestrogen Receptor into the Nucleus

2.26.1 In Vitro

Granulosa cells were harvested from 28 day old rat ovaries into chilled Medium 199 in embryo dissecting dishes placed on ice. The cells were collected after centrifugation for 10 min at 800 x g at 4°C and resuspended in 20 ml of Medium 199. The cell suspension was divided into four 5-ml portions and oxygen/carbon dioxide (95% : 5%) bubbled into the Medium 199. The cell suspensions were incubated in the presence of 10nM cold E_2 or DES at 37°C for 1 hr or without any steroid at 4 and 37°C for 1 hr respectively. After incubation, the cell suspensions were kept on ice for 5 to 10 min and the cells collected by centrifugation at 800 x g for 10 min at 4°C. The cells were then washed 4 times with TED buffer and cytosol and nuclear suspensions prepared as described earlier. The cytosol was treated with DCC to remove free steroids prior to incubation with 10nM [^{3}H]-oestradiol in the presence or absence of 1µM DES. Cytosol and nuclear oestrogen receptor binding was estimated as described earlier.

2.26.2 In Vivo

Groups of 28 day old rats were injected intraperitoneally as follows

(i) 2.5, 5.0 or 10µg of cold oestradiol or DES 60 min prior to sacrifice

(ii) 50.0μg of cold oestradiol, 15, 30, 45, 60 and 120 min prior to sacrifice

(iii) 50.0μg of DES or testosterone, 60 or 120 min prior to sacrifice

Concentrations of these steroids (200μg/ml) were prepared in absolute ethanol and the required dose constituted in 0.1 ml saline prior to injection. Control animals received 0.1 ml saline only. The granulosa cells from ovaries of control and injected animals were harvested, cytosol and nuclear suspensions prepared as described previously. As usual, the cytosol was treated with DCC to remove endogenous steroids before incubation with 10nM [^{3}H]-oestradiol in the presence or absence of OE_2, DES or DHT under conditions previously described. Macromolecular bound [^{3}H]-oestradiol was separated from free steroids by Sephadex LH-20 chromatography. Radioactivity was counted in 4 ml Unisolve I scintillant.

Nuclear oestrogen binding sites were determined with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM OE_2, DES or DHT at 37°C for 1 hr. In some experiments, the proportion of nuclear unoccupied oestrogen binding sites were also determined at 4°C, 1 hr.

2.27 Effect of Chaotropic Salts, Protease, Protease and Phosphatase Inhibitorson Binding of Oestrogen to cytosol components

2.27.1 Chaotropic salts and Phosphatase Inhibitor

Cytosol was prepared from granulosa cells from 24 to 28 day old rat ovaries in 90% of the required volume of TED buffer. Portions of the cytosol were made up to the required ionic strengths with 10% of the final volume of 1.5M or 4M KCl or 1.5M or 4M NaSCN/ TED buffer. In the case of $NaMoO_4$, the cytosol was prepared in TED buffer containing 20mM $NaMoO_4$. The cytosols were incubated for 18 hr at 4°C with 10nM [^{3}H]-oestradiol in the presence or absence of 1µM DES or OE_2 and the macromolecular bound [^{3}H]-oestradiol was separated from free steroids with the appropriate buffer (TED/KCl/NaSCN/$NaMoO_4$) on sephadex LH-20 and radioactivity counted in Unisolve I scintillant.

2.27.2 Protease and Protease Inhibitor

To assess the effect of protease and protease inhibitors on cytosol components, cytosol was prepared in 90% of the required TED buffer and divided into five portions. To each portion was added 10% of the final volume of either TED buffer or stock solutions of Trypsin, bacitracin or phenylmethylsulphonyl-fluoride (PMSF) in TED buffer (1 mg/ml) respectively to obtain the required volume of cytosol at a concentration of 100µg/ml of the protease and protease inhibitors.

The cytosols were incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM OE_2 or DES for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated from free steroids on Sephadex LH-20 and radioactivity counted in Unisolve I scintillant.

2.28 Studies on the ontogeny of the Oestrogen Binding Species

Cytosol and nuclear suspensions were prepared from 14 groups of rats aged 8, 12, 15, 18, 19, 20, 21, 22, 24, 26, 28, 32, 35 and on the day of vaginal patency (40 ± 2 days). Granulosa cells removed from 8 and 12 day old rats were homogenised in half the usual volume of TED buffer (i.e. cells from 8 ovaries to 1 ml TED buffer).

2.28.1 Cytosol

Duplicate 0.2 ml aliquots were incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or E_2 for 18 hr at 0-4°C. Macromolecular bound [^{3}H]-oestradiol was separated by sephadex LH-20 gel filtration and radioactivity counted in Unisolve I scintillant.

2.28.2 Nuclear fraction

Triplicate 0.2 ml aliquots were also incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES at 37°C for 1 hr. Nuclear oestrogen receptor binding was determined as described previously,

counting the radioactivity in butyl PBD/Toluene scintillant. Specific oestrogen binding sites in cytosol and nuclear fractions were expressed as

i) Receptor Concentration (femtomoles) per mg DNA

ii) Receptor concentration (femtomoles) per granulosa cell

(iii) Receptor concentration (femtomoles) per mg cytosol protein

(iv) Receptor concentration (femtomoles) per ovary, based on the assumption that all receptor-containing granulosa cells have been removed.

(v) Receptor Sites/cell

2.29 Effect of Gonadotrophins (FSH/LH) on Oestrogen Binding Sites

FSH and LH were reconstituted as a mixture in the proportion 70 : 30% and the mixture prepared into 0.1 ml aliquots containing 5 I.U. of gonadotrophin. Five groups of rats of various ages (20, 24, 26, 30 and 33 days) were injected with 5 I.U. of the gonadotrophin preparation saline intraperitoneally for 2 consecutive mornings (48 hr) prior to sacrifice. The oestrogen binding species were assayed in both the cytosol and nuclear fraction of the granulosa cells as described in the above section.

Four separate experiments were conducted for each group. Fourteen rats (for 8 and 12 day olds) and 8 rats for the other age groups were used for each determination.

2.30 Statistical Analysis of Data

Statistical Analysis of data, where necessary were performed using Students' t-test (two tail) and p-values greater than 0.1 were considered insignificant.

CHAPTER THREE

RESULTS

3.1 Ovarian Follicular Development

Two basic functions of the ovary are recognised: production and release both of viable ova and of hormones, especially oestrogens and progesterone, which have important regulatory roles in growth and development of the animal. Follicular growth is involved in both functions and is a process which, once initiated, continues until the follicle either ovulates or undergoes atresia (Peters et al., 1975). The initiation of follicular growth and the formation of small primary follicles is thought to be independent of pituitary gonadotrophins but subject to intra-ovarian controls (Williams, 1940; Greep et al., 1942; Peters et al., 1975). To determine when follicular growth was initiated in the rat ovary longitudinal sections of the ovary of rats aged 8, 28 and 40 days were prepared, stained in Ehrlich's haematoxylin and eosin and viewed at 25 times magnification (Plates 1,2 and 3). The ovary of 8 day old rats was found to be a solid translucent body weighing less than 1 mg and measuring less than 2 mm along its longest axis. The appearance of granulosa cells in the ovary on day 8 led to the organisation of follicles (with 1 to 4 rows of granulosa cells). These follicles were embedded in the stromal tissue and dispersed both in the cortical and medullary regions of the

ovary (see plate 1). No evidence was found to suggest that granulosa cells were differentiated from cells of the surface epithelium or the hilar region (Franchi et al., 1962; Odor & Blandau, 1969; Byskov et al., 1977). Rather it seemed more likely that the granulosa cells had been differentiated from the multi-potential stroma as suggested by Peters & Pederson (1969). Between day 8 (appearance of the granulosa cells) and day 28 (described as the pre-pubertal peak by Jones & Krohn, 1961) the ovary underwent over a 800% enlargement due principally to enlarging follicles (Plate 2). The smooth surface of the ovary became invaded by bulging follicles as the animal grew older. Between day 28 and the day of vaginal patency (the animal's first pro-oestrus as suggested by Ramirez & Sawyer, 1965), the ovary underwent about a 75% increase in size due both to an increasing number of follicles and the enlargement of some of these follicles (Plate 3). Follicular development appeared to consist of two concurrent phases:

(a) the oocytes underwent an increase in size (compare Plates 4 and 5)

(b) the formation of new follicles (acquisition of granulosa cells and the thecal tissue) and the enlargement of some of these follicles (granulosa cell hyperplasia). The follicles present at any one age could be divided into 2 groups: the non-proliferating and the proliferating follicles. The non-



Plate 1a. Longitudinal section of an ovary removed from an 8 day-old rat. Stained with Haematoxylin & Eosin (H&E) x 25.



Plate 1b. Longitudinal section of an ovary removed from an 8 day-old rat. (oo)naked oocytes (f)follicles with 1-2 rows of granulosa cells. H&E stain(x60)

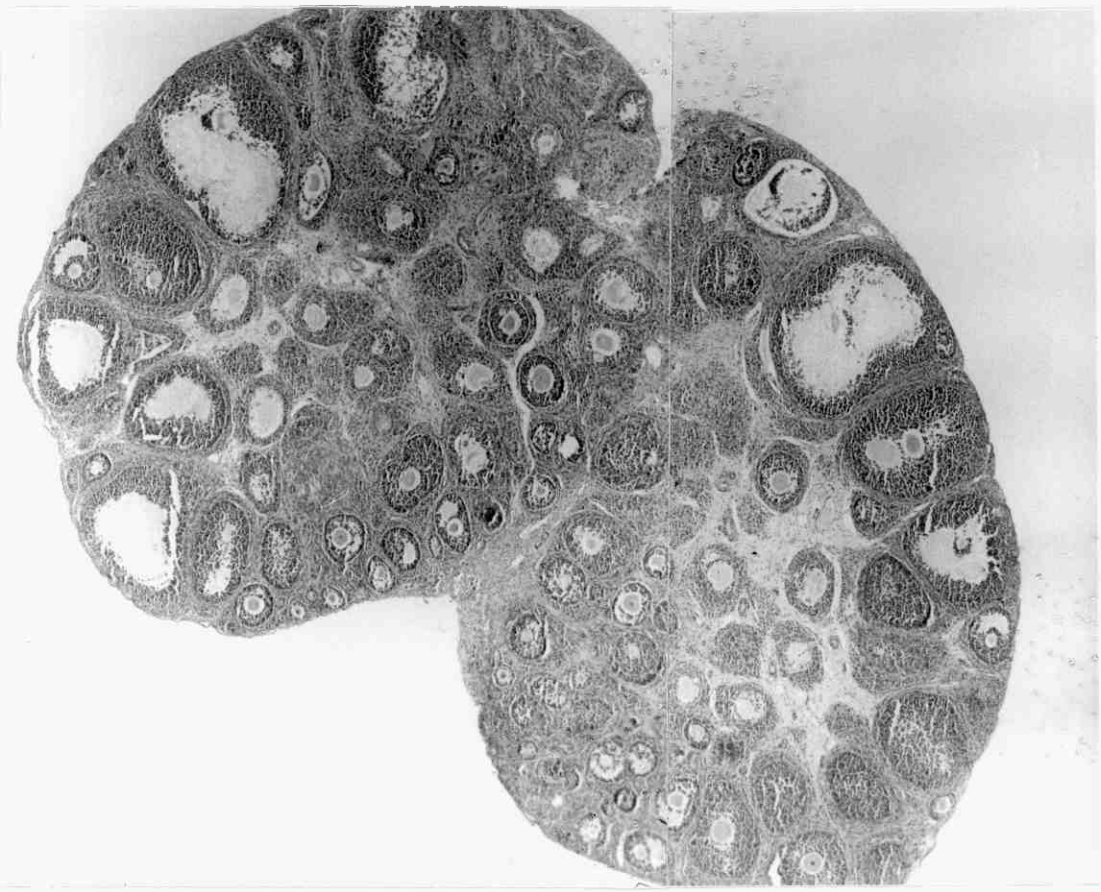


Plate 2. Longitudinal section of an ovary removed from a 28 day-old rat. H&E stain (x 25).

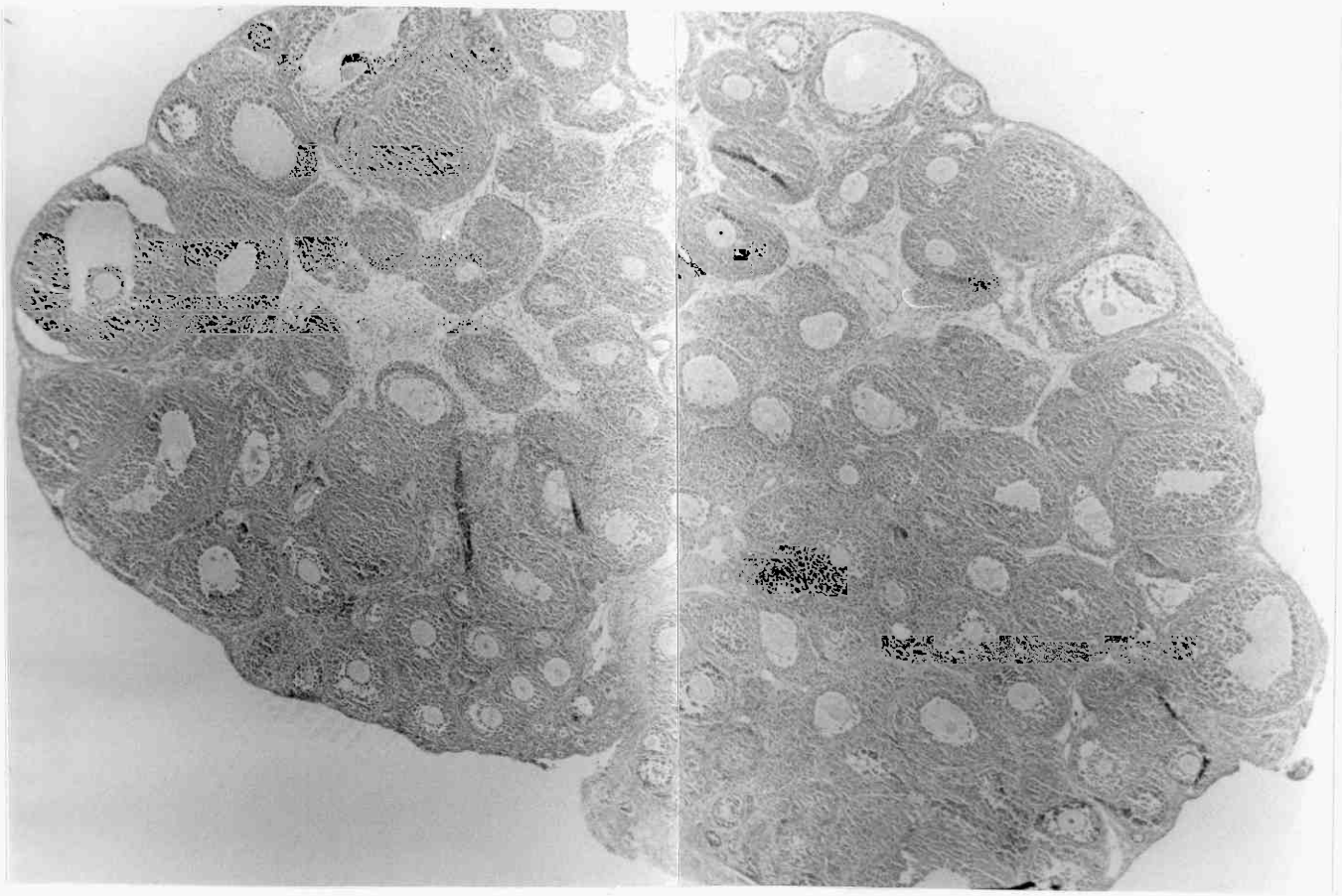


Plate 3. Longitudinal section of an ovary removed from a 40 day-old rat. H&E stain (x 25).

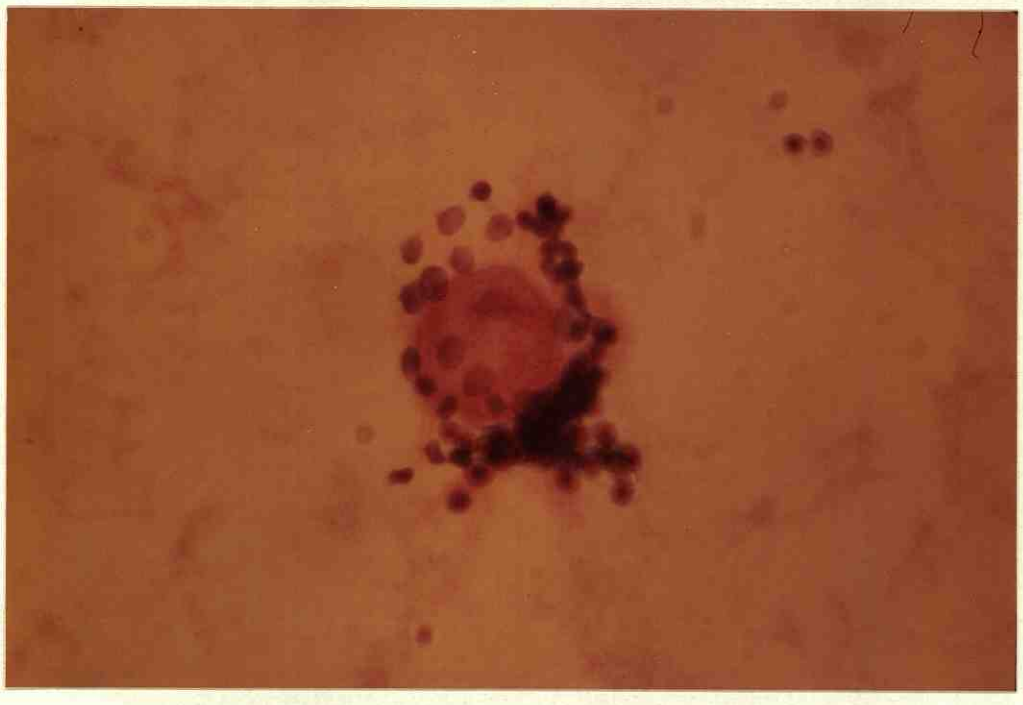


Plate 4. Smear of granulosa cells isolated from ovaries of rats aged 8 days. Note the presence of an oocyte. H&E stain (x180).

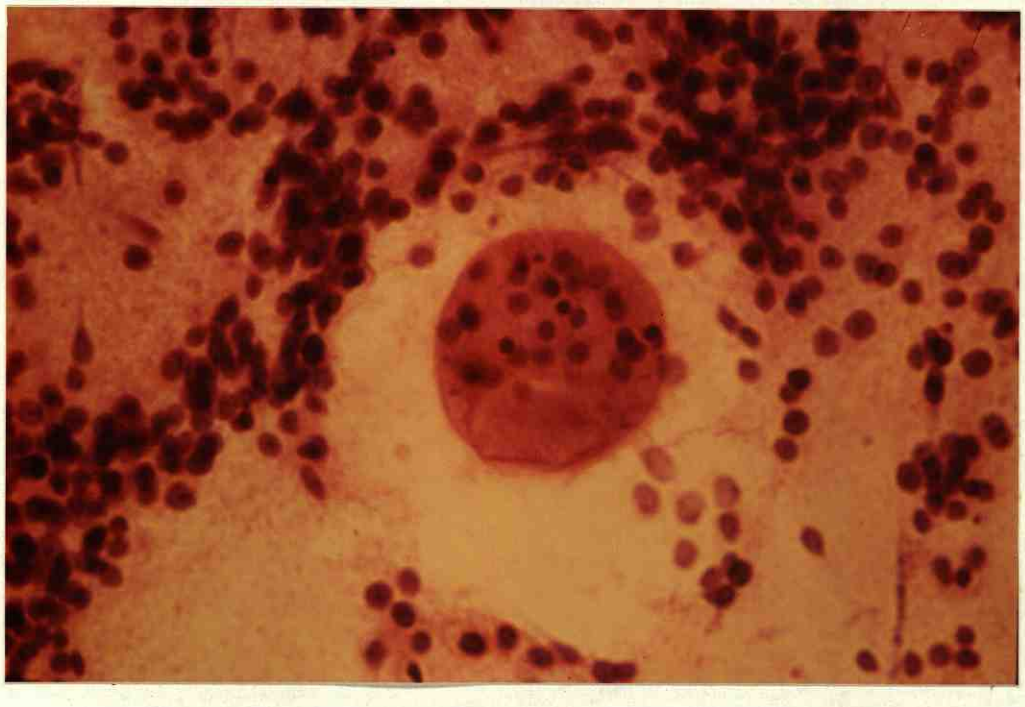


Plate 5. Smear of granulosa cells isolated from ovaries of rats aged 28 days. Note the presence of an oocyte. H&E stain (x180).

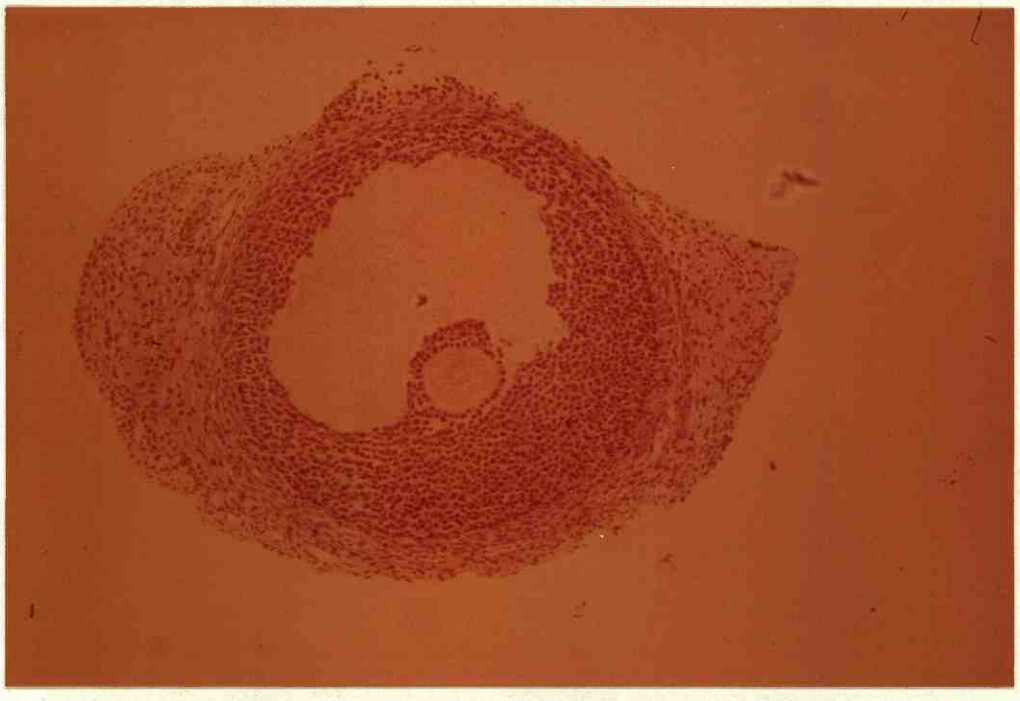


Plate 6.

A cross-section through a dominant antral follicle excised from a 28 day old-old rat ovary. H&E stain(x30)

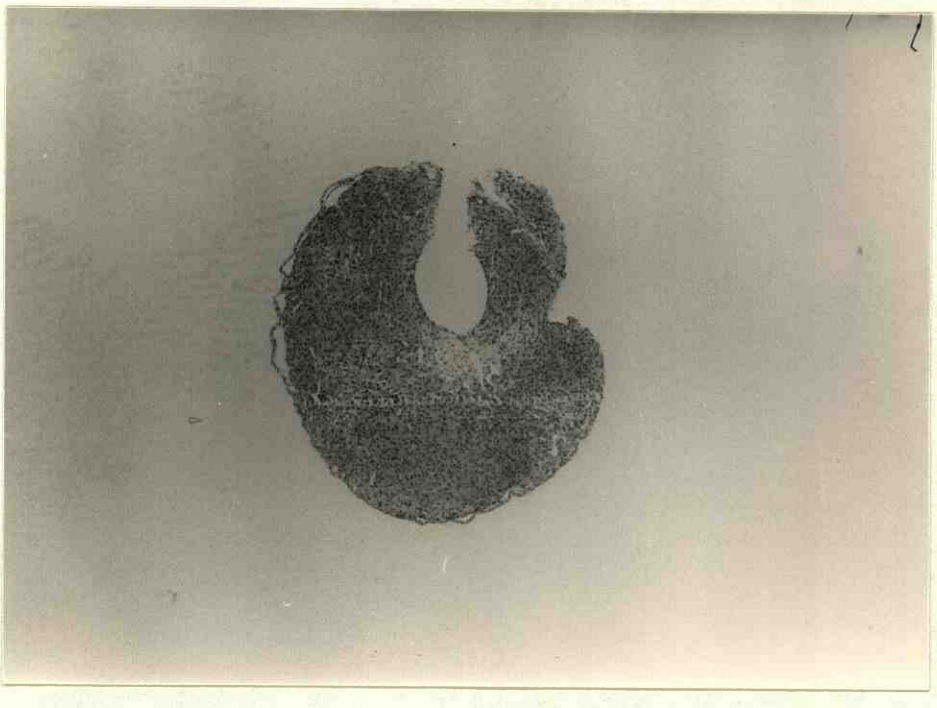


Plate 7.

A cross-section through a corpus luteum excised from an ovary of a rat two days after vaginal opening. H&E stain (x30).

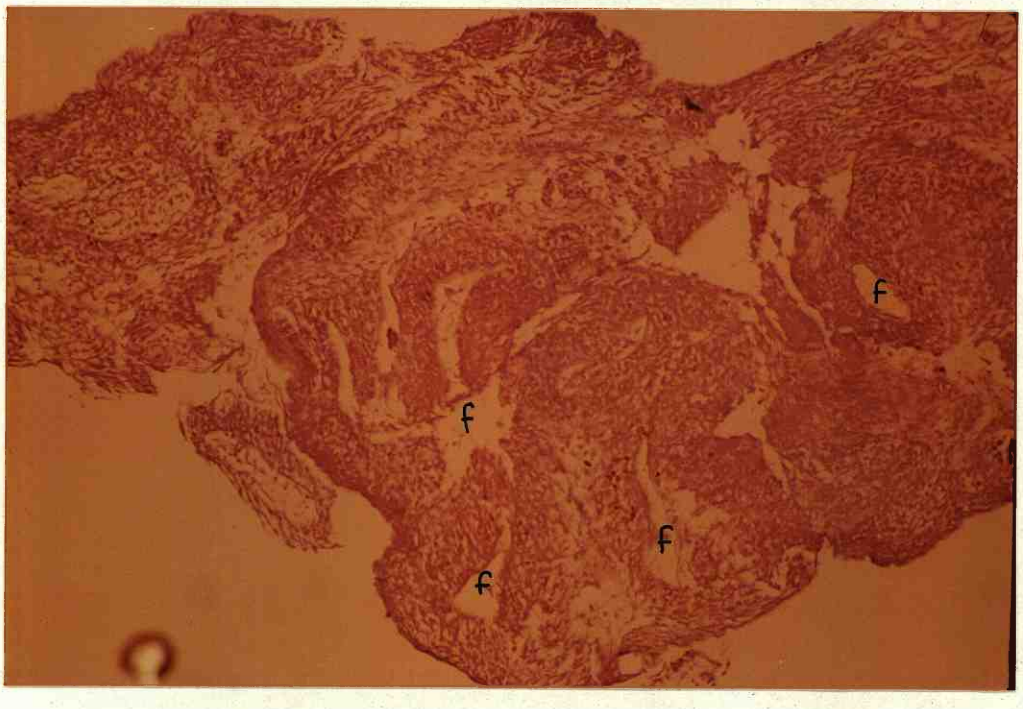


Plate 8. Longitudal section of a 28 day-old rat ovary after isolation of granulosa cells. Note the 'empty' follicles(f). H&E stain.(x25).

proliferating group consists of almost all the small follicles (usually 1 to 4 rows of granulosa cells) and they form a pool from which follicles apparently leave to start their development. These follicles were located predominantly in the medullary region of the ovary. The proliferating group contains all the larger follicles (in the preantral and antral stage of development) which continue to grow until ovulation or degeneration (Peters, 1969; Peters et al., 1975). These follicles were usually limited to the cortical region of the ovary. Extensive granulosa cell hyperplasia and accumulation of follicular fluid in the antral follicles caused them to bulge beyond the surface, interrupting the smooth contour. This makes it possible to excise an intact follicle free from the rest of the ovary (Plate 6). Towards maturity three to four of these large follicles become very prominent in each ovary, having been selected for ovulation. Plate 7 shows a longitudinal section through a corpus luteum excised from a 42 day old rat ovary, 2 days after vaginal opening.

3.2 Preparation of Isolated Granulosa Cells

Granulosa cells were isolated from ovaries of rats aged between 8 and 42 days. As expected only very few granulosa cells could be removed from ovaries of 8 day old animals. As the animal grew older both the number of granulosa cells and the ease of removing them increased. Plate 5 demonstrates the homogeneity

of the isolated cell preparation with oocytes being the major contaminants. Because of the tendency of excessively bulging follicles from older rats to squash and tear off the ovary when squeezed, ovaries from animals aged 28 days were found to be the easiest to work with. Moreover, using ovaries from 28 day old animals ensured that only granulosa cells were isolated and not lutein cells. Plate 8 demonstrates the efficiency with which granulosa cells can be isolated from the ovary (compare with plate 6).

3.3 Estimation of DNA content of the Granulosa Cell

The DNA content of known numbers of granulosa cells were determined by the method of Burton (1956) and a standard curve was constructed by plotting absorbance at 280nm against cell number (Fig 3.1A). This curve was compared with another standard curve obtained by plotting absorbance at 280nm against DNA concentration, using Calf Thymus DNA as standard (Fig 3.1B).

One granulosa cell was estimated to contain 8.00 ± 0.26 pg DNA (n = 3) which was consistent with the estimation of 6 pg DNA made by Richards (1975). For routine analysis of receptor data, each granulosa cell was assumed to contain 8 pg DNA irrespective of the age of the animal from which it was isolated.

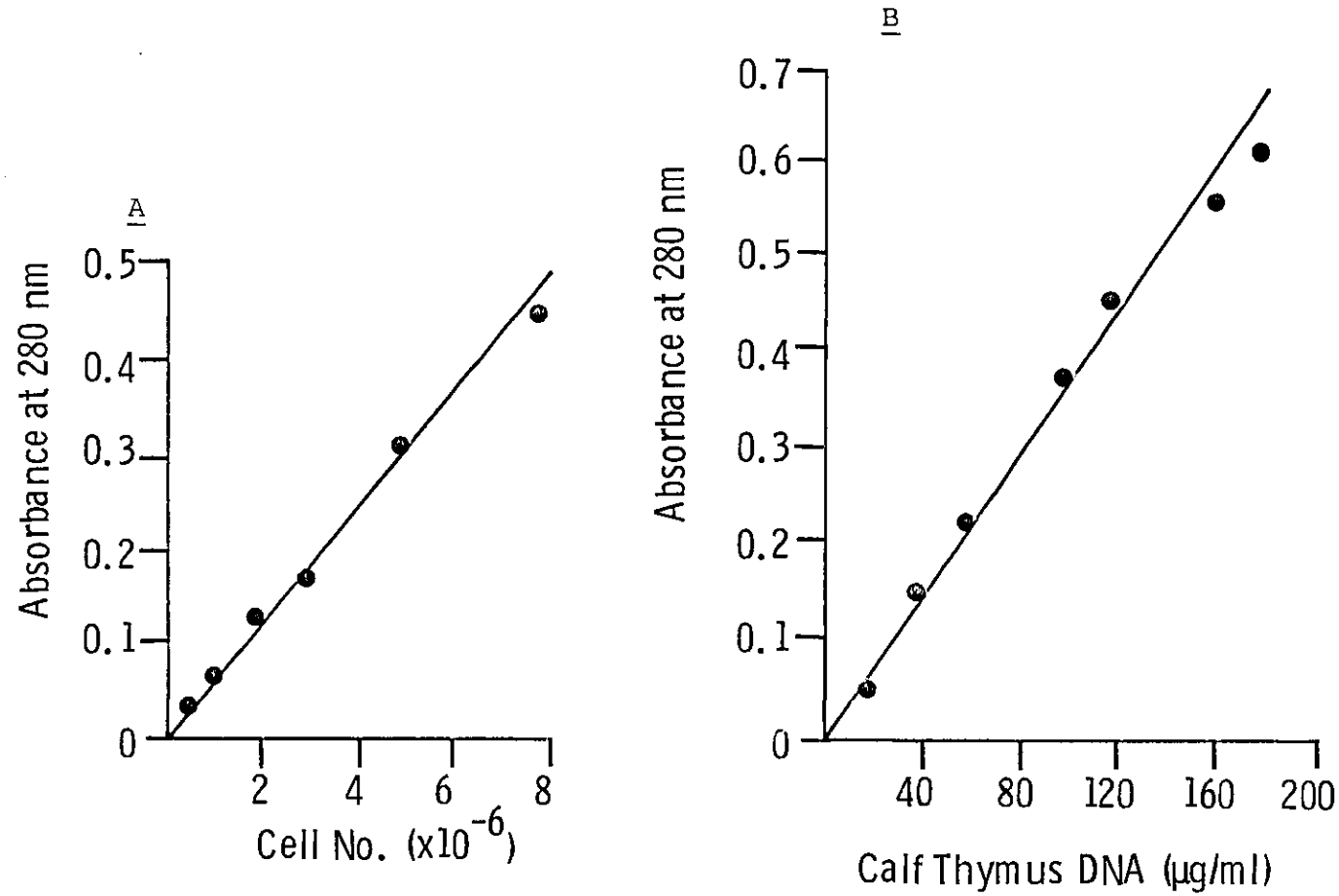


Fig. 3.1. Determination of DNA content of granulosa cells.
Duplicate aliquots containing $5x10^5$ to $8x10^6$ granulosa cells were hydrolysed in 0.5 ml 0.5M PCA. The DNA content of the hydrolysate was determined by the method of Burton (1956) and absorbance measured at 280 nm(A). This was then compared with a standard curve constructed using calf thymus DNA as a reference (B). DNA content/granulosa cell was computed using the 2 curves. Experiments were repeated three times and each experimental group contained 30 rats, aged 24-28 days.

3.4 Characterisation of Ovarian Oestrogen Receptor Species

3.4.1 Whole Ovary

Ovaries removed from rats aged 28 days were used for routine experiments as the levels of nuclear and cytosol oestrogen receptor was found to be fairly constant between 24 and 32 days of age (Section 3.30).

3.4.2 Saturation analysis of the Cytosol Oestrogen Receptor

The binding of [^{3}H]-oestradiol to whole ovarian cytosol was studied using a range of concentrations (0.1 to 8nM) each in the absence or presence of a 100 fold excess of DES. Apparent saturation of binding sites was seen at 4nM [^{3}H]-oestradiol. Scatchard analysis (Scatchard, 1949) revealed a single class of oestrogen binding species with an apparent Kd of 0.39 ± 0.03nM (n = 3) and the binding capacity was 350 ± 40 fmol/mg cytosol protein (Fig 3.2).

3.4.3 Saturation analysis of the Nuclear Oestrogen Receptor

The binding of [^{3}H]-oestradiol to whole ovarian tissue nuclei was studied using a range of concentrations (0.5 to 16nM) each in the absence or presence of a 100 fold excess of DES. Saturation was apparent at 8nM [^{3}H]-oestradiol with a Kd of 0.35 ± 0.09nM and a binding capacity of 208 ± 78 fmol/mg DNA (n = 3) (Fig 3.3).

The values for dissociation constant are in agreement with the reports of Saiduddin & Zassenhaus (1977).

Ovarian oestrogen receptor is thought to be located predominantly in the granulosa cells (Stumpf, 1969). Because the ovary is a heterogeneous tissue, expression of the receptor content per mg DNA, per mg tissue cytosol protein or per ovarian cell is not very meaningful (Saiduddin & Zassenhaus, 1977).

In order to rationalise the measurement of the ovarian oestrogen receptor system, the studies were performed using isolated granulosa cells.

3.5 The Distribution of Oestrogen Receptor In Ovarian Cells

Comparisons of oestrogen receptor measurements made in whole ovarian tissue with that of granulosa cells and residual ovarian tissue indicated that the ovarian oestrogen receptor was probably located exclusively in the granulosa cell (Table 1). The residual ovarian tissue, after removal of the granulosa cells, contained only approximately 6% of the receptor content of the intact ovary.

Receptor recovery in the granulosa cell fraction compared with the intact ovary, was 75.7 ± 1.1% and 95.75 ± 15.75% (n = 3) for the cytosol and nuclear fractions respectively. There is however, an apparent anomaly when the data is expressed as cytosol receptor

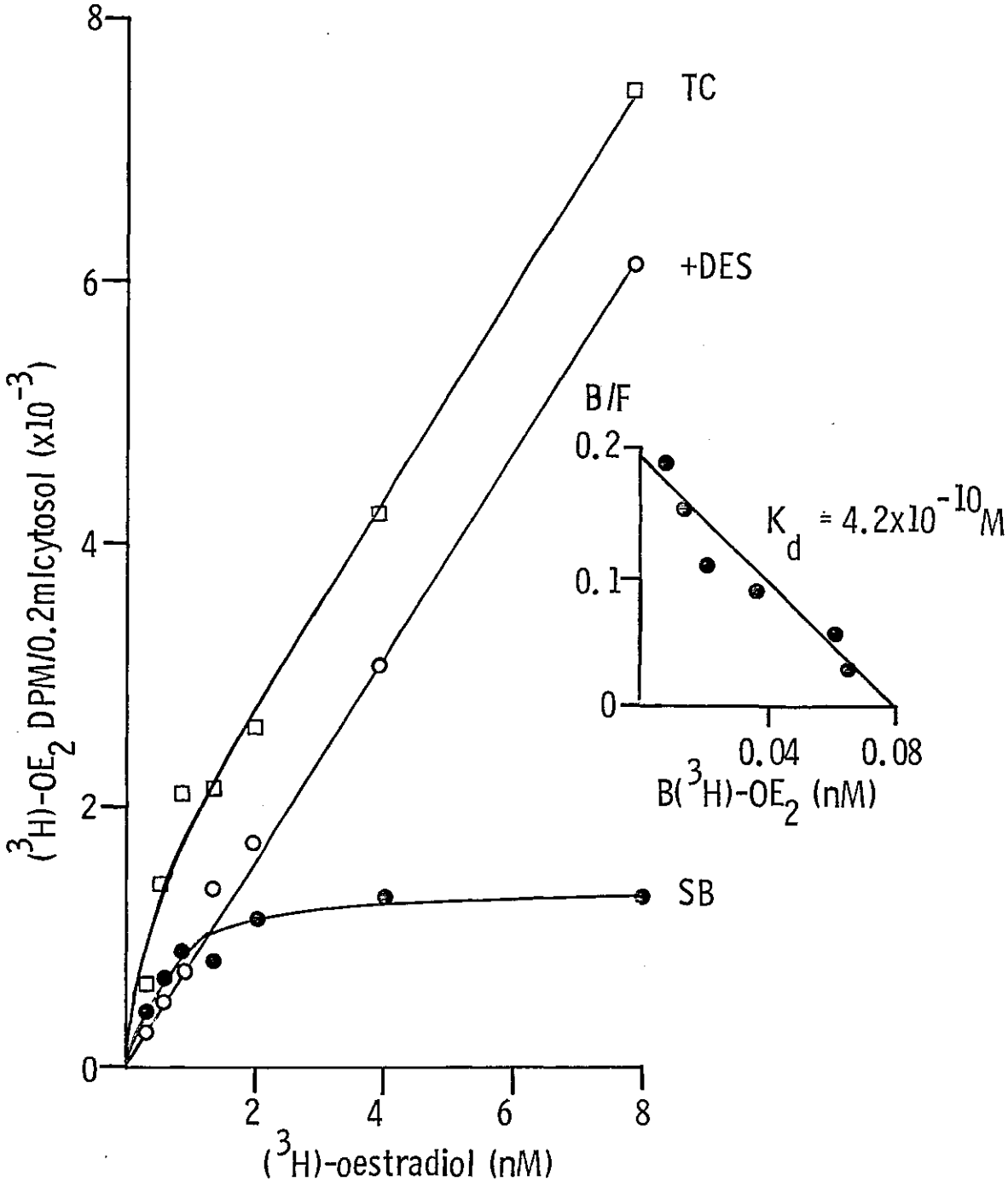


Fig.3.2. Saturation analysis of ovarian cytosol oestrogen receptor. Cytosol (0.2 ml) was incubated with 0.1 to 8 nM [^{3}H]-oestradiol in the absence (TC) or presence of 100 fold excess of DES at 4°C for 18 hr. The dissociation constant(Kd) was calculated by the method of Scatchard (1949). Cytosol protein was approx. 2 mg/ml. Determinations were conducted in duplicate and each experiment repeated three times. Each experimental group contained 10 rats aged 28 days.

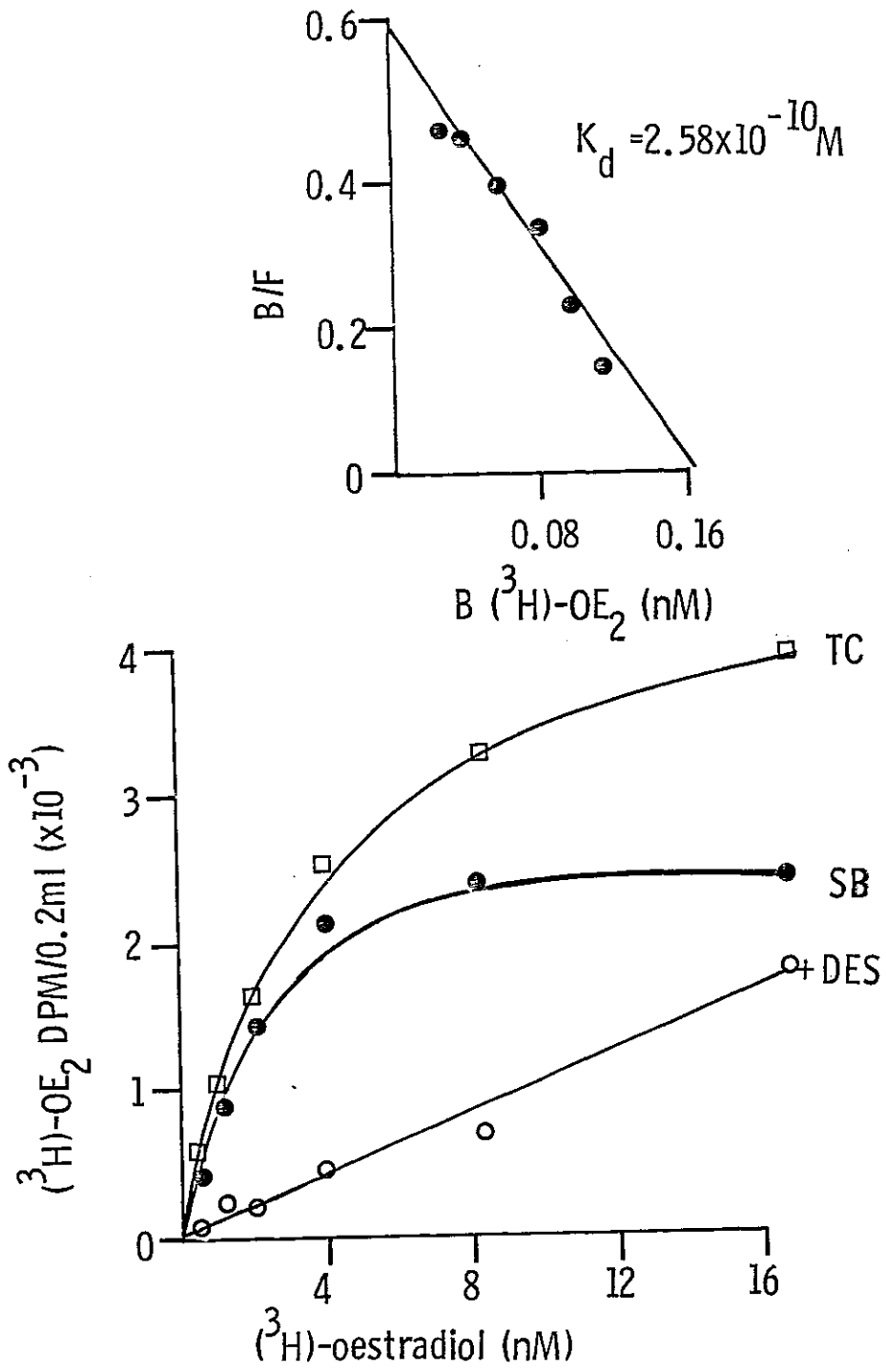


Fig.3.3. Saturation analysis of ovarian nuclear oestrogen receptor. Nuclear suspension (0.2 ml) was incubated with 0.5 to 16 nM [^{3}H]-oestradiol in the absence(TC) or presence of 100 fold excess of DES at 37°C for 1 hr. The dissociation constant(Kd) was calculated by the method of Scatchard (1949). The nuclear suspension contained approx. 0.7 mg DNA/ml. Determinations were conducted in triplicate and each experimental group contained 10 rats,aged 24-28 days.

TABLE 1.

Distribution of Oestrogen Receptor in Ovarian Cells

	RECEPTOR CONCENTRATION			
	CYTOSOL		NUCLEAR	
Tissue	fmol/mg cyt. protein	Fmol/ovary	f/mol/mg DNA	fmol/ovary
Whole ovary	180.48 ± 17.11	94.04 ± 7.60	115.60 ± 11.20	16.07 ± 4.9
Granulosa cell	696.58 ± 63.11	71.13 ± 4.72	406.00 ± 53.00	14.90 ± 2.24
Residual ovarian Tissue (ROT)	15.63 ± 4.46	5.37 ± 1.62	7.90 ± 1.62	1.76 ± 028
RECEPTOR RECOVERY				
Granulosa cell	-	75.70 ± 1.10%	-	95.75 ± 15.75%
ROT	6.83 ± 1.66%	5.64 ± 1.27%	6.77 ± 0.75%	11.31 ± 1.78%

Duplicate aliquots (0.2ml) of cytosol and triplicate aliquots of the nuclear suspensions of either whole ovary, granulosa cell or residual oavrian tissue were incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES for 18 hr at 4°C (cytosol) or 1 hr at 37°C (nuclear suspension). Specific [^{3}H]-oestradiol binding was expressed as receptor concentration (specific activity 100 Ci/mmol; 222,000 d.p.m. = 1 pmol). Ovarian cytosol protein concentration was approx. 2mg/ml and DNA concentration approx. 0.15mg/ml. Each experiment was repeated three times (values = mean ± S.D.) and each experimental group contained 10 rats, aged 28 days.

per mg cytosol protein or nuclear receptor per mg DNA because of the heterogeneity of the ovarian tissue and the homogeneity of the granulosa cell preparation. The 24% loss of the cytosol receptor may have occurred during the isolation of the cells when some of the granulosa cells burst releasing their cellular contents.

3.6 Granulosa Cell Oestrogen Binding Studies

3.6.1 Estimation of Equilibrium Dissociation Constants

3.6.1(a) Nuclear Oestrogen Receptor

The analysis of oestrogen receptor binding using a range of [^{3}H]-oestradiol concentrations from 0.3 to 10nM in the presence or absence of a 100 fold excess of DES showed saturation of the nuclear oestrogen receptor at 5nM [^{3}H]-oestradiol (Fig 3.4). Scatchard analysis revealed a single class of oestrogen binding species with an apparent dissociation constant (Kd) of 1.64 ± 0.92 nM and a binding capacity of 440 ± 93 fmol/mg DNA (n = 3). The value for the Kd agrees with reports published for the granulosa cell nuclear receptor (Richards, 1977) and for other oestrogen target tissues (King & Mainwaring, 1974; Buller & O'Malley, 1976) and was not significantly different from that measured in the whole ovarian nuclei (Section 3.4.2). Binding of [^{3}H]-oestradiol to granulosa cell nuclear suspension was equally displaced by a 100 fold excess of either OE_2 or DES (Fig 3.4).

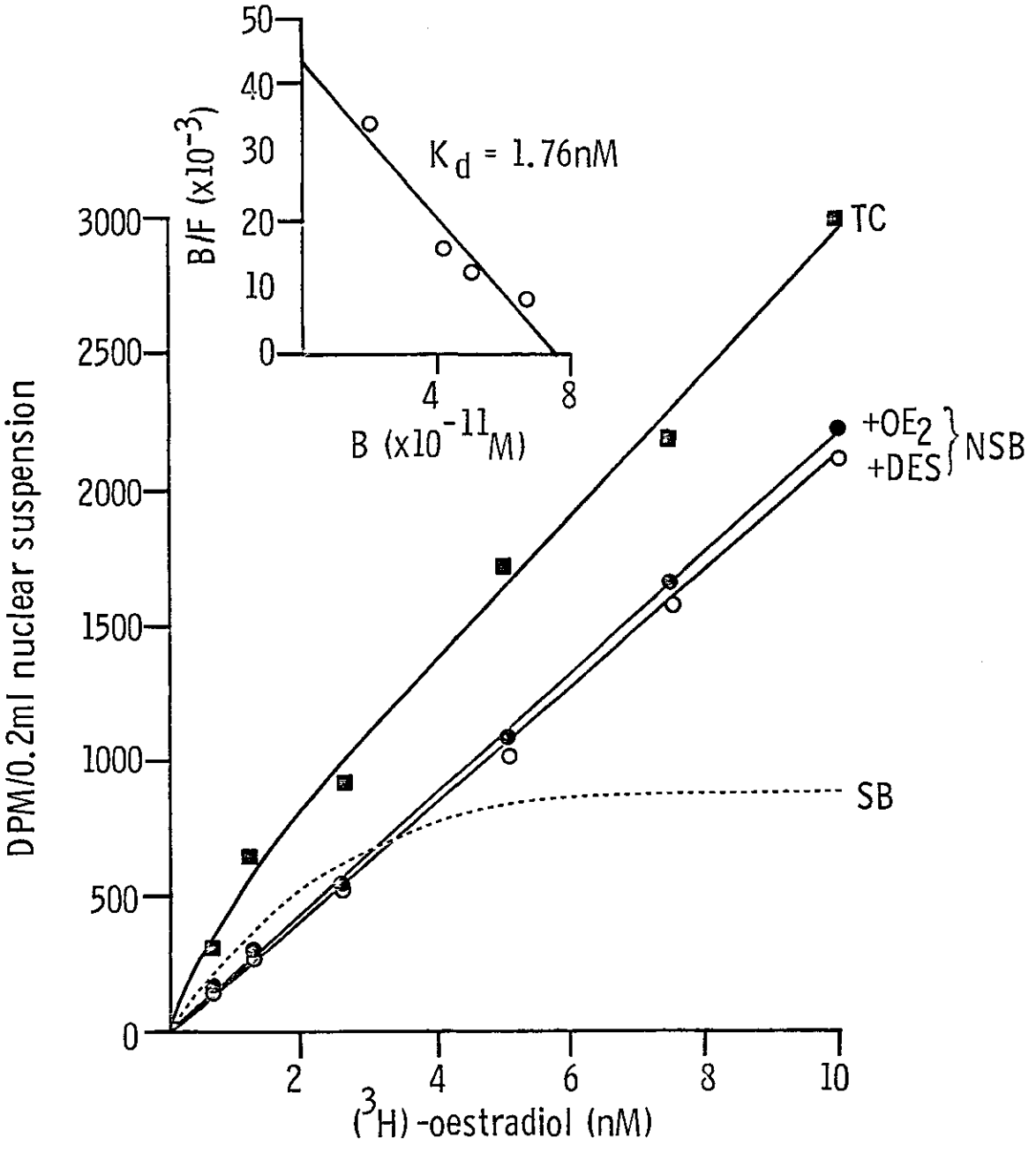


Fig.3.4. Saturation analysis of granulosa cell nuclear oestrogen receptor. Nuclear suspension (0.2ml) were incubated with 0.3 to 10 nM [^{3}H]-oestradiol in the absence (TC) or presence of 100 fold excess of DES or OE_2 at 37°C for 1 hr. K_d was calculated by the method of Scatchard (1949). The nuclear suspension contained approx. 150 ug DNA/ml. Determinations were conducted in triplicate and each experiment repeated three times. Each experimental group contained 25 rats, aged 28 days.

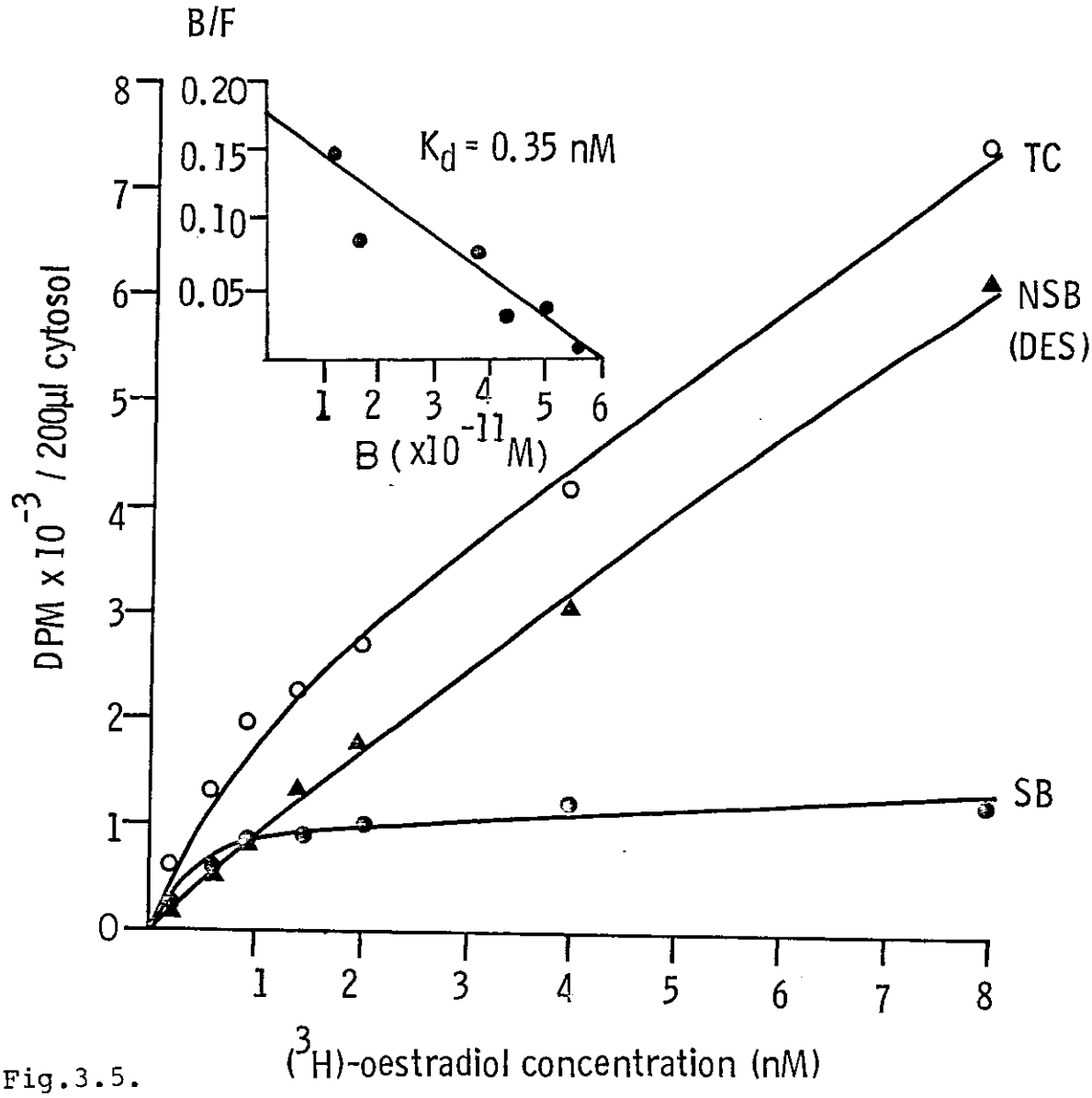


Fig.3.5.

Saturation analysis of granulosa cell cytosol oestrogen receptor. Cytosol (0.2ml), pretreated with DCC, was incubated with 0.2 to 8nM [^{3}H]-oestradiol in the presence or absence of DES(NSB) at 4°C for 18 hr. Kd was calculated by the method of Scatchard (1949). Cytosol protein was approx. 200 µg/ml. Determinations were conducted in duplicate and each experiment repeated three times. Each experimental group contained 25 rats, aged 28 days.

3.6.1(b) Cytosol Oestrogen Receptor

When a range of concentrations of [^{3}H]-oestradiol (from 0.1 to 8nM) with a 100 fold excess of DES as competitor was employed, saturation of the granulosa cell cytosol oestrogen receptor was seen at 2nM [^{3}H]-oestradiol. The apparent Kd for the oestrogen receptor was 0.58 ± 0.22 nM (n = 3) which was similar to that measured in whole ovarian cytosol. The concentration of binding sites was 2780 ± 200 fmol/mg cytosol protein (Fig 3.5).

3.7 Ligand - Competition Studies

The competition of various steroids for the binding of [^{3}H]-oestradiol to the granulosa cell oestrogen receptor was determined in cytosol and nuclear suspension. These were labelled with 10nM [^{3}H]-oestradiol with or without a 100 fold excess of the various competitors at 4°C for 18 hr and 37°C for 1 hr respectively.

3.7.1 Nuclear Oestrogen Receptor

Oestradiol-17β and DES were equally effective in displacing [^{3}H]-oestradiol from the oestrogen receptor (Table 2). Oestriol and oestrone were 50 to 70% as effective as oestradiol. Progesterone, cortisol and testosterone could compete for only approximately 20% of [^{3}H]-oestradiol binding. The androgen metabolite 5α-dihydrotestosterone (DHT) did not compete for the nuclear oestrogen receptor.

TABLE 2. LIGAND COMPETITION STUDIES

COMPETITOR	CYTOSOL	NUCLEI
17β-oestradiol	100 (n=10)	100 (n=5)
Diethylstilboestrol	36.42 ± 4.11 (n=10)	120.3 ± 16.41 (n=5)
Oestrone	68.10 ± 0.69 (n=3)	47.3 ± 9.27 (n=3)
Oestriol	26.69 ± 1.09 (n=3)	64.11 ± 15.72 (n=3)
Testosterone	33.29 ± 5.77 (n=5)	16.65 ± 1.57 (n=3)
5α-Dihydrotestosterone	52.72 ± 4.97 (n=9)	2.76 ± 0.23 (n=3)
Progesterone	55.65 ± 4.69 (n=3)	26.38 ± 2.63 (n=3)
Cortisol	16.98 ± 2.22 (n=3)	20.83 ± 1.23 (n=3)
Tamoxifen	105.50 ± 10.60 (n=3)	-
Clomiphene citrate	124.62 ± 13.72 (n=3)	-
Nafoxidine	119.89 ± 15.50 (n=3)	-
R1881	66.55 ± 14.61 (n=3)	-
17β-oestradiol + 5α-dihydrotestosterone	102.28 ± 2.32 (n=7)	104.88 ± 8.64 (n=7)
5α-dihydrotestosterone + diethylstilboestrol	64.08 ± 4.76 (n=7)	137.08 ± 7.78 (n=7)

Aliquots (0.2ml) of cytosol and nuclear suspension were incubated with 10nM $[^3H]$-OE_2 in the absence or presence of 1μM concentrations of various competitors for 18 hr at 4°C. Displacement by 1 μM oestradiol = 100%. Where two competitors were used together, each was maintained at 1μM concentration (values = Mean ± SEM). Cytosol experiments were performed in duplicate and nuclear suspension in triplicate (n = number of experiments).

3.7.2 Cytosol Oestrogen Receptor

The binding of [^{3}H]-oestradiol in the granulosa cell cytosol was displaced more effectively by OE_2 than by DES. At equivalent concentration DES was only 36% as effective as oestradiol while oestrone, oestriol and progesterone displaced approximately 68, 27 and 56% respectively, of [^{3}H]-oestradiol binding. Testosterone competed for 33% and DHT competed for 53% of the [^{3}H]-oestradiol binding whilst the synthetic androgen methyltrienolone (R1881) displaced 67% and cortisol 17% of the [^{3}H]-oestradiol binding. Anti-oestrogens tamoxifen, clomiphene citrate and nafoxidine were as effective as oestradiol in displacing [^{3}H]-oestradiol binding in the granulosa cell cytosol.

When both DHT and DES were used together as competitors (each of 100 fold excess) there was an apparent additive displacement of [^{3}H]-oestradiol (Tables 2 and 3). This implied that the two steroids perhaps displaced [^{3}H]-oestradiol from different binding sites and using DES suppression to denote the oestrogen receptor, it can be concluded that DHT is competing for binding other than to the oestrogen receptor. When OE_2 and DHT were used together however, there was no apparent increase in displacement of [^{3}H]-oestradiol binding compared to oestradiol alone. Therefore OE_2 and DHT appeared to displace [^{3}H]-oestradiol from the same binding species. The ability of the synthetic androgen R1881, which is a specific ligand for the androgen receptor (Dubé et al.,1980),

TABLE 3. Competition by DES, DHT and R1881 for [^{3}H]-oestradiol Binding to Cytosol Oestrogen Receptor.

	[^{3}H]-oestradiol binding		
	Dpm/0.2ml cytosol	Displaceable specific counts	Oestrogen receptor
Control	30046 ± 1750		
+ R1881	24007 ± 840	4875 ± 664	
			5374 ± 463
+ DES + R1881	18633 ± 422	11413 ± 1398	
+ DES	26640 ± 1651	3406 ± 137	3406 ± 137
+ DHT	22501 ± 766	7545 ± 1309	
			3710 ± 179
+ DHT + DES	18791 ± 591	11255 ± 1183	

Cytosol (0.2ml) was incubated with 10nM [^{3}H]-oestradiol in the absence or presence of 1μM R1881, R1881 + DES, DES, DHT, DHT + DES for 18 hr at 4°C. Where two competitors were used, each was maintained at 1μM concentration. Specific binding was determined by Sephadex LH-20 chromatography. The experiments were conducted in duplicate, each experiment repeated three times (values = Mean ± SEM) and each experimental group containined 15 rats aged 28 days.

to displace [^{3}H]-oestradiol is similar to that of DHT. However, like DHT, R1881 did not apparently compete for the oestrogen receptor (Table 3). The R1881-displaced [^{3}H]-oestradiol binding might be due to binding to the androgen receptor (Schreiber & Ross, 1976; Schreiber et al., 1976). The apparent inability of DHT to bind to the oestrogen receptor (although being 30% more potent than testosterone, King & Mainwaring, 1974) reinforces the observation made in Fig 3.10 where testosterone did not compete for the oestrogen receptor binding sites in vitro even when a 1000 fold excess was employed over the [^{3}H]-oestradiol concentration. As oestradiol appeared to be more effective in binding to the granulosa cell cytosol oestrogen binding species than DHT or R1881, it did not appear that the oestradiol was binding to the androgen receptor. Both oestradiol and DES have been shown to be equally effective in displacing [^{3}H]-oestradiol from the oestrogen receptor (Sudo et al., 1983). As there is no evidence in the literature to explain the observed difference in oestradiol and DES displacement of [^{3}H]-oestradiol in the granulosa cell cytosol, the difference was attributed to an additional oestrogen binding species. This has been quantitatively estimated as the oestradiol-displaced counts minus DES-displaced counts (oestrogen receptor) and has been termed the second oestrogen binding (SOB) component of the granulosa cell cytosol. This quantitative difference was observed at all concentrations of DES and OE_2 up to 1000 fold excess when

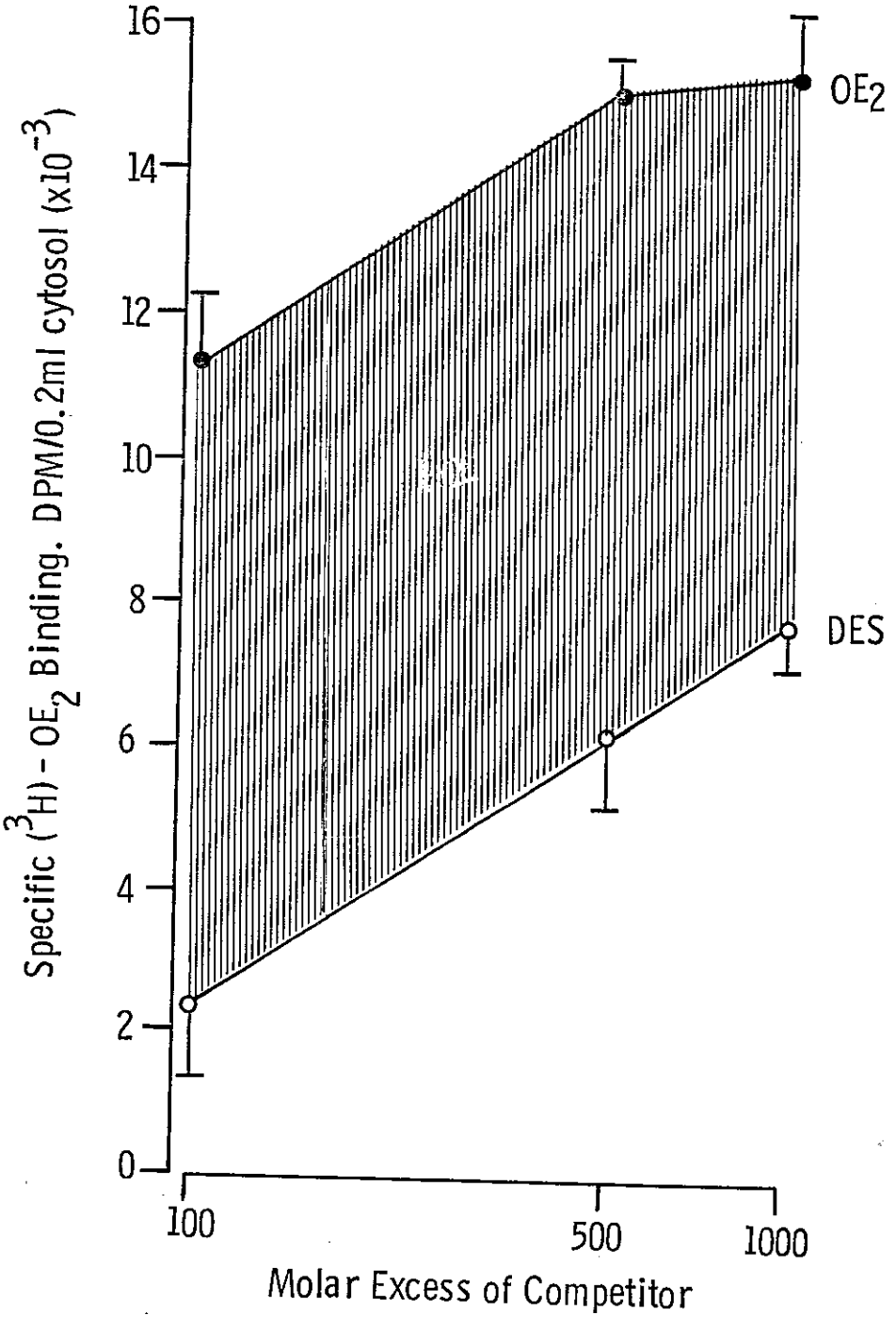


Fig.3.6. Efficiency of DES and OE_2 in displacing [^{3}H]-OE_2 binding in granulosa cell cytosol.
Cytosol (0.2 ml) was incubated in the presence or absence of 1 μM, 5 μM and 10 μM DES or OE_2 with 10 nM [^{3}H]-OE_2 for 18 hr at 4°C. Specific DES and OE_2 binding was assessed using Sephadex LH-20 chromatography. Experiments were conducted in duplicate and each experiment repeated three times (values=mean ± S.D.). Each experimental group contained 10 rats, aged 28 days.

granulosa cell cytosol was radiolabelled with 10nM [^{3}H]-oestradiol (Fig 3.6). Thus in the granulosa cell cytosol 65% of macromolecular bound [^{3}H]-oestradiol was associated with SOB(at a concentration of 10nM [^{3}H]-oestradiol)when the determination was performed using Sephadex LH-20 to separate macromolecular bound from free steroid. Oestrogen binding species with characteristics similar to SOB (with regard to its ability to bind oestrogens, progesterone and androgens) have been described for other oestrogen target tissues. Dickson et al.(1978) found a sex-steroid binding protein in male but not female rat liver cytosol which bound oestradiol and [^{3}H]-5α-dihydrotesterone rapidly at the same site. This protein did not bind DES. In the chick oviduct cytosol, Taylor & Smith (1982) found an oestrogen binding protein which bound progesterone, R5020, testosterone and DHT but not DES. Dubois (1980) described a similar oestrogen binding protein in the rat prostate cytosol which did not bind DES. The SOB also binds the anti-oestrogens nafoxidine, clomiphene citrate and tamoxifen (Table 2). Sudo et al. (1983) have described a protein present in the ovary and other oestrogen target organs which binds anti-oestrogens but not oestradiol or DES.

3.8 Determination of the Dissociation Constant of the Granulosa Cell Cytosol Second Oestrogen Binding Species (SOB)

Following the observation that most of the bound [^{3}H]-oestradiol was associated with the SOB rather than with the classical oestrogen receptor, an extended range of [^{3}H]-oestradiol concentrations (0.1 to 36nM) was employed in saturation analysis with a 100 fold excess of OE_2 as competitor. A biphasic saturation curve was revealed with apparent saturation at both 4nM and at greater than 30nM [^{3}H]-oestradiol. Double reciprocal analysis of the data revealed the presence of two oestrogen binding components (Fig 3.7). The higher affinity species had an apparent Kd of 2.6 ± 1.51nM (not significantly different from that measured using DES as competitor, section 3.6.1b) and the lower affinity species had an apparent Kd of 45.78 ± 6.88nM (n = 3). The higher affinity binder appeared to have a binding capacity of 4660 ± 245 fmol/mg cytosol protein and the lower affinity binder a capacity of 16.63 ± 2.44 pmol/mg cytosol protein. The value of the Kds measured for the lower affinity binder, which was thought to represent SOB, was similar to those measured for the non-DES suppressible oestrogen binding species described by Dickson *et al* (1978), Dubois (1980) and Taylor & Smith (1982).

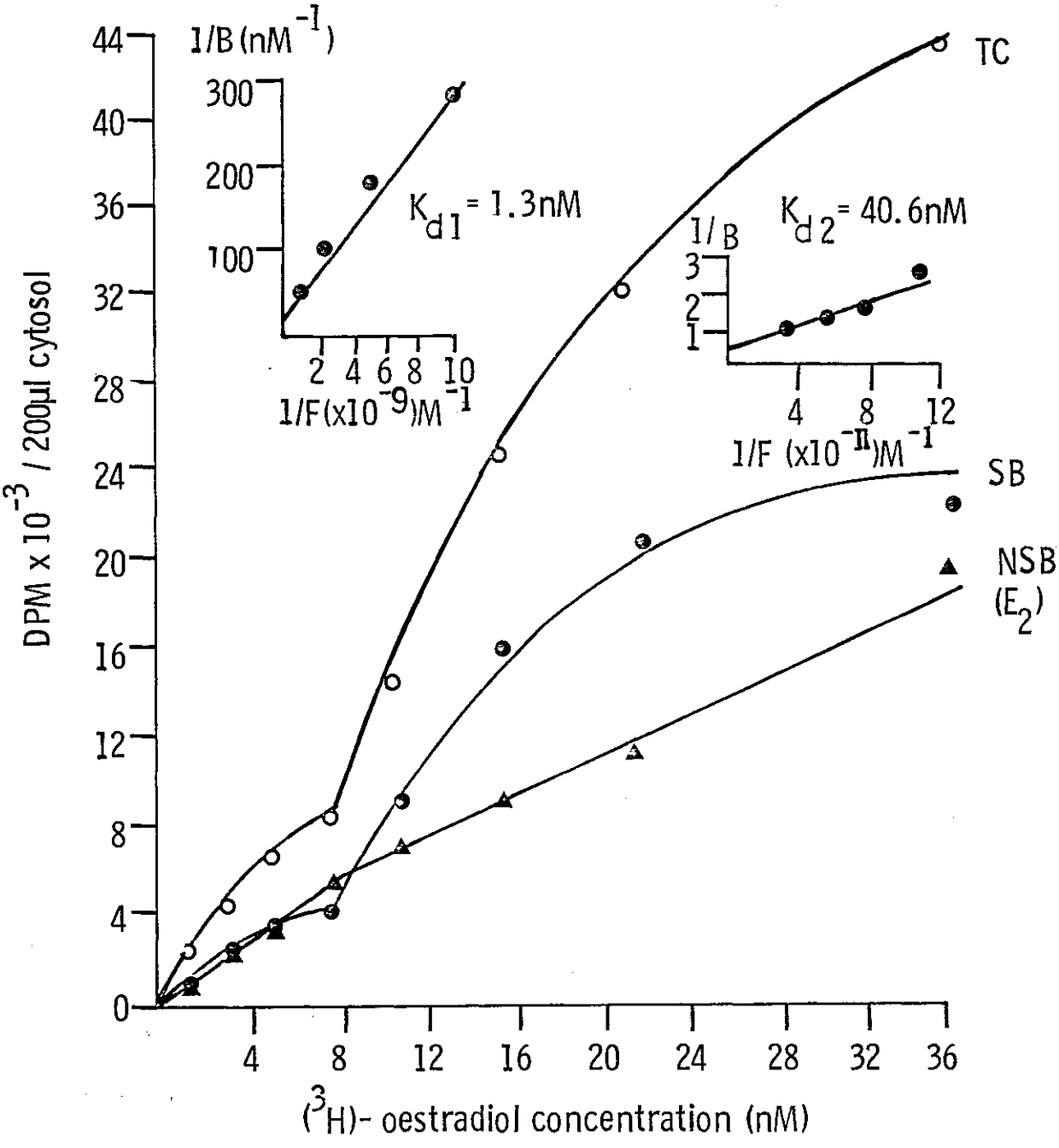


Fig.3.7. Saturation analysis of granulosa cell cytosol oestrogen binding species. Duplicate DCC-treated cytosol was incubated with 0.1 to 36nM [^{3}H]-OE_2 in the absence (TC) or presence of OE_2 for 18 hr at 4°C. Dissociation constants (K_ds) were calculated using double-reciprocal analysis. Cytosol protein was approx. 0.2 mg/ml. Each experiment was repeated three times and each experimental group contained 25 rats, aged 28 days.

3.9 Characterisation of Granulosa Cell Oestrogen Binding Species

3.9.1 Cytosol Oestrogen Binding Species

In an attempt to distinguish between the putative oestrogen receptor and SOB, it was elected to firstly determine the optimal conditions for the measurement of the oestrogen receptor.

3.9.2 Optimisation of Oestrogen Receptor Measurement

3.9.2(a) Removal of Endogenous Steroids

The ovary synthesises androgens and oestrogens with the granulosa cell being the predominant site of oestrogen synthesis (Dorrington & Armstrong, 1979; Richards, 1980). It seemed likely that high local concentration of oestrogens could occupy available binding sites and in addition, dilute the [^{3}H]-oestradiol concentration used in receptor assays. As shown in Fig 3.8, over 90% of the available binding sites were occupied or were not measured and only after prior treatment with dextran-coated charcoal (DCC) for 10 min at 4°C to remove endogenous steroids were the sites made available for binding to [^{3}H]-oestradiol at 4°C.

3.9.2(b) Assay of Oestrogen Receptor

3.9.2b.1 Separation of Bound and Free Steroid with Dextran-Coated Charcoal (DCC)

One method routinely employed in many laboratories for separation of macromolecular bound [^{3}H]-oestradiol from free ligand makes use of DCC (McGuire *et al.*,

1977; Powell et al., 1981). When this method was used to evaluate granulosa cell cytosol oestrogen receptor, no specific [^{3}H]-oestradiol binding was observed. Powell et al.(1981) observed that with cytosol protein concentrations of below 1 mg/ml, breast cancer tissue cytosol oestrogen receptor levels were underestimated. The inability to obtain specific oestrogen binding when DCC was employed might therefore be due to the fact that the granulosa cell cytosol protein concentration was low (approximately 200 µg/ml).

3.9.2b.2 Sephadex LH-20 Chromatography

Separation of macromolecular bound [^{3}H]-oestradiol from free ligand by Sephadex LH-20 chromatography was found to be simple and reproducible. To optimise the conditions for separation of the oestrogen receptor, granulosa cell cytosol was prelabelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1µM DES for 18 hr at 4°C. The radiolabelled receptor complex was then applied to the Sephadex LH-20 gels for periods ranging from 15 to 210 min at 4°C, prior to elution with TED buffer as shown in Fig 3.9. There was a progressive fall in [^{3}H]-oestradiol binding to the cytosol components without any reduction of the quantity of specific macromolecular bound [^{3}H]-oestradiol. The oestrogen receptor - [^{3}H]-oestradiol complex was thus stable on the Sephadex LH-20 gels at 0 to 4°C for up to 210 min.

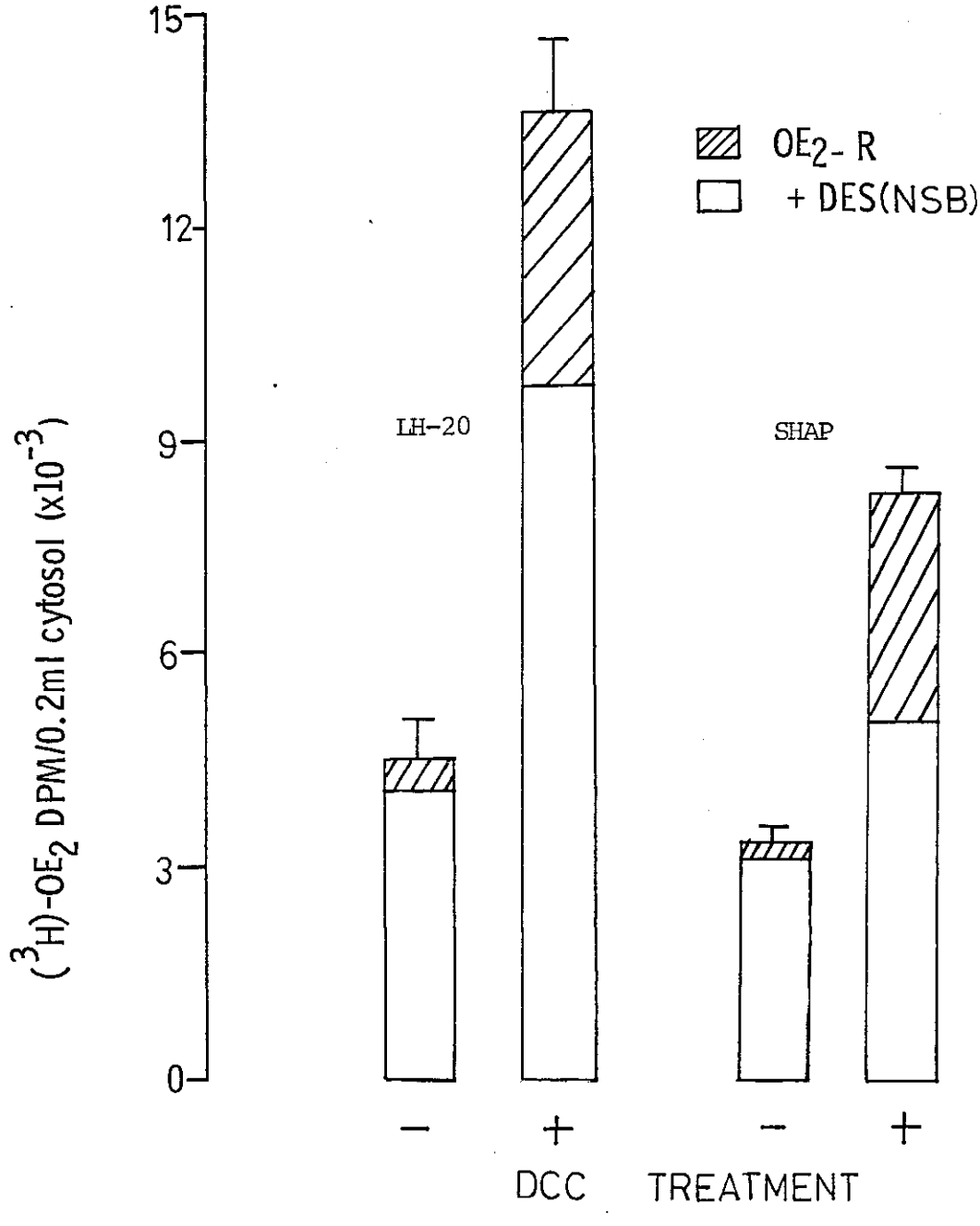


Fig.3.8. Effect of removal of endogenous steroids from granulosa cell cytosol on oestrogen receptor measurement. Cytosol was either treated with DCC for 10 min at 4°C (+DCC) or was not treated (-DCC) prior to incubation with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES for 18 hr at 4°C. Specific binding was assessed by either Sephadex LH-20 chromatography (LH-20) or spheroidal hydroxyl-apatite (SHAP) technique. Determinations were conducted in duplicate, each experiment repeated three times (values = mean ± S.D.) and each experimental group contained 10 rats, aged 24-28 days.
(Note: total binding is whole histogram
open area is nonspecific binding (NSB)
hatched area is oestrogen receptor (OE_2-R))

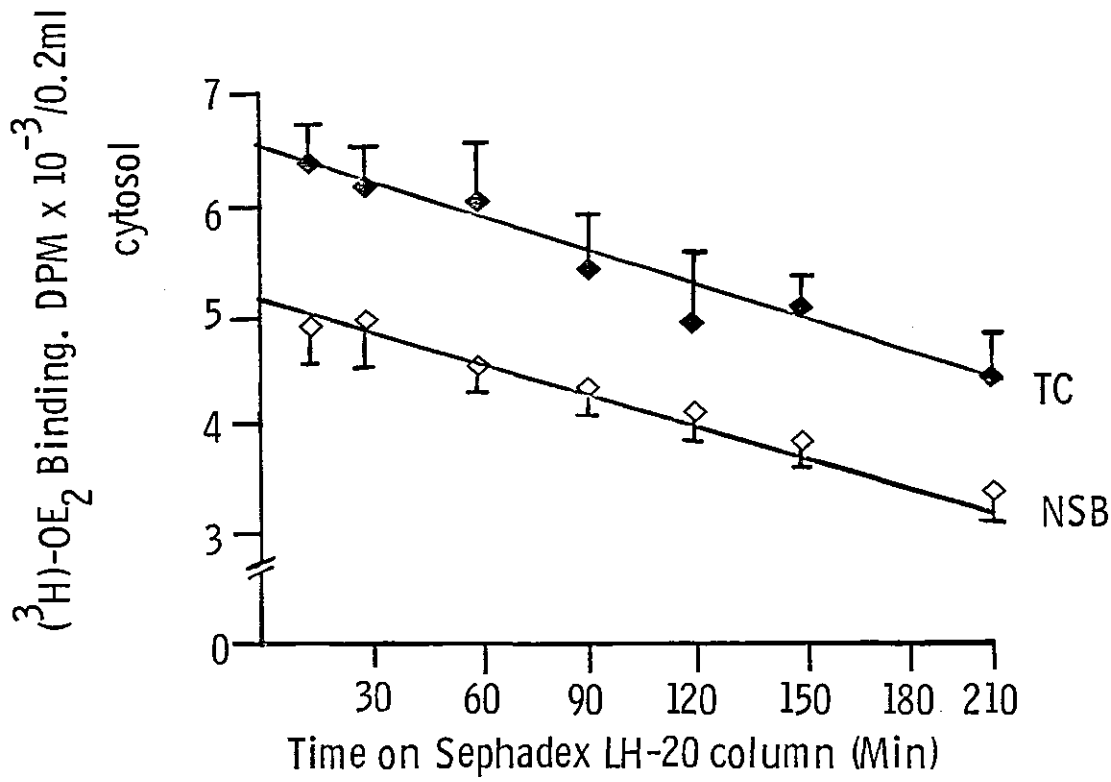


Fig.3.9. Effect of prolonged exposure of oestrogen receptor to Sephadex LH-20 gels. DCC-treated cytosol (0.2 ml) was incubated in duplicate with 10 nM [^{3}H]-OE$_2$ in the absence (TC) or presence of 1 μM DES (NSB) for 18 hr at 4°C. To separate macromolecular bound [^{3}H]-OE$_2$ from free steroid, the cytosol reaction mixture was applied to Sephadex LH-20 gels for 15 to 210 min. The macromolecular bound [^{3}H]-OE$_2$ was eluted with 1.2 ml TED buffer after exposure to the gels and radioactivity counted in Unisolve 1 scintillant. Each experiment was repeated three times (values = mean ± S.D.) and each experimental group contained 10 rats, aged 24-28 days.

The granulosa cell/ovarian cytosol contains a very high level of nonspecific oestrogen binding component comprising about 65 to 80% of the total [^{3}H]-oestradiol binding. In an attempt to reduce this nonspecific binding, receptor measurement was performed in the presence of 20, 50, 100, 500 and a 1000 fold excess of testosterone with respect to 10nM [^{3}H]-oestradiol. As shown in Fig 3.10, testosterone displaced [^{3}H]-oestradiol from low or non-specific binding sites (possibly SOB) without any reduction in the quantity of the oestrogen receptor measured. The Sephadex LH-20 technique offered a more sensitive separation which detected binding species which were otherwise lost when DCC separation was employed. It was therefore used routinely for measurements of the granulosa cell cytosol oestrogen binding species. Interassay differences in the concentrations of oestrogen binders measured were found to be due primarily to the differences in the number of granulosa cells that could be removed from the ovaries from experiment to experiment, or the age of the ovaries from which the granulosa cells were isolated. Inspite of the successful reduction of the nonspecific binding by inclusion of testosterone in incubations (Fig 3.10) no testosterone was added to the cytosol in routine assays as the SOB was found to bind androgens. When OE_2 was used as competitor the level of the 'nonspecific' binding was lowered.

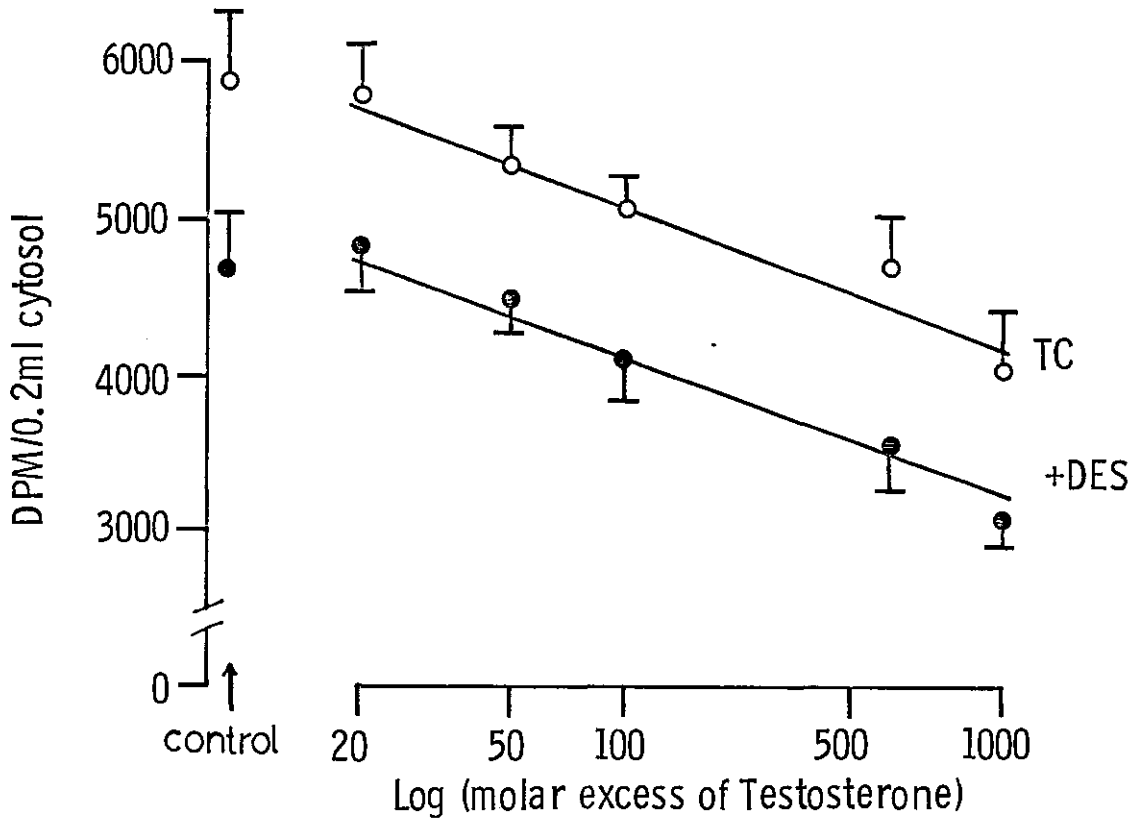


Fig.3.10. Effect of testosterone on measurement of oestrogen receptor of the granulosa cell cytosol.
DCC-treated cytosol (0.2 ml) was incubated in duplicate with 10 nM [^{3}H]-oestradiol in the absence (TC) or presence of 1 μM DES, all in the presence of 20,50,100,500 and 1000 fold molar excess of testosterone for 18 hr at 4°C. Specific binding was assessed after separation of bound from free steroid using Sephadex LH-20 chromatography. Experiments were repeated three times (values = mean ± S.D.) and each experimental group contained 12 rats, aged 24-28 days.

3.9.2c Optimal Temperature/Time for Radiolabelling the Oestrogen Receptor

Granulosa cell cytosol which had been pretreated with DCC to remove endogenous steroid was incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES at 4°, 25° or 37°C for 1, 2, 4, 6 or 18 hr. As shown in Fig 3.11 all the receptor sites available were labelled with [^{3}H]-oestradiol after 4 hr incubation at 4°C. Extended periods of incubation at 4°C added no further increase to the quantity of receptor measured in the cytosol. It was found that even though at elevated temperatures the receptor was rapidly labelled (within the first two hours), there was no observable difference in the receptor concentrations measured in incubations for 4 to 18 hr at 4°C or 4 hr at 25°C. Oestrogen receptor was rapidly labelled at 37°C but progressive loss of measurable receptor was seen with prolonged incubations at 37°C. For routine assays of oestrogen receptor content, the cytosol was incubated at 4°C for 18 hr with [^{3}H]-oestradiol.

3.10 Distinction between Cytosol Oestrogen Receptor and the Second Oestrogen Binding Species

After establishing the optimal conditions for measuring the concentration of oestrogen receptor, attempts were made to determine the distinguishing characteristics of the two oestrogen binding species of the rat granulosa cell cytosol. When the spheroidal hydroxylapatite method of measuring the receptor is

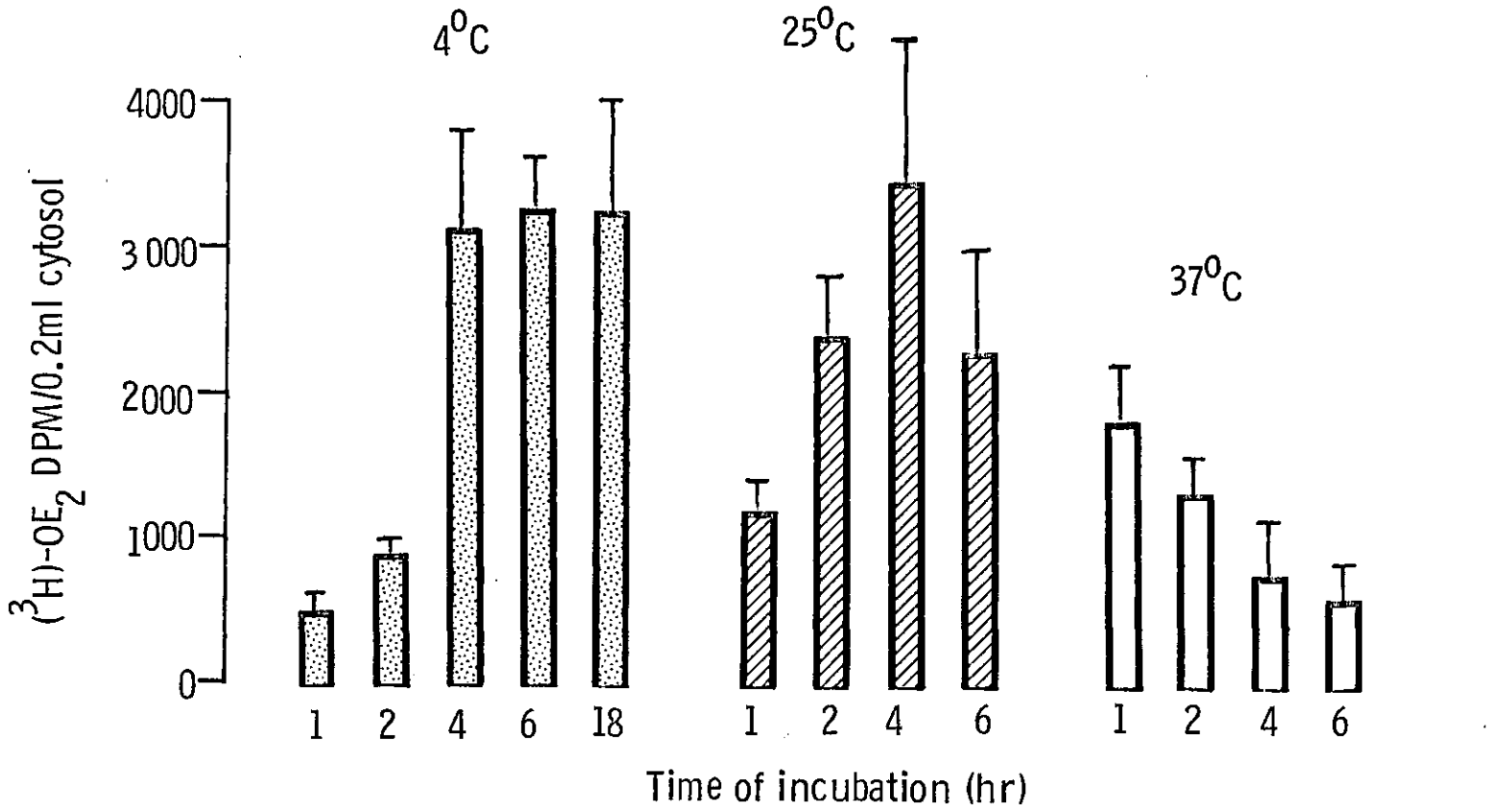


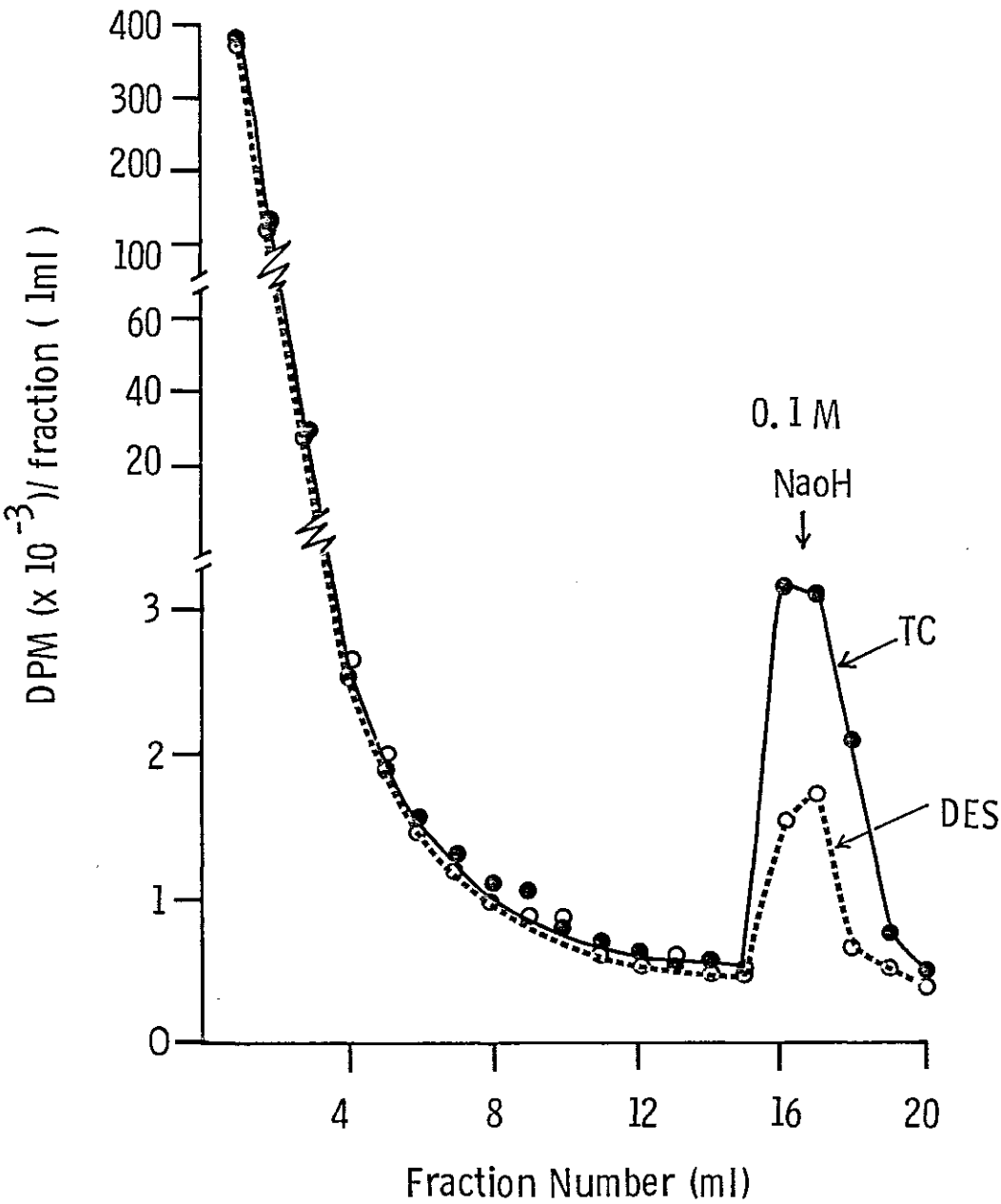
Fig.3.11. Optimal temperature/time for radiolabelling granulosa cell cytosol oestrogen receptor in vitro. DCC-treated cytosol(0.2ml) was incubated in duplicate with 10 nM [^{3}H]-oestradiol with or without 1 μM DES at 4,25 or 37°C for 1,2,4 or 6 hr or for 18 hr at 4°C. Specific binding was assessed following Sephadex LH-20 chromatography. Experiments were repeated three times(values = mean ± S.D.) and each experimental group contained 25 rats, aged 28 days

employed, unbound cytosol components are washed off the hydroxlapatite gel before incubation of receptor, which is retained on the gel, with [^{3}H]-oestradiol (Thrower & Lim, 1980). Therefore it was hoped that this method could enable characterisation of the receptor in the absence of the second binder.

3.11 Spheroidal Hydroxylapatite (SHAP) Chromatography

The characteristic elution pattern of oestrogen receptor complexes from SHAP using this technique is illustrated in Fig 3.12. All the macromolecular bound radioactivity on the column was eluted by a 3 ml 0.1M NaOH wash. Binding data from this technique was treated in the same way as that obtained using the Sephadex LH-20 technique to give estimates of receptor content. Maximum binding of receptor complexes to SHAP was achieved in 30 to 45 min (Fig 3.13). Thrower & Lim (1980) found that binding was complete within 30 min for hypothalamic cytosol and within 5 min for uterine cytosol. No significant differences was observed when the receptor content was measured by either Sephadex LH-20 or by SHAP chromatography (Table 4). However, 60% of the second oestrogen binding species (SOB) was lost when quantified by SHAP binding. These experiments also confirmed that DHT can partially displace [^{3}H]-oestradiol, probably from the second oestrogen binding species and confirmed also that DES was less effective in displacing [^{3}H]-oestradiol than oestradiol from

Fig.3.12. The elution of granulosa cell cytosol oestrogen receptor from spheroidal hydroxylapatite(SHAP). Cytosol(0.2) was applied to SHAP and incubated for 30 min. Unbound components were washed out with TED buffer. Bound components were labelled with 10 nM [^{3}H]-oestradiol in the absence (TC) or presence of 1 µM DES for 18 hr at 4°C. Free steroid was washed out of the SHAP with TED buffer (8 ml) and bound [^{3}H]-oestradiol eluted with 0.1M NaOH (3ml). Specific binding = TC - DES.



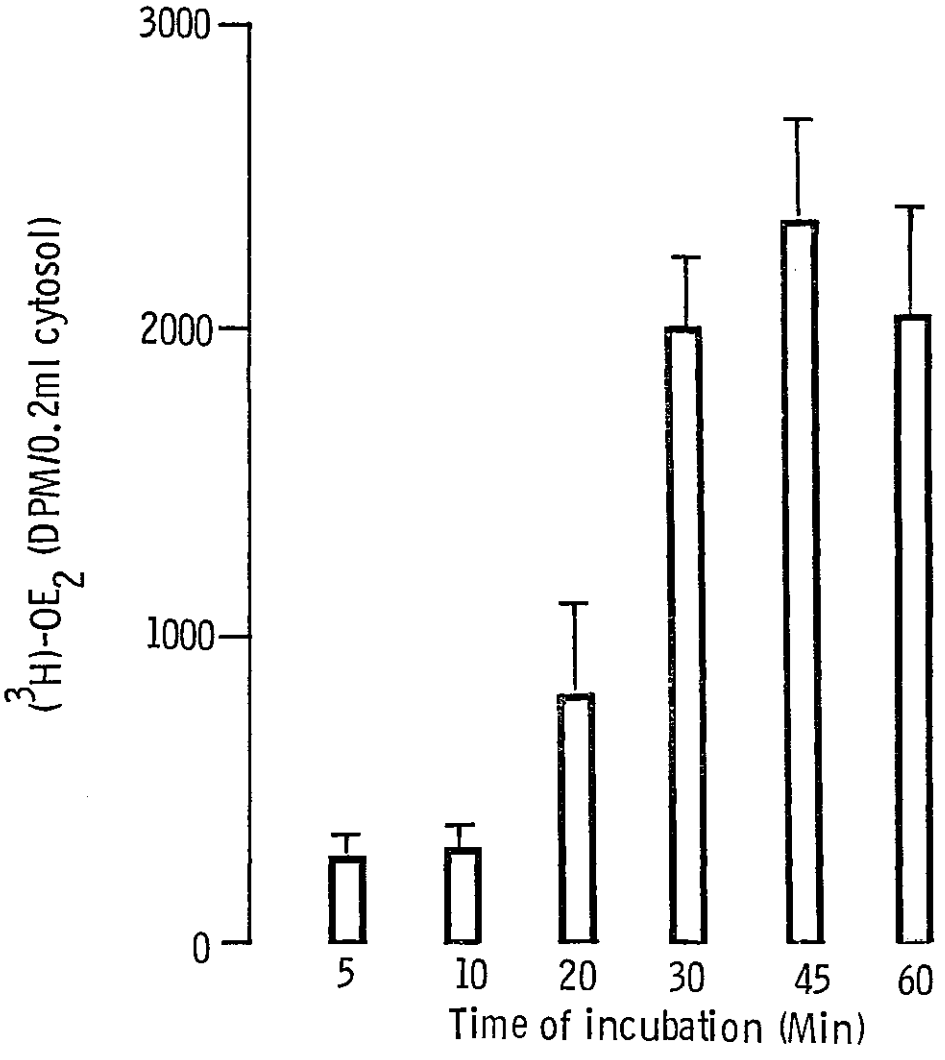


Fig.3.13. Time course of oestrogen receptor binding to spheroidal hydroxylapatite (SHAP).
Cytosol (0.2 ml) was incubated in duplicate on SHAP gels for 5,10,20,30,45 and 60 min at 4°C. Unbound components were washed out of the gels with TED buffer (1ml) and the bound components incubated with 10 nM [^{3}H]-oestradiol in the absence (TC) or presence of 1 µM DES at 4°C for 18 hr. Experiments were repeated three times and each experiment contained 15 rats, aged 28 days. (values = mean ± S.D.)

TABLE 4.

A. Measurement by Sephadex LH-20 Chromatography

	Dpm/0.2ml Cytosol (Mean ± S.D.)		
		Displaceable Counts	Second binder
TC	14452 ± 1270		
+ DES	11234 ± 1542	3232 ± 540	
+ OE_2	4998 ± 610	9469 ± 889	6237 ± 1099
+ DHT	9850 ± 1101	4616 ± 888	
+ DHT/DES	8045 ± 1552	6421 ± 1078	
+ DHT/OE_2	4931 ± 455	9535 ± 927	

B. Measurement by SHAP Chromatography

TC	8175 ± 445		
+ DES	5464 ± 150	2710 ± 488	
+ OE_2	3429 ± 87	4754 ± 388	2043 ± 229
+ DHT	4595 ± 185	3943 ± 780	
+ DHT/DES	5800 ± 575	2374 ± 142	
+ DHT/OE_2	3790 ± 67	4127 ± 325	

Cytosol (0.2ml) was incubated with 10nM $[^3H]$-oestradiol in the absence (TC) or presence of 1 μM DES, OE_2, DHT. DES + DHT, or DHT + OE_2. Macromolecular bound $[^3H]$-oestradiol was assessed either using Sephadex LH-20 chromatography (A) or Spheroidal hydroxylapatite technique (B). The experiments were conducted in duplicate and those performed on Sephadex LH-20 repeated seven times. Measurements on SHAP were repeated three times. Values = Mean ± SD. Each experimental group contained 20 rats, aged 28 days.

granulosa cell cytosol oestrogen binding components. The reduced difference between oestradiol and DES binding observed on SHAP indicates that SOB may have been washed off the gels prior to incubation with [^{3}H]-oestradiol.

3.12 Ammonium Sulphate Fractionation of Granulosa Cell Cytosol

Ammonium sulphate has been used at concentrations varying from 30 to 70% saturation to partially purify oestrogen receptors. Puca *et al.*(1971) used 30% saturation for purification of the calf uterine cytosol oestrogen receptor and Taylor & Smith (1982) used the same concentration to preferentially precipitate chick oviduct progestin and oestrogen receptors. Using 30, 50 and 70% ammonium sulphate fractionation procedures only 3 - 10% recovery of oestrogen receptor was obtained (Table 5).Taylor & Smith (1982) obtained 23 - 39% and Myatt *et al.*(1982) obtained 81 and 53% recovery of the mature rat uterine cytosol oestrogen receptor at 30 and 50% ammonium sulphate saturation. Since all these authors used cytosol containing 3 to 5 mg protein per ml, it seemed likely that the poor yield in the case of the granulosa cell cytosol was the result of its low protein concentration. The cytosol was therefore augmented with a carrier protein BSA, to a final concentration of 2 mg/ml. This led to an improved yield (Table 5). In the presence of the exogenous protein in the unfractionated cytosol only 50% of the receptor present (in the absence of

BSA) could be measured. However, when the cytosols were fractionated at 30, 50 and 70% ammonium sulphate saturation, 110, 152 and 164% yields, respectively, were obtained compared with BSA-augmented control cytosol. Augmenting the cytosol protein concentration with BSA did not alter the level of SOB significantly when compared to the control untreated cytosol. When the BSA-augmented cytosol was fractionated at 30, 50 and 70% ammonium sulphate saturation, 37, 53 and 136% SOB yields respectively, were obtained. Thus in the presence of a sufficient quantity of carrier protein, greater yields of the receptor and SOB was obtained. Moreover, it was possible to demonstrate preferential precipitation of the receptor and SOB with increasing ammonium sulphate concentration until at 70% saturation both were precipitated to about the same extent.

3.13 The Binding of Granulosa Cell Cytosol Oestrogen Binding Species to Concanavalin (Con)-A Sepharose

The finding that the second oestrogen binding species (SOB) of the granulosa cell cytosol bound both oestrogens and androgens suggested that it might be similar or identical to the blood-borne Testosterone-oestradiol binding globulin (TeBG) (Anderson, 1974). A solid phase method used for measurement of serum TeBG binding capacity involves exposure of the plasma to Con-A Sepharose to which the TeBG binds (Nisula et al., 1978). Con-A is a carbohydrate specific adsorbent which binds glycoproteins but not albumin.

Preferential binding of SOB to Con-A Sepharose may have enabled isolation and proper characterisation of the oestrogen receptor to be undertaken in SOB-depleted cytosol. However, both SOB and the oestrogen receptor bound to Con-A Sepharose (87.29 ± 8.72 and 46.28 ± 3.22%, respectively; Table 6a) which was unexpected. The binding of the oestrogen receptor to Con-A Sepharose was confirmed using immature rat uterine cytosol. In this case 87.88 ± 0.87% (n = 3) of the receptor content bound to Con-A Sepharose. Out of the amount that bound to Con-A sepharose, 98.03 ± 1.38 and 87.53 ± 5.57% of the receptor and SOB respectively, could be measured whilst still bound to the gels (Tables 6a and 6b). The Follicular fluid fraction was also incubated with Con-A Sepharose to ascertain whether the SOB present in granulosa cell cytosol was a contaminant from the follicular fluid. As Table 6a shows, the follicular fluid fraction contained only 12.22 ± 0.63% (n = 3) of the SOB concentration found in the granulosa cell cytosol and this possibly was a contaminant from the granulosa cells. This view was strengthened by the finding that DES-specific binding sites were detectable in the follicular fluid which could have been the result of granulosa cells bursting during their removal from the ovaries. These results suggest that both the oestrogen receptor and the second oestrogen binding species might be glycoproteins or may possess carbo-hydrate residues.

TABLE 5. Ammonium Sulphate Fractionation of Granulosa Cell Cytosol and the Effect of the Presence of a Carrier Protein.

Specific [^{3}H] -oestradiol binding

Untreated	Native Cytosol	% yield	Cytosol + 2mg BSA/ml	% yield
OE_2	12201 ± 533	-	7888 ± 1170	-
DES	6368 ± 485	100	3047 ± 512	100
SOB	5833 ± 250	100	4871 ± 704	100
30% $(NH_4)_2SO_4$				
OE_2	348 ± 108	-	4874 ± 83	-
DES	215 ± 92	3.3	3155 ± 170	110.15 ± 20.36
SOB	133 ± 18	2.4	1713 ± 101	36.68 ± 4.90
50% $(NH_4)_2SO_4$				
OE_2	974 ± 90		6824 ± 272	
DES	626 ± 74	10.58 ± 1.54	4365 ± 362	151.87 ± 30.86
SOB	314 ± 67	4.87 ± 1.57	2459 ± 92	52.85 ± 7.22
70% $(NH_4)_2SO_4$				
OE_2	1764 ± 146	-	109.86 ± 380	-
DES	622 ± 67	10.05 ± 1.95	4660 ± 400	163.99 ± 33.60
SOB	1147 ± 122	19.56 ± 1.34	6326 ± 242	135.52 ± 16.55

Dextran-coated charcoal (DCC) treated cytosol was either augmented with 2mg/ml BSA or remained untreated (native cytosol). Portions of these cytosol were then brought to 30,50 or 70% $(NH_4)_2SO_4$ saturation using solid $(NH_4)_2(SO_4$. After intermittent shaking for 1 hour at 4°C, the samples were centrifuged at 20,000 x g for 30min to sediment precipitates. These were then redissolved in the original volume of TED buffer. Aliquots (0.2ml) were incubated with 10nM [^{3}H] -oestradiol in the absence or presence of 1µM OE_2 or DES. Specific binding was assessed on Sephadex LH-20. SOB = OE_2 specific binding - DES specific binding. Determinations were conducted in duplicate, experiments repeated thrice (Values = Mean ± SD). Each experimental group contained 20 rats, aged 28 days.

TABLE 6. Binding of Granulosa Cell Cytosol, follicular fluid fraction and immature rat uterine cytosol oestrogen binding components to Concanavalin-A Sepharose.

A. Specific [^{3}H] -oestradiol/0.2ml cytosol

			% yield
i.	Control cytosol		
	OE_2	16427 ± 1426	-
	DES	8194 ± 922	100
	SOB	7210 ± 400	100
ii.	Con-A Sepharose Treated Cytosol		
	OE_2	6906 ± 392	-
	DES	4387 ± 177	
	SOB	1707 ± 238	
iii.	Calculated loss of OE_2 - binding sites		
	OE_2	10331 ± 1116	-
	DES	3812 ± 689	46.28±3.22
	SOB	6526 ± 1188	87.29±8.72
iv.	Recovery on Con-A Sepharose gels		
	OE_2	9923 ± 160	-
	DES	3791 ± 560	98.03±1.38
	SOB	6132 ± 398	87.53±5.57
v.	Control Follicular Fluid fraction (rFF)		
	OE_2	2240 ± 35	
	DES	1383 ± 75	
	SOB	857 ± 70	
vi.	Con-A Sepharose treated		
	OE_2	1728 ± 143	
	DES	1688 ± 83	
	SOB	Nil	

TABLE 6 (cont) Binding of Immature Rat Uterine Cytosol to Concanavalin-A Sepharose

B.

	Specific [^{3}H]-oestradiol binding/0.2ml cytosol	
		% yield
i. Control cytosol	8433 ± 1360	100
ii. Con-A Sepharose Treated cytosol	1013 ± 88	87.88 ± 8.87
iii. Recovery on Con-A	5571 ± 36	67.19 ± 10.55

Cytosol or follicular fluid fraction was incubated with 50% slurries of Con-A Sepharose for 60min at 4°C. Aliquots (0.2ml) were then incubated with 10nM [^{3}H]-oestradiol in the absence (TC) or presence of 1μM DES or OE_2 for 18 hr at 4°C. Specific binding was assessed using Sephadex LH-20 to separate macromolecular bound [^{3}H]-oestradiol from free steroid. The Con-A Sepharose gels were washed with TED buffer and 0.2ml aliquots of the slurry incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2 for 18 hr at 4°C. Free unbound radioactivity was washed off the gel with TED buffer and TED buffer containing 1% BSA (see Materials and Methods). Determinations were conducted in duplicate. Values = Mean ± SD for three experiments. Each experimental group contained 15 rats, aged 28 days.

3.14 The Effect of Chaotropic Salts on Binding of [^{3}H]-oestradiol in Granulosa Cell Cytosol

Sato et al.(1981) reported the presence of a Mouse Leydig Cell tumour cytosol component which bound oestradiol but not DES. Exposure of this cytosol to a chaotropic salt (0.4M NaSCN) converted the species to a form which then bound both oestradiol and DES with high affinity. Chaotropic salts act by breaking protein - protein bonds in aggregated molecules and it appeared that NaSCN exposed binding sites which hitherto were masked. It was considered that the SOB might have been a receptor with masked DES-binding sites. Therefore granulosa cell cytosol was exposed to 0.15M or 0.4M NaSCN, 0.15M or 0.4M KCl and then incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2 for 18 hr at 4°C. No significant alteration in the concentration of oestrogen binding was observed for either the receptor or SOB (Table 7). Thus either all oestrogen binding sites were already exposed or if masked binding sites were present, they were apparently resistant to the action of the chaotropic salts.

3.15 The Effect of Protease and Protease Inhibitors on Binding of [^{3}H]-oestradiol in Granulosa cell cytosol

To examine whether the second oestrogen binding species was a product of endogenous proteolysis during cytosol preparation resulting in a subsequent alteration of steroid specificity, the granulosa cell cytosol was exposed to 100μg/ml of protease, or protease inhibitors. The cytosol was then incubated

for 18 hr at 4°C with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2. As illustrated in Fig 3.14 the presence of the protease inhibitor phenylmethylsulphonylfluoride (PMSF) caused 55 and 50% loss of the receptor and the second oestrogen binder respectively. The protease inhibitor bacitracin however enhanced receptor binding by 86% but did not significantly alter the concentration of SOB. Trypsin increased receptor and SOB binding by 176 and 93% respectively. Agarwal & Paillard (1979) observed that protease inhibitors as well as proteases increased oestrogen binding in the male liver cytosol. Petterson et al.(1982) also observed that trypsin increased oestrogen receptor binding in breast cancer tissue cytosol.

3.16 The Effect of Sodium Molybdate on Binding of [^{3}H]-oestradiol in Granulosa Cell cytosol

Moncharmont et al.(1982) found that sodium molybdate prevented the formation of receptor aggregates in the presence of oestradiol. To find out if receptor aggregation in the granulosa cell cytosol might involve masking of the steroid binding site, the cytosol was prepared in TED buffer containing 20mM Sodium molybdate. The cytosol was then incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2 for 18 hr at 4°C. No significant increase in oestrogen receptor or SOB binding of [^{3}H]-oestradiol was observed (Table 7). This indicated that any aggregation that might be formed in vitro probably

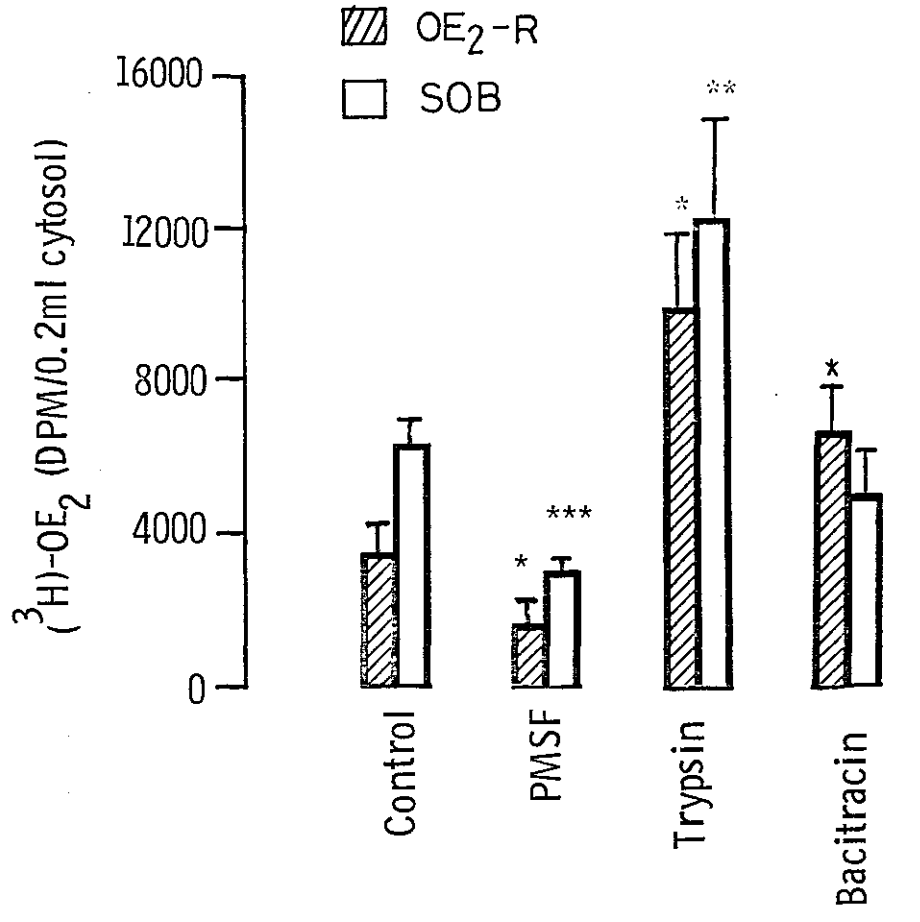


Fig.3.14. Effect of protease and protease inhibitors on [^{3}H]-oestradiol binding in granulosa cell cytosol. Cytosol (0.2ml), pretreated with 100 µg/ml of phenylmethylsulphonyl-fluoride(PMSF), trypsin or bacitracin, was incubated with 10 nM [^{3}H]-oestradiol with or without 1 µM DES or OE_2 for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was determined by Sephadex LH-20 chromatography. DES-suppressible counts = receptor, OE_2- suppressible - DES-suppressible counts = SOB. Experiments were repeated three times (values = mean ± S.D.) and each experimental group contained 15 rats, aged 28 days.

(*) significantly different from control ($p<0.01$)
(**) significantly different from control ($p<0.02$)
(***) significantly different from control ($p<0.001$)

Table 7. Effect of NaSCN, KCl and $NaMoO_4$ on binding of $[^3H]$- oestradiol to granulosa cell cytosol oestrogen binding components.

	Specific $[^3H]$- oestradiol Binding (d.p.m./0.2ml cytosol)		
	$+OE_2$	+DES	SOB
Control cytosol	9254 ± 1039	3481 ± 667	5774 ± 947
+ NaSCN (0.15M)	8419 ± 871	3229 ± 884	5190 ± 893
+ NaSCN (0.4M)	9052 ± 919	3848 ± 829	5204 ± 785
+ KCl (0.15M)	8557 ± 1129	3069 ± 1003	5489 ± 1313
+ KCl (0.4M)	8878 ± 1131	3876 ± 1003	5002 ± 903
+ $NaMoO_4$ (20mM)	9482 ± 972	3782 ± 507	5686 ± 602

Cytosol (0.2ml) containing 0.15M or 0.4M NaSCN, 0.15 or 0.4M KCl or 20mM $NaMoO_4$ was incubated with 10nM $[^3H]$-oestradiol with or without 1μM DES or OE_2 for 1 hr at 4°C. Specific binding (oestrogen receptor and SOB) was assessed using Sephadex LH-20 chromatography. Determinations were conducted in duplicate and each experiment repeated three times (Values = Mean ± S.D.). Each experimental group contained 20 rats, aged 28 days.

did not involve either the thiol group or the steroid binding site (Ikeda, 1982) at which the molybdate ion might act.

3.17 Thermal Stability of the Cytosol Oestrogen Binding Species

The thermal stability of the oestrogen receptor-oestradiol interaction is well documented and so experiments were performed to establish if the granulosa cell cytosol receptor and the scond oestrogen binding species could be distinguished on the basis of their ability to resist thermal denaturation.

Granulosa cell cytosol was prelabelled with 10nM [^{3}H]-oestradiol for 18 hr at 4°C in the presence or absence of 1μM DES or OE_2 and then subsequently heated for 30 min at 37°, 55° and 65°C. The results are illustrated by the histograms in Fig 3.15. The oestrogen receptor was found to be completely denatured by exposure to temperatures of 65°C for 30 min. This is consistent with the observation of Myatt et al.(1982) for the mature rat uterine cytosol oestrogen receptor and with other reports (Puca et al., 1971; King & Mainwaring, 1974). The interaction of SOB with [^{3}H]-oestradiol however, was more stable and under similar conditions (65°C, 30 min) 30% of the control concentrations were measured. Sudo et al. (1983) have described an anti-oestrogen binding protein in the ovary as well as other oestrogen target tissues which resisted thermal degradation at temperatures up to 100°C. The occupied oestrogen

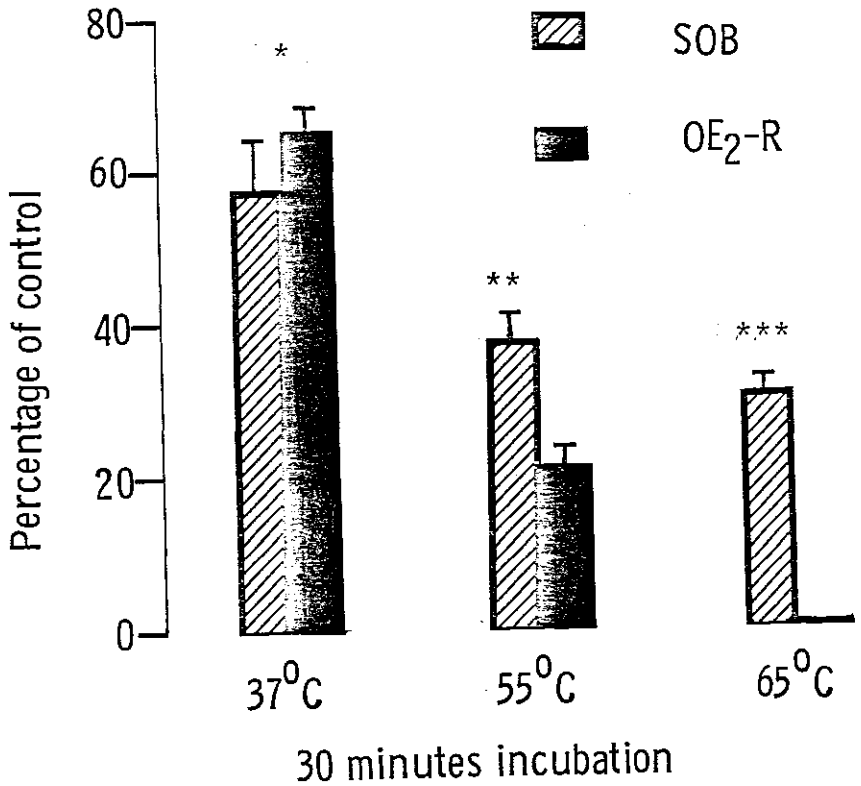


Fig.3.15. Effect of incubating granulosa cell cytosol oestrogen binding species at elevated temperatures. Cytosol (0.2 ml) was incubated with 10 nM [^{3}H]-oestradiol in the presence or absence of 1 μM DES or OE_2 for 18 hr at 4°C (= control binding). In addition some samples were heated at 37,55 or 65°C for 30 min prior to chromatography on Sephadex LH-20. DES-suppressible counts = receptor(OE_2-R) and SOB = OE_2 - DES-suppressible counts. Control binding = 100%. Each determination was conducted in duplicate and the experiment repeated three times(values = mean ± S.D.)(*) statistically, no differential effect on SOB/OE_2-R, (**) is significant at $p<0.05$ and (***) is significant at $p<0.001$. (Students' t-test).

binding species in the cytosol of the granulosa cell could therefore be distinguished on the basis of the relative thermal stabilities of their interaction with [^{3}H]-oestradiol.

3.18 Kinetic Studies of the Receptor-/SOB-Oestradiol Interaction

As the oestrogen receptor and the second oestrogen binding species were shown to have different equilibrium dissociation constants (Kd), association and dissociation rates were studied to find out if these could also offer further parameters for distinguishing between these two oestrogen binding species.

3.18.1 Association Rate Measurements

Granulosa cell cytosol was incubated with [^{3}H]-oestradiol at 0-4°C or 25°C and the specific oestradiol and DES-suppressible binding assessed at 15 min intervals for up to 120 minutes. From this data the concentration of the oestrogen receptor and SOB was determined. At 0°C, the apparent association rate constant (Ka) determined from the slopes of the second-order plots was $7.2 \pm 0.25 \times 10^7$/mol/hr (n = 3) for the oestrogen receptor which agrees with that for the pituitary and hypothalamic oestrogen receptor (Clark et al., 1982). The Ka for the second oestrogen binding species was $1.72 \pm 0.38 \times 10^8$/mol/hr (n = 3) (Fig 3.16). At 25°C the Ka for the receptor was $1.23 \pm 0.26 \times 10^8$/mol/hr (n = 3) and that for the second binder was $4.5 \pm 0.62 \times 10^8$/mol/hr (n = 3) (Fig 3.17).

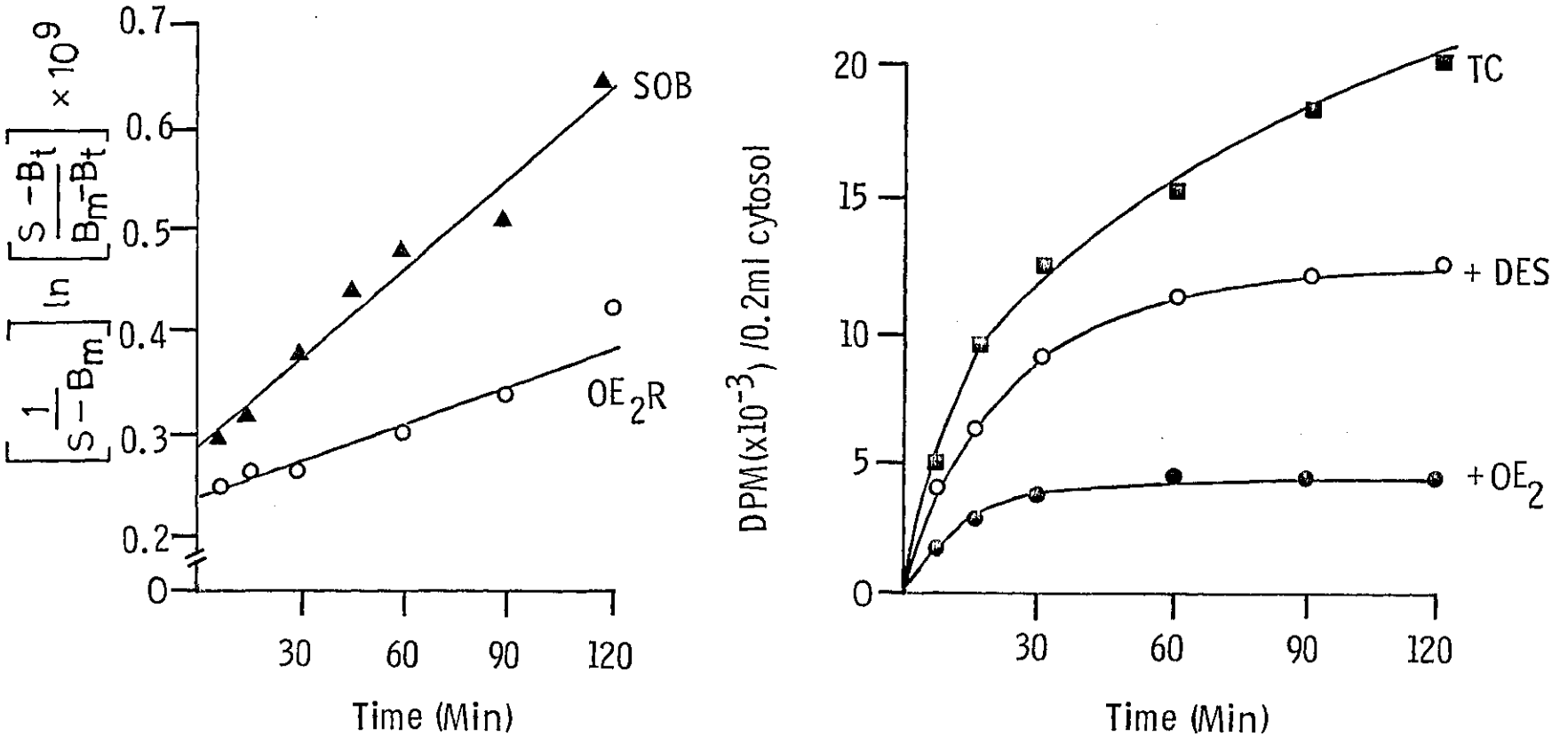


Fig.3.16. Measurement of association rates of SOB and oestrogen receptor with [^{3}H]-oestradiol at 0-4°C. DCC-treated cytosol was incubated with 10 nM [^{3}H]-oestradiol in the absence(TC) or presence of 1 μM DES or OE_2 at 0-4°C. Duplicate aliquots were taken at 10,15,30,60, 90 and 120 min. The DES- and OE_2-suppressible binding was assessed using Sephadex LH-20 chromatography. SOB binding = OE_2 - DES specific binding at each time point.

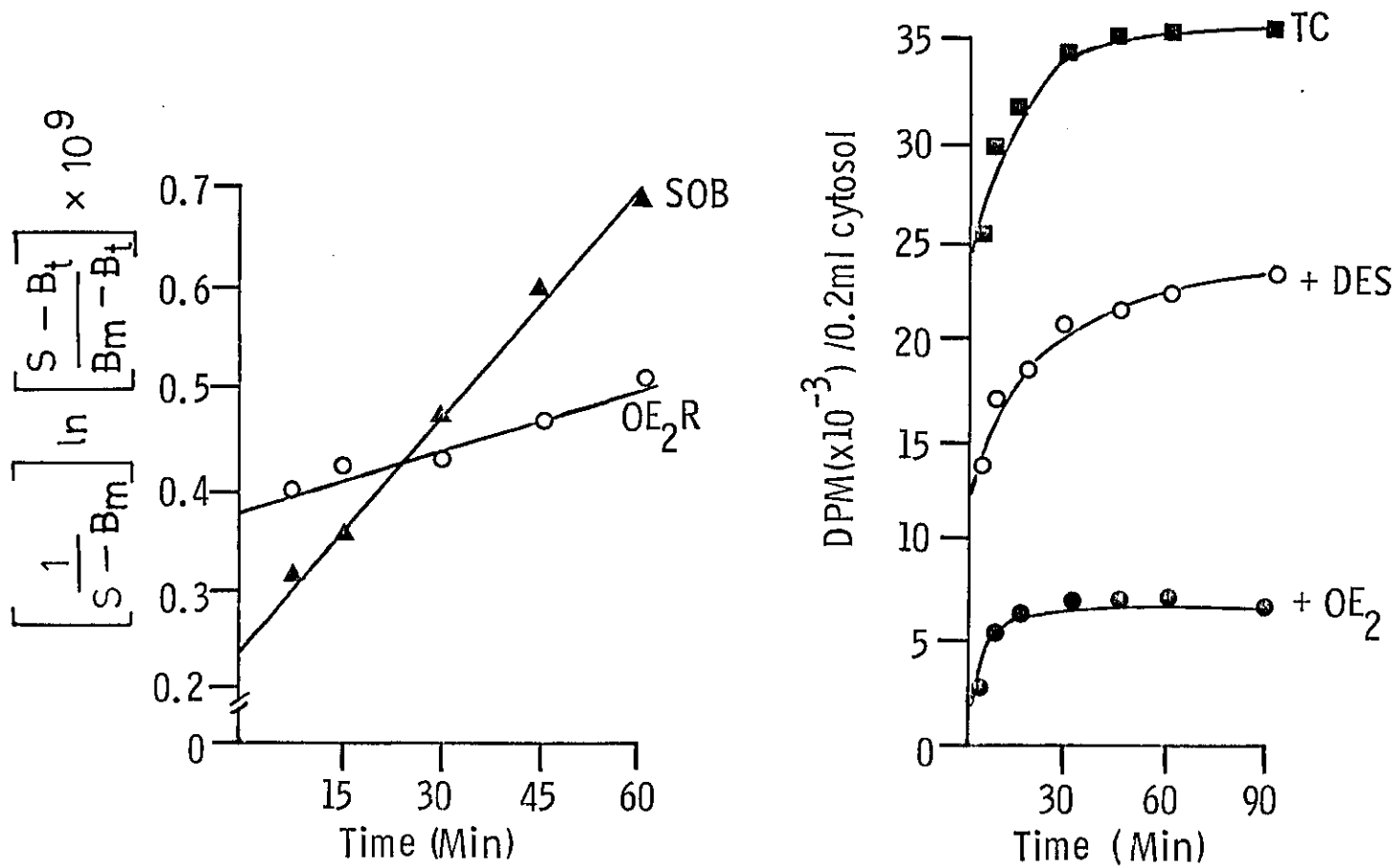


Fig.3.17. Measurement of association rates of SOB and oestrogen receptor with [^{3}H]-oestradiol at 25°C. DCC-treated cytosol was incubated with 10 nM [^{3}H]-oestradiol in the absence(TC) or presenceof 1 μM DES or OE_2 at 25°C. Duplicate aliquots were taken at 10,15,20,30, 45,60 and 90 min. The DES- and OE_2-suppressible binding was assessed using Sephadex LH-20 chromatography. SOB = OE_2 - DES specific binding at each time point.

3.18.2 Dissociation Rate Measurements

The dissociation of [^{3}H]-oestradiol from both DES-suppressible and oestradiol-suppressible binding sites at 25°C was a biphasic phenomenon which followed two first-order kinetics. The initial rapid phase of dissociation was followed by a slower second phase. (Fig 3.18). To calculate the true rate constants of the dissociation process, the contribution of the slow dissociating form was subtracted from the rapid dissociating component (Weichman & Notides, 1977).

3.18.2a Dissociation of [^{3}H]-oestradiol from DES-Suppressible Binding Sites at 25°C

X-ray analysis has shown that oestrogens such as oestradiol and diethylstilboestrol (DES) exhibit the same coefficient of binding to the oestrogen receptor (Hospital et al., 1975). Because DES does not bind to plasma proteins such as albumin and alpha-fetoprotein, it has been preferred to oestradiol for the mesurement of oestrogen receptor. The dissociation rate constant (K_{-1}) of the rapid dissociating component at 25°C was found to be 0.30 ± 0.07 min^{-1} using a 1000 fold excess of DES to initiate dissociation of [^{3}H]-oestradiol from DES-suppressible binding sites, previously incubated with 10nM [^{3}H]-oestradiol. This rapid dissociating component had a half-life ($t_{\frac{1}{2}}$) of 2.5 ± 0.5 min and comprised about 70% of the receptor content. The slow dissociating component had a rate constant (K_{-2}) of 3.73 ± 0.57 x 10^{-3} min^{-1} and a half-life of 59.30 ± 1.15 min (n = 3) at 25°C

(Fig 3.18A). Incubation of the cytosol at 25°C for 30 min to activate the oestrogen receptor prior to dissociation at 25°C reduced the magnitude of the rapid dissociating component to less than 25% of the receptor content (Fig 3.20B). After subtracting the contribution of the second component, the first component (the rapid dissociating form) could not be accurately determined due to the small number of time points. The rate constant of the second component was found to be $4.22 \pm 0.32 \times 10^{-3}$ min $^{-1}$ with a half-live of 62 ± 25 min (n = 3). These values were found not to be significantly different from those obtained for the second component in the 'non-activated' cytosol.

Pre-incubation of the cytosol with 20nM [^{3}H]-oestradiol had no effect on the rate constants or half-lives when dissociation was performed at 25°C.

3.18.2b Dissociation of [^{3}H]-oestradiol from Oestradiol-Suppressible Binding sites (SOB + Oestrogen Receptor) at 25°C

In the experiments described above, dissociation of [^{3}H]-oestradiol from DES-suppressible (oestrogen receptor) sites was initiated using a 1000 fold excess of cold DES. To find out whether the presence of the second oestrogen binding species in the cytosol would have any effect on the rate of dissociation of [^{3}H]-oestradiol from the receptor, the cytosol was pre-incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM OE_2 for 18 hr at 4°C. Dissociation

at 25°C was initiated using a 1000 fold excess of oestradiol, which in theory would cause dissociation of [^{3}H]-oestradiol from both the receptor and SOB. The apparent dissociation rate constant for the rapid dissociating component (K_{-1}) at 25°C was found to be 0.23 ± 0.05 and the half-life ($t_{\frac{1}{2}}$) was 3.16 ± 0.65 min (n = 3). This component comprised 50% of the oestradiol-suppressible binding. The slow dissociating component had a rate constant (K_{-2}) of 3.07 ± 1.34 x 10^{-3} min^{-3} and a half-life ($t_{\frac{1}{2}}$) of 66.67 ± 3.2 min (n = 3) (Fig 3.19A). Comparison of these values with those obtained when [^{3}H]-oestradiol was dissociated from DES-suppressible sites with DES shows that the presence of SOB does not alter the rate of [^{3}H]-oestradiol dissociation from the oestrogen receptor. In fact [^{3}H]-oestradiol dissociation from OE_2-suppressible sites still followed a biphasic curve.

Warming the reaction mixture at 25°C for 30 min to activate the oestrogen receptor prior to dissociation at 25°C did not alter the magnitude of the fast dissociating component (Fig 3.19B). When the oestrogen receptor was studied (DES-suppressible sites dissociated with DES) under idential conditions, a 50% reduction of the magnitude of the fast component was observed (Fig 3.18B) representing a significant difference ($p < 0.001$) due to the presence of SOB. The rate constant (K_{-2}) obtained was 3.72 ± 0.68 x 10^{-3} min^{-1} and the half-life ($t_{\frac{1}{2}}$) was 63.52 ± 6.3 min (n = 3). These values were not significantly different from

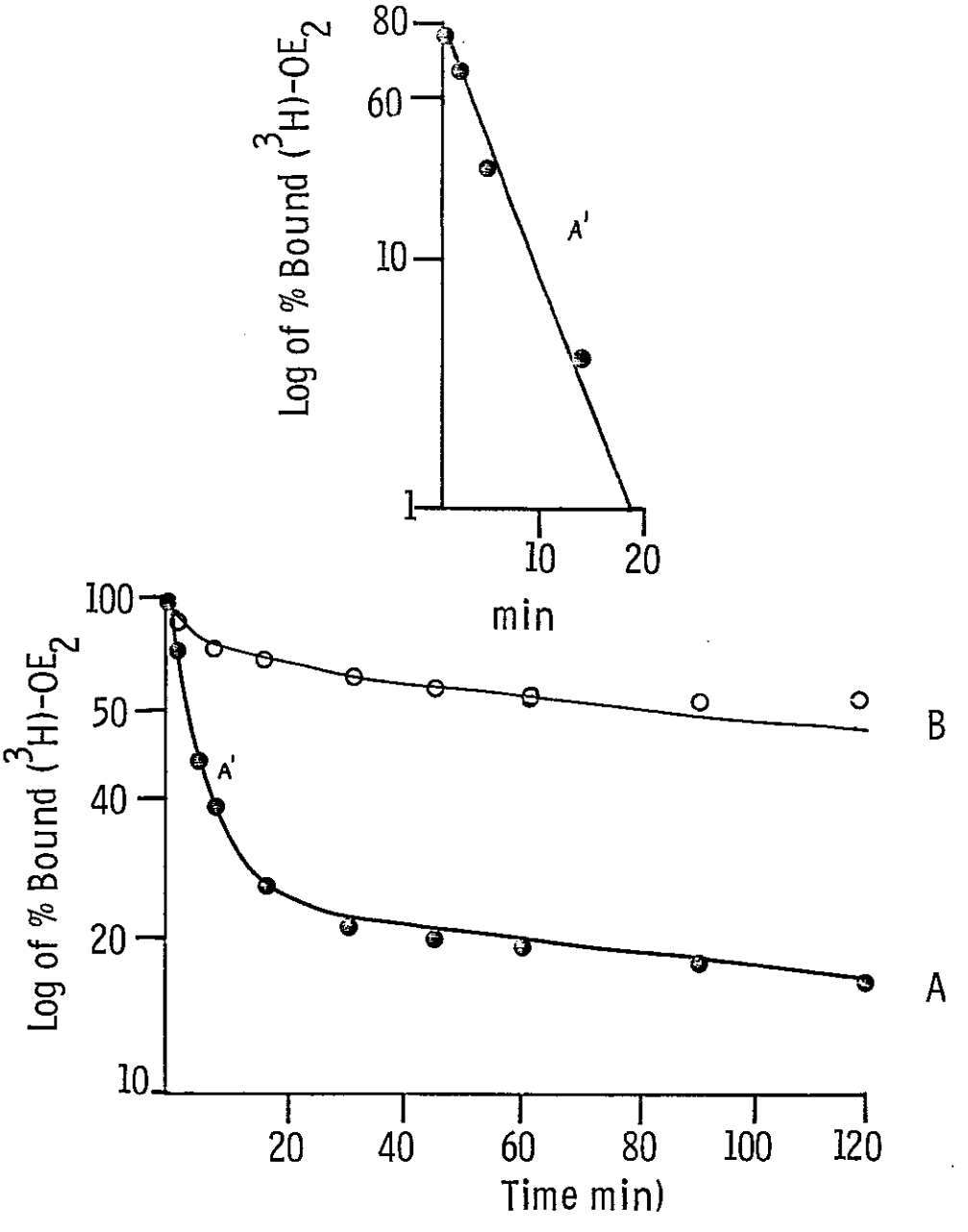


Fig.3.18. Dissociation of [^{3}H]-oestradiol from oestrogen receptor in the granulosa cell cytosol.
DCC-treated cytosol was incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1µM DES for 18 hr at 4°C only (A) or plus 30 min at 25°C (B). Macromolecular bound [^{3}H]-OE$_2$ was separated from free steroid on Sephadex LH-20 gels. Dissociation of [^{3}H]-OE$_2$ from the receptor (DES-suppressible sites) was initiated by adding 10uM DES at 25°C. Duplicate aliquots (0.2ml) were taken at times 0,1,3,7,15,30,45, 60,90 and 120min. [^{3}H]-OE$_2$ binding at time 0 = 100%. Rate constants were determined from the slopes of the dissociation curves (see section 2.24.2)

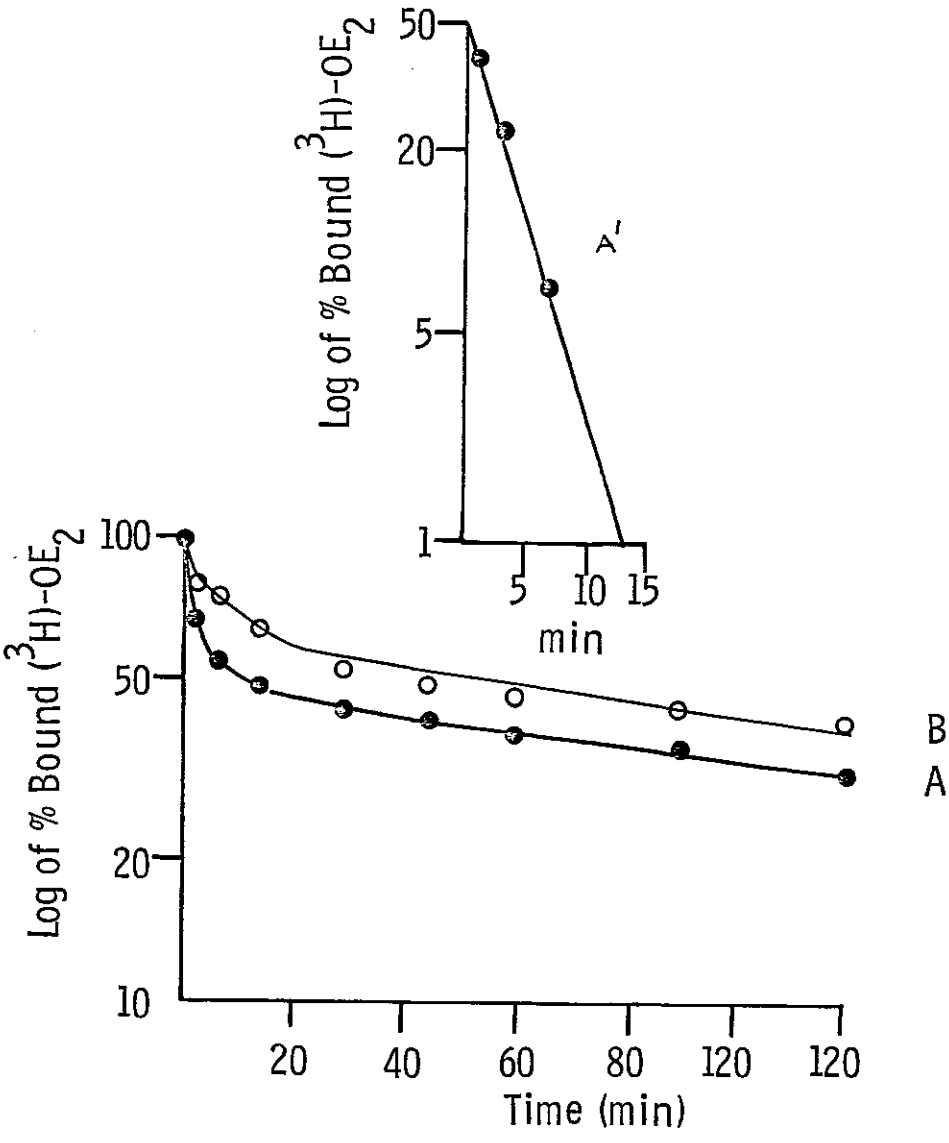


Fig.3.19. Dissociation of [^{3}H]-oestradiol from OE_2-suppressible sites in the granulosa cell cytosol. DCC-treated cytosol was incubated with 10nM [^{3}H]-OE_2 in the presence or absence of 1μM OE_2 for 18 hr at 4°C only (A) or plus 30 min at 25°C (B). Macromolecular bound [^{3}H]-OE_2 was separated from free steroid on Sephadex LH-20 gels. Dissociation of [^{3}H]-OE_2 from oestrogen binding sites was initiated by addition of 10μM OE_2 at 25°C. Duplicate 0.2ml aliquots were taken at times 0,1,3,7,15,30,45,60,90 or 120 min. [^{3}H]-OE_2 binding at time 0 = 100%. Rate constants were calculated from the slopes of the dissociation curves(see section 2.24.2).

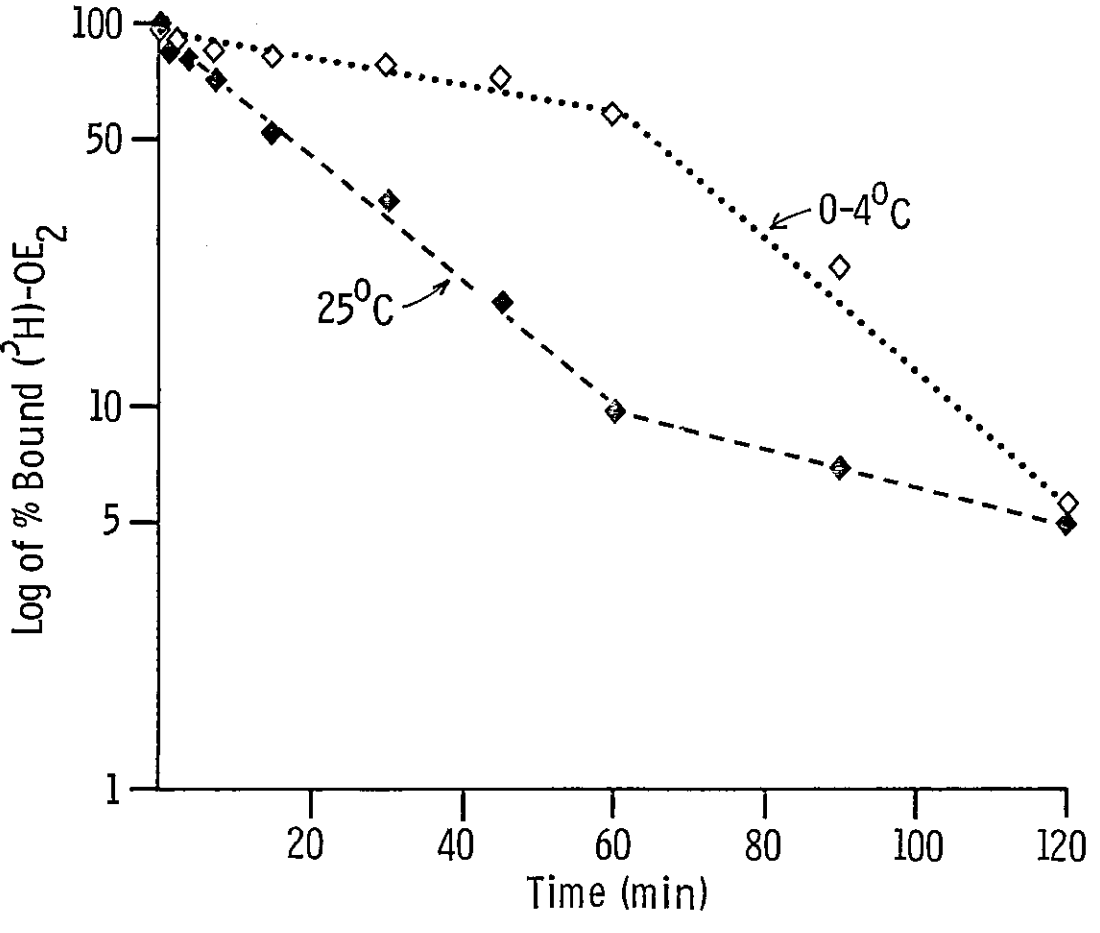


Fig.3.20. Dissociation of [^{3}H]-oestradiol from the oestrogen receptor of immature rat uterine cytosol. The cytosol was labelled with 10 nM [^{3}H]-OE$_2$ in the presence or absence of 1 μM DES at 4^{0}C for 18 hr. Macromolecular bound [^{3}H]-OE$_2$ was separated from free steroid using Sephadex LH-20 chromatography. Dissociation of [^{3}H]-OE$_2$ from the oestrogen receptor was determined at 25^{0}C and at 0-4^{0}C by adding 10 uM DES. Duplicate aliquots(0.2ml) were taken at times 0,1,3,7,15,30,45,60,90 and 120 min. [^{3}H]-OE$_2$ binding at 0 = 100%. Rate constants were determined from the slopes of the curves(see section 2.24.2).

those obtained for the second component present in 'non-activated' cytosol. Again the rate constant of the first part of the curve (i.e. the rapid dissociating component) could not be accurately determined.

The inclusion of the second oestrogen binding species in the dissociation kinetic studies therefore appeared to have an effect only on the proportion of fast and slow components measured in the cytosol.

3.18.2c Dissociation Kinetics of Immature Rat Uterine Cytosol Oestrogen Receptor at 25°C

Dissociation kinetic studies were also performed at 25°C using immature rat uterine cytosol receptor pre-labelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES for 18 hr at 4°C. This was to ascertain whether there were any similarities with the granulosa cell cytosol receptor dissociation kinetics. At 25°C, [^{3}H]-oestradiol dissociation occurred in two phases: the rate constant of the rapid dissociating component (K_{-1}) was 0.13 ± 0.02 and half-life ($t_{\frac{1}{2}}$), 5.43 ± 0.81 min (n = 3); The rate constant of the second component (K_{-2}) was 4.20 ± 0.18 x 10^{-3} min^{-1} with a half-life ($t_{\frac{1}{2}}$) of 70 ± 9.20 min (n = 3) (Fig 3.20). Comparison of these values with those of the granulosa cell cytosol showed that [^{3}H]-oestradiol dissociation from the fast dissociating component (K_{-1}) of granulosa cell cytosol oestrogen receptor was twice as rapid as that from the immature

rat uterine cytosol oestrogen receptor. All other kinetic parameters were identical for both the granulosa cell and immature rat uterine cytosol receptors.

3.18.2d Dissociation Kinetics of the Granulosa Cell Oestrogen Binding Species at 0-4°C

When the dissociation kinetic studies were performed at 25°C, using either DES or OE_2 to initiate dissociation of [^{3}H]-oestradiol, no unique differences were found which could be attributed to SOB. It was considered that because SOB is a low affinity binder, it is likely that [^{3}H]-oestradiol would dissociate more rapidly from it than from the oestrogen receptor at elevated temperatures. The dissociation studies were therefore conducted at 0 - 4°C with the idea of establishing (i) if the slow dissociating form was already present at the start of the experiment or was formed as the experiment progressed at 25°C, (ii) if a unique dissociation curve could be found which would be attributable to SOB - [^{3}H]-oestradiol dissociation.

Dissociation of [^{3}H]-oestrdiol from DES-suppressible sites (prelabelled with 10nM [^{3}H]-oestradiol) using a 1000 fold excess of DES at 4°C showed that 30% of the [^{3}H]-oestradiol present at the beginning of the experiment dissociated within three minutes (Fig 3.21). Thereafter the [^{3}H]-oestradiol-oestrogen receptor dissociation became slow with a rate constant 5.79 ± 1.31 x 10^{-3} min^{-1}, until after 45 min when rapid

dissociation occurred. The rate constant of this rapid dissociation of [^{3}H]-oestradiol was 0.12 ± 0.02 min and complete dissociation was achieved at 90 minutes. As can be seen from Fig 3.21, dissociation of [^{3}H]-oestradiol from OE_2-suppressible sites using a 1000 fold excess of OE_2 at 4°C was also initially rapid, losing about 20% radioactivity within the first three min. The SOB-oestradiol receptor - [^{3}H]-oestradiol dissociation then became slow with a rate constant of $4.06 \pm 1.76 \times 10^{-3}$ min^{-1} until after 60 min when [^{3}H]-oestradiol dissociation hastened to a rate of 0.086 ± 0.028 min^{-1}. Complete dissociation of [^{3}H]-oestradiol occurred by 120 minutes. The rapid dissociation of [^{3}H]-oestradiol from the oestrogen receptor at 0 - 4°C after 45-60 min from DES - and OE_2 - suppressible binding sites were idential and might be a reflection of cooperative binding interaction. The dissociation phenomenon appears to go in the opposite direction at low temperatures i.e. the loss of [^{3}H]-oestradiol enhancing rapid dissociation from other sites. No significant differences were observed in the rate constants of the slow dissociating forms of both DES - and OE_2-suppressible sites at 0 - 4°C.

Pre-labelling the OE_2 - and DES - suppressible sites with higher concentrations of [^{3}H]-oestradiol (20nM or more) somehow stablized the receptor -, SOB -[^{3}H]-oestradiol complexes so that a slow monophasic dissociation of [^{3}H]-oestradiol, with rate constant $2.56 \pm 0.86 \times 10^{-3}$ min^{-1}, was obtained at 0 - 4°C.

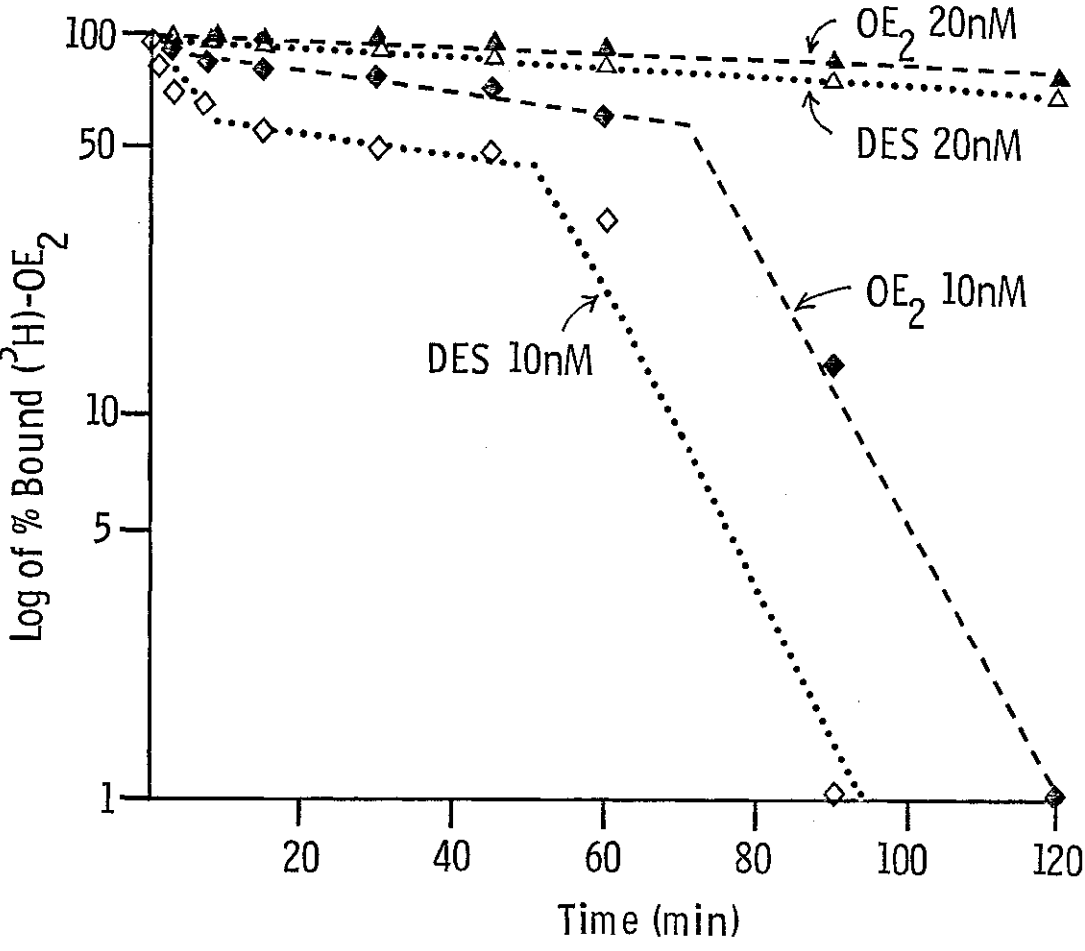


Fig.3.21 Dissociation of [^{3}H]-OE$_2$ from oestrogen binding sites of the granulosa cell cytosol at 0-4°C. DCC-treated cytosol was labelled with 10 or 20 nM [^{3}H]-OE$_2$ in the presence or absence of 1 or 2 μM DES or OE$_2$ at 4°C for 18 hr. Macromolecular bound [^{3}H]-OE$_2$ was separated from free steroid using Sephadex LH-20 chromatography. Dissociation of [^{3}H]-OE$_2$ from oestrogen binding sites was initiated by adding 10 or 20 μM DES or OE$_2$ at 0-4°C. Duplicate aliquots (0.2ml) were taken at times 0,1,3,7,15,30, 45,60,90 and 120 min. [^{3}H]-OE$_2$ binding at time 0 = 100%. Rate constants were determined from slopes of the curves(see section 2.24.2).

3.18.2e Dissociation Kinetic of the Immature Rat Uterine Cytosol Receptor at 0 - 4°C

Immature rat uterine cytosol was pre-labelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES and the DES- suppressible binding dissociated with a 1000 fold excess of DES at 0 - 4°C. The dissociation of [^{3}H]-oestradiol was initially slow with a rate constant of $3.91 \pm 0.52 \times 10^{-3}$ min^{-1}.(Fig 3.20) The rate constant of the fast dissociating form after 60 min was 0.036 ± 0.012, which was significantly different ($p < 0.001$) from the fast dissociating form of the granulosa cell cytosol oestrogen receptor at 0 - 4°C. Only 8% [^{3}H]-oestradiol remained associated with the oestrogen receptor by 120 minutes. This is in agreement with the observation made with the granulosa cell cytosol oestrogen binding species at 0 - 4°C labelled with 10nM [^{3}H]-oestradiol.

3.19 Intracellular Movement of Receptor in Granulosa Cells

Competition studies in granulosa cell nuclear suspension revealed that both oestradiol (OE_2) and DES were equally effective in displacing [^{3}H]-oestradiol from the nuclear oestrogen receptor (Table 2). Scatchard analysis revealed the presence of only one class of oestrogen binding sites in the granulosa cell nuclei (Fig 3.4) i.e. the oestrogen receptor. It was therefore hoped that translocation of the oestrogen receptor from the cytosol leaving the SOB in that fraction would enable proper characterization of SOB. Saiduddin & Zassenhaus (1977) however, observed no

significant degree of translocation of oestrogen receptor in the ovary of intact immature rats injected with 10µg oestradiol. At this dose, complete translocation of the oestrogen receptor was obtained in the uterus. Thus it appeared that a factor(s) which militated against translocation resided in the ovary, possibly in the granulosa cells. An attempt was therefore made to study the intracellular movement of the receptor in the granulosa cells.

3.19.1 Whole Cell Uptake of [^{3}H]-oestradiol In Vitro

Whole granulosa cells were incubated with 5, 10 or 20nM [^{3}H]-oestradiol in the presence or absence of a 100 fold excess of testosterone or DES at 4°, 25° or 37°C. Unbound [^{3}H]-oestradiol was washed off the cells using 1% BSA in TED buffer and the bound [^{3}H]-oestradiol counted. It was observed that [^{3}H]-oestradiol uptake into the granulosa cells increased with increasing temperature and with increasing concentration of [^{3}H]-oestradiol (Table 8). However, the uptake of [^{3}H]-oestradiol was extremely low. It could also be observed that tesosterone competed as efficiently as DES for [^{3}H]-oestradiol binding in the granulosa cells.

3.19.2 In vitro Translocation of the Oestrogen Receptor

As only minimal [^{3}H]-oestradiol was taken up by the granulosa cells, it was postulated that high local concentration of steroids in the granulosa cell may perhaps have militated against exchange for [^{3}H]-oestradiol on available oestrogen binding sites or

may have prevented [^{3}H]-oestradiol diffusion against a concentration gradient. In an attempt to identify and overcome these influences, granulosa cells were next incubated with 10nM cold oestradiol or DES or with no steroid at all for 1 hr at 37°C. Another portion of the granulosa cells was left on ice for 1 hr without any steroid treatment prior to analysis for oestrogen receptors in both the cytosol and nuclear fractions. The cytosol was treated with DCC to remove endogenous steroid before estimation of the receptor content with 10nM [^{3}H]-oestradiol in the presence or absence of 1µM DES. No depletion of the cytosol receptor was observed after incubating the cells with either 10nM DES or OE_2 at 37°C. Rather, OE_2 induced over a 100% increase in cytosol receptor and over a 200% decrease of the nuclear oestrogen receptor (Fig 3.22). No significant alteration of the cytosol or nuclear receptor content of the cells was observed after incubation with DES. Incubation of the cells at 37°C in the absence of steroid led to a 38% reduction of the cytosol oestrogen receptor content over the receptor concentration at 4°C ($p < 0.005$). This decrease might be due to thermal degradation of the cytosol receptor. Thus no positive translocation (i.e. depletion of cytosol receptor and a corresponding accumulation of nuclear receptor) was observed and this might be a reflection of the minimal oestrogen uptake into the cells (see section 3.19.1).

TABLE 8. Granulosa Cell Uptake of [^{3}H] -oestradiol in vitro

[^{3}H] -oestradiol concentration		Specific [^{3}H] -oestradiol uptake (DpM/10^6 cells) 4°C	25°C	37°C
5nM	± 0.5µM DES	0	86 ± 38	92 ± 22
	± 0.5µM testosterone	0	80 ± 47	103 ± 50
10nM	± 1.0µM DES	0	126 ± 42	133 ± 28
	± 1.0µM testosterone	0	177 ± 48	156 ± 62
20nM	± 2.0µM DES	37 ± 13	116 ± 30	173 ± 48
	± 2.0µM testosterone	58 ± 32	186 ± 88	206 ± 42

Granulosa cells were incubated with 5, 10 or 20nM [^{3}H] -oestradiol in the presence or absence of a 100 fold excess of testosterone or DES at 4, 25 or 37°C. The cells were processed for radioactivity counting as described in the materials and methods. Determinations were made in triplicate and the experiment repeated three times (Values = Mean ± S.D.). The granulosa cells were isolated from rats aged 24-28 days.

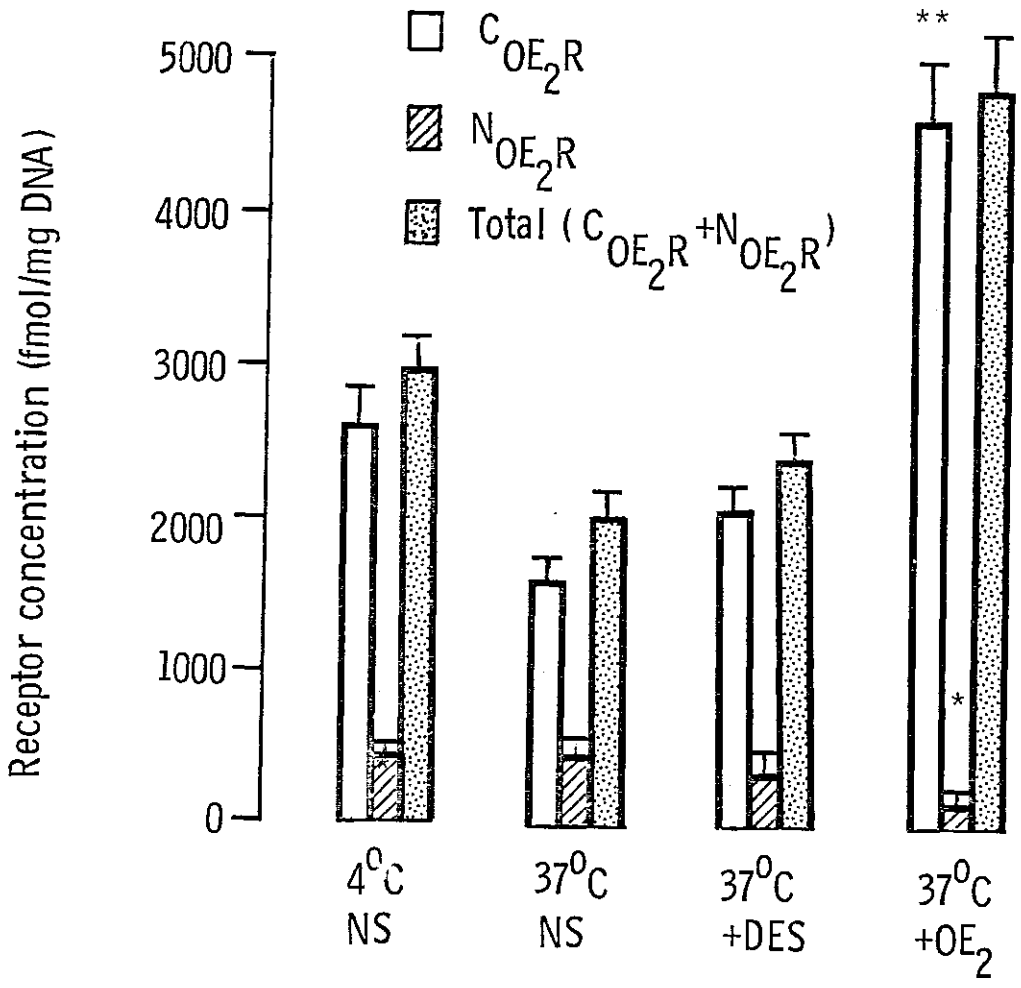


Fig.3.22. Oestrogen receptor translocation in granulosa cells in vitro. Isolated granulosa cells were incubated with 10 nM OE_2 or DES at 37°C or without steroid (NS) for 1 hr at 4 & 37°C Duplicate DCC-treated cytosol aliquots(0.2ml) and triplicate aliquots(0.2ml) of the nuclear fraction were incubated with 10 nM [^{3}H]-OE_2 in the presence or absence of 1 μM DES to estimate oestrogen receptor content(see materials and methods). Experiments were repeated three times(values = mean ± S.E.M.). Granulosa cells were isolated from rats aged 28 days. Each cell contains approx. 8pg DNA.

(*) significantly different from 37°NS($p<0.001$)

(**) significantly different from 4°C,NS;37°C,NS and 37°C,DES ($p<0.001$)

3.19.3 In Vivo Translocation of the Oestrogen Receptor

In an attempt to induce intracellular movement of the oestrogen receptor in granulosa cells in vivo, 28 day old rats were injected with either 2.5 µg, 10 µg or 50 µg cold oestradiol 1 hr prior to sacrifice. The cytosol was treated with DCC as usual prior to estimation of oestrogen binding sites with 10nM [^{3}H]-oestradiol in the presence or absence of 1µM DES or OE_2. The nuclear oestrogen receptor content was also estimated using the same concentration of radioligand and DES. Neither depletion of cytosol receptor nor accumulation of nuclear receptor was observed. As it was likely that alpha-fetoprotein (AFP) bound the oestradiol and reduced the level of the steroid reaching the ovary, experiments were repeated using DES which does not bind to AFP. The rats were injected with 50 µg DES intraperitoneally 30, 45, 60 or 120 min prior to sacrifice. As shown in Fig 3.23 there was very little change in cytosol oestrogen receptor content during the first 30 min after injection. By 45 min, the cytosol receptor content had doubled but by 60 and 120 min after the injection, the cytosol oestrogen receptor had decreased appreciably ($p < 0.001$). No alteration in the concentration of SOB was observed up to 120 min after the injection of 50 µg DES. Significant reductions in nuclear oestrogen receptor were observed 30 and 45 min after injection ($p < 0.05$ and $p < 0.005$ respectively) but by 60 min and at 120 min after the injection, the nuclear oestrogen receptor concentration had returned to the control level and no

significant difference could be observed compared to the untreated animals (Fig 3.24). At no time point however was accumulation of nuclear oestrogen receptor over control levels observed.

Intraperitoneal injection of 28 day old rats with 50µg testosterone resulted in only 20% of the control concentration of cytosol receptor being measured 60 min after injection but 80% being measured at 120 min after injection. SOB concentrations were not significantly different at 60 and 120 min but about 20% more SOB was measured compared to the control untreated rats (Fig 3.24). No significant alteration in the nuclear oestrogen receptor was observed when the rats were treated with 50µg testosterone.

Injection of 50µg oestradiol to 28 day old rats had no effect on cytosol receptor concentration at 60 min but a complete depletion of receptor was seen at 120 min (Fig 3.24). No significant alteration of SOB concentration was observed as a result of the oestradiol administration. Nuclear oestrogen receptor accumulation was observed only at 60 min when a 20% increase over untreated concentrations ($p < 0.05$) was obtained. No corresponding depletion of cytosol receptor was observed at this time.

3.20 Oligo (dT) - Cellulose Binding

The inability of the oestrogen receptor to accumulate in the granulosa cell nucleus even after complete

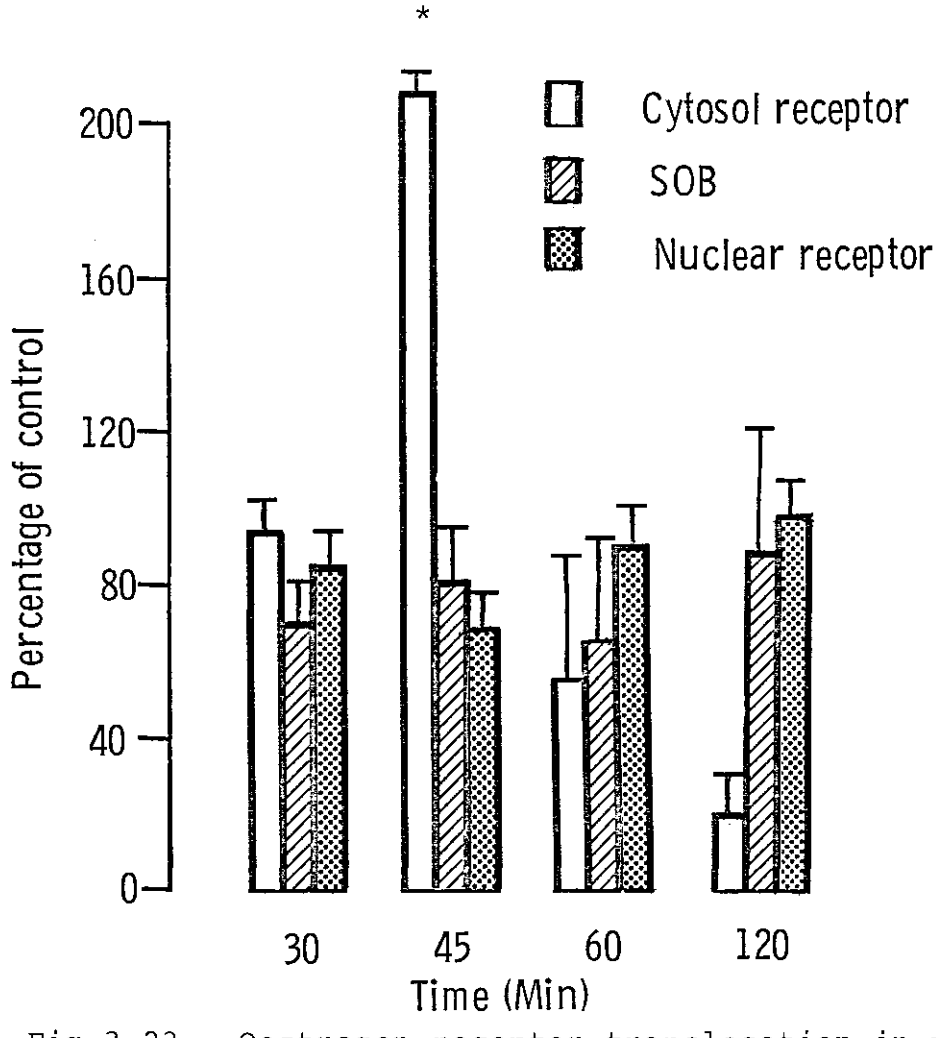


Fig.3.23. Oestrogen receptor translocation in vivo with DES. Rats, aged 28 days, were injected with 50µg DES in 0.1ml saline (control rats had saline only) 30,45, 60 and 120 min prior to sacrifice. Duplicate DCC-treated cytosol aliquots(0.2ml) and triplicate 0.2ml nuclear fraction were incubated with 10 nM [^{3}H]-OE_2 in the presence or absence of 1 µM DES (cytosol and nuclear) and 1 µM OE_2(cytosol).to estimate specific OE_2,DES and SOB binding(SOB = OE_2 - DES). Experiments were repeated four times(values = mean ± S.D.). Each experimental group contained 25 rats.

(*) significantly different from 30,60,120 min ($p<0.001$)

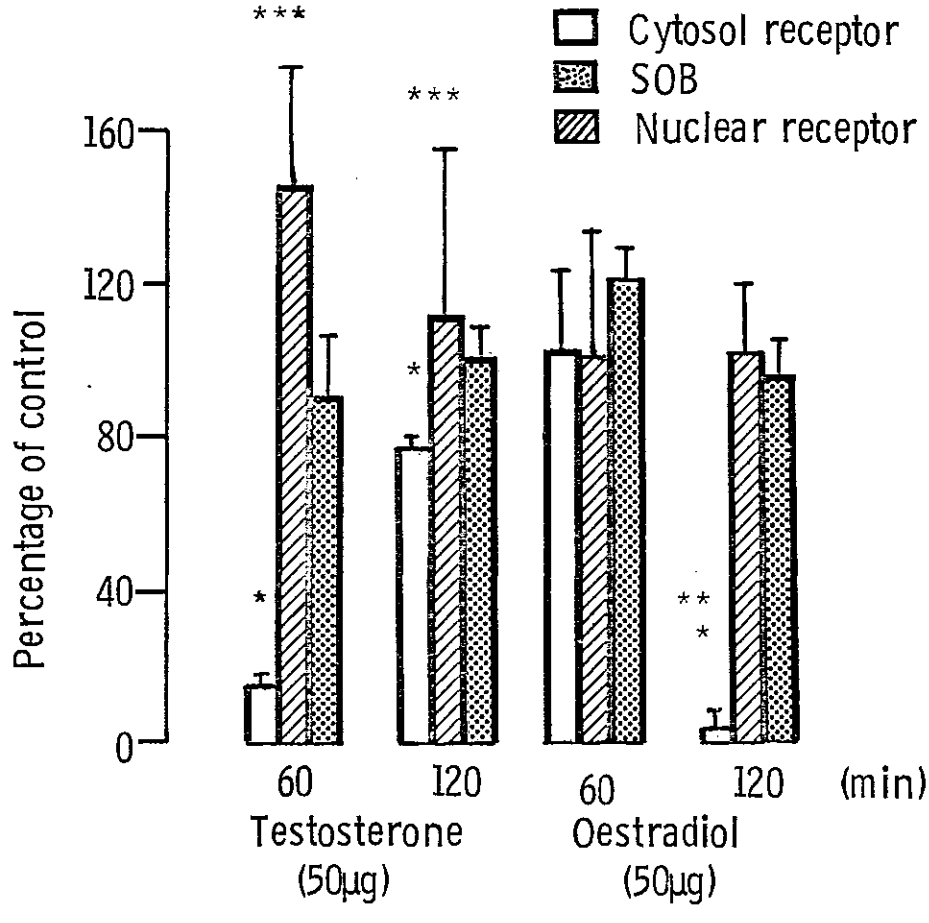


Fig.3.24. Oestrogen receptor translocation in vivo with testosterone and oestradiol.
Rats, aged 28 days, were injected with 50ug oestradiol or testosterone in 0.1ml saline (control rats received saline only) 60 or 120 min prior to sacrifice. Duplicate cytosol (0.2ml) and triplicate 0.2ml nuclear fraction were incubated with 10nM [^{3}H]-OE_2 in the absence or presence of 1µM DES or OE_2 (cytosol) or DES only (nuclear) to estimate specific OE_2, DES and SOB binding (SOB = OE_2-DES). Experiments were repeated four times (values ± S.D.). Each experimental group contained 25 rats.

(*) significantly different from control ($p<0.001$)
(**) significantly different from 60 min value ($p<0.001$)
(***) significantly different from control ($p<0.05$)

TABLE 9 . Binding of Granulosa Cell Cytosol Oestrogen Binding Components to Oligo (dT)-Cellulose

	Sephadex LH-20 Measurement (Dpm/0.2ml cytosol)		Oligo (dT)-Cellulose Binding (Dpm/0.2ml Cytosol)		
		Suppressible Binding		Suppressible Binding	Percentage Binding to Oligo (dT)-cellulose
TC	12807 ± 1694		730 ± 134		
+OE_2	6418 ± 845	6390 ± 898	565 ± 107	165 ± 28	2.79 ± 0.43
+DES	9703 ± 868	3099 ± 888	675 ± 124	56 ± 34	1.77 ± 0.97

DCC treated cytosol (0.2ml) was incubated with 10nM [^{3}H] -oestradiol in the absence (TC) or presence of 1μM DES or OE_2 for 18 hr at 4°C. Macromolecular bound [^{3}H] -oestradiol was assessed using Sephadex LH-20 chromatography. The proportion which binds to oligo (dT)-cellulose was determined by applying the previously incubated cytosol (0.2ml) on oligo (dT)-cellulose for 45 min, at 4°C. Unbound radioactivity was washed off with 15ml TED buffer. Bound radioactivity was eluted with 4 x 1ml 1.2M KCl in TED buffer.

depletion of the cytosol receptor was surprising. Using similar conditions to those used for rat uterine oestrogen receptor (Myatt et al.,1982) it was observed that the cytosol receptor could not bind to oligo (dT)-cellulose in vitro (Table 11). In fact, only 1 to 2% of the receptor could bind compared to 30% in the rat uterine cytosol (Thrower et al., 1976; Myatt et al., 1982). It appeared therefore that the granulosa cell cytosol receptor was "non-functional" in terms of oligo (dT)-cellulose binding capability and might have to be converted from that state to an active DNA binding species if it is to elicit genomic responses.

3.21 Molecular Forms of the Granulosa Cell Oestrogen Binding Species

Examination of the literature reveals that sucrose density gradient centrifugation and gel filtration techniques have been widely used to determine molecular weights and sizes of the oestrogen receptor and other steroid binding species. These techniques were therefore employed to ascertain if the granulosa cell cytosol oestrogen binding species, identified on the basis of their dissociation constants and steroid specificities could be further distinguished on the basis of their molecular forms.

3.21.1 Nuclear Oestrogen Receptor

3.21.1a Sucrose Density Gradient Centrifugation

Oestrogen receptor extracted from granulosa cell nuclei with 0.4M KCl was labelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1 M DES for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated from free steroid by Sephadex LH-20 chromatography and 0.2 ml fractions of the void volume containing receptor were run on 5-30% sucrose density gradients containing 0.4M KCl. Using BSA as a standard molecular weight marker, the samples were centrifuged at 300,000 x g for 16.5 hr at 2°C.

The oestrogen receptor sedimented with a sedimentation coefficient of 4.6S with molecular weight 69-72,000 daltons. (Fig 3.25).

3.21.1b Gel Filtration

The oestrogen receptor extracted from the nuclei with 0.4M KCl was labelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated by Sephadex LH-20 chromatography and 1 ml samples chromatographed on 30 x 1.5 cm Sephacryl S-200 super-fine columns. The oestrogen receptor eluted as a single peak with a Stoke's radius of 35 Å and molecular weight 69,000 daltons (Fig 3.26).

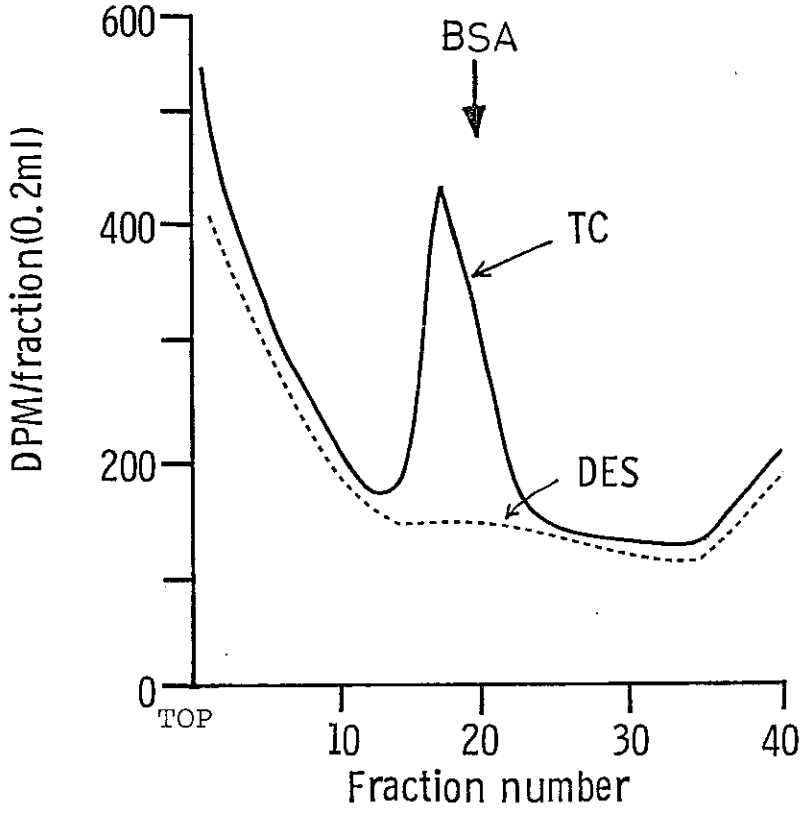


Fig.3.25. Analysis of granulosa cell nuclear oestrogen receptor by sucrose density centrifugation. Oestrogen receptor was extracted from nuclei with 0.4M KCl/TED buffer at 4°C. Aliquots(0.2ml), prelabelled with 10nM [^{3}H]-OE_2 in the absence (TC) or presence of 1μM DES for 18 hr at 4°C, was chromatographed on Sephadex LH-20. Macromolecular bound [^{3}H]-OE_2 in 0.2ml was then layered on 5-30% sucrose gradients containing 0.4M KCl and centrifuged at 300,000 x g for 16.5 hr at 2°C. BSA(mol. wt. 69,000,4.6S)was run a separate gradient concurrently with the cytosol as a reference molecular weight marker. Forty 0.1ml fractions were collected and counted for radioactivity measurement but the absorbance of the reference protein was determined directly during fractionation of the gradients.

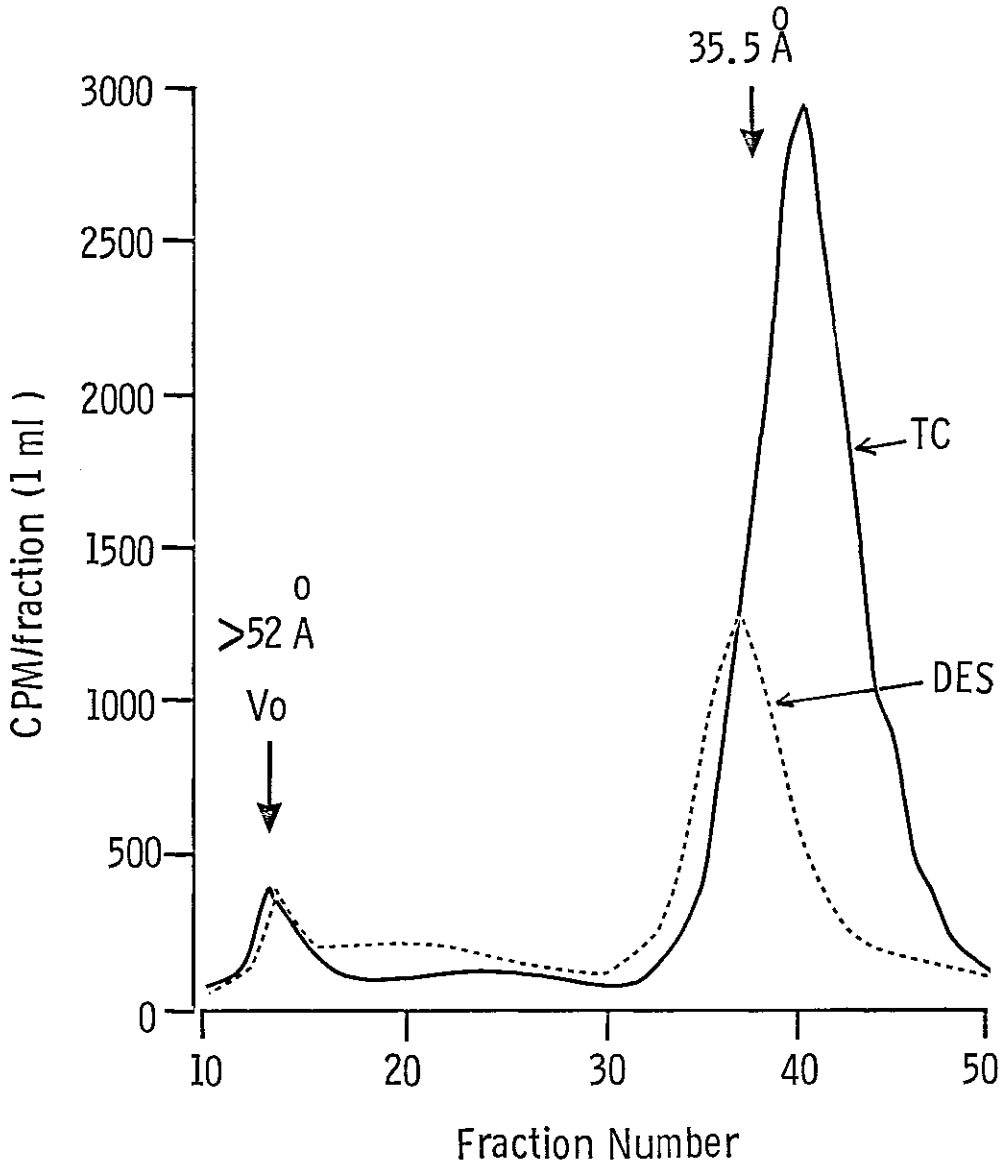


Fig.3.26. Fractionation of granulosa cell nuclear receptor on Sephacryl s-200. Oestrogen receptor was extracted from nuclei with 0.4M KCl/TED buffer at 4°C. Nuclear extract(0.2ml) was labelled with 10 nM [^{3}H]-OE$_2$ in the absence(TC) or presence of 1 μM DES for 18 hr at 4°C and chromatographed on Sephadex LH-20. The break-through volume(1.2ml) containing macromolecular bound [^{3}H]-OE$_2$ was applied to Sephacryl s-200(30x1.5cm column) and 50 22-drop fractions(1 ml) collected over 3-3.5 hr. Column void volume was determined using Blue Dextran 2000 and BSA(69,000 M.W.,Stoke's radius, 35.5 Å) was used as a reference molecular weight marker.

3.21.2 Cytosol Oestrogen Binding Species

3.21.2a Sucrose Density Gradient Centrifugation (SDG)

Granulosa cell cytosol, pretreated with DCC to remove endogenous steroid, was labelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2 for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated from free steroid using Sephadex LH-20 chromatography. Samples (0.2 ml) containing the macromolecular bound [^{3}H]-oestradiol were centrifuged on 5-30% sucrose density gradients in TED buffer at 300,000 x g for 16.5 hr at 2°C. The cytosol oestrogen binding species produced two peaks of [^{3}H]-oestradiol binding (Fig 3.27). One peak had an apparent molecular weight of 212-214,000 dalton and a sedimentation coefficient of 10.35 using catalase and BSA (M.W., 230,000, 11.3S and 69,000, 4.6S respectively) as standard molecular weight markers. This peak was suppressed by both a 100 fold excess of OE_2 and DES. The other peak was observed in the region of 2 to 3S (M.W., 30 - 45,000) and this was suppressible by a 100 fold excess of OE_2 but apparently not by DES (Fig 3.27). Granulosa cell cytosol was also prepared in TED buffer containing 0.4M KCl and labelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM OE_2 or DES for 18 hr at 4°C. Two peaks of [^{3}H]-oestradiol were observed after centrifugation at 300,000 x g for 16.5 hr at 2°C on 5 - 30% sucrose density gradients containing 0.4M KCl (Fig 3.28). The peak of 4.4S (M.W. 64,000) was suppressed by both OE_2 and DES but the more prominent peak at 5.8S (M.W. 110,000) was suppressed by OE_2 but not DES.

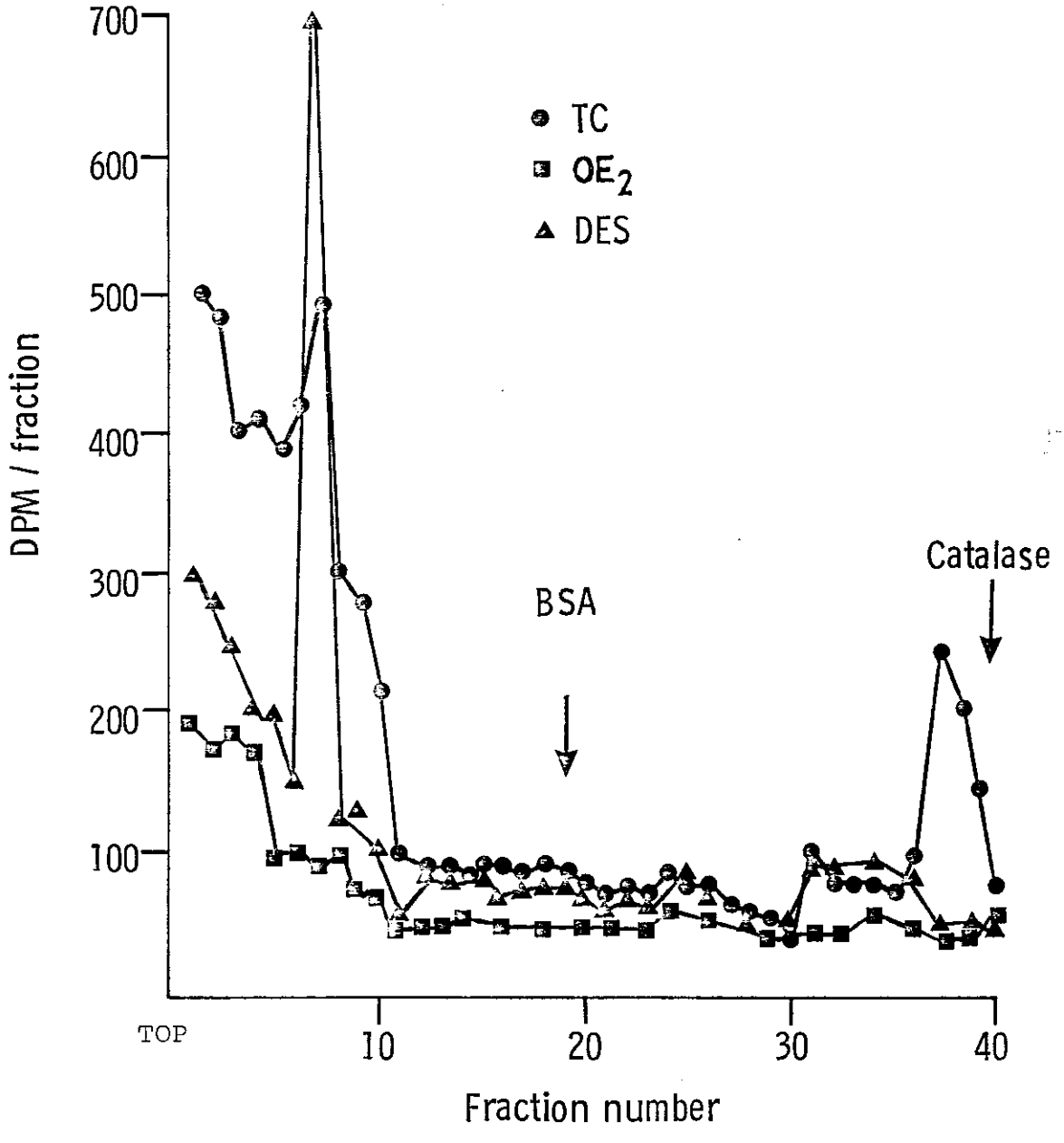


Fig.3.27. Analysis of granulosa cell cytosol oestrogen binding species by sucrose density centrifugation under no salt conditions. DCC-treated cytosol was incubated with 10nM [^{3}H]-OE_2 in the absence(TC) or presence of 1μM $_3$DES or OE_2 ^{2}for 18 hr at 4°C. Macromolecular bound [^{3}H]-OE_2 was seperated from free steroid using Sephadex LH-20 chromatography. Aliquots (0.2ml) were layered on 5-30% sucrose gradients containing no KCl and centrifuged at 300,000 x g for 16.5 hr at 2°C. BSA and catalase(69,000,4.6S; 232,000,11.3S) were run on separate gradients concurrently with the cytosol as reference molecular weight markers. Forty 0.1ml fractions were collected for measurement of radioactivity but the absorbance of the reference proteins were determined directly during fractionation of the gradients.

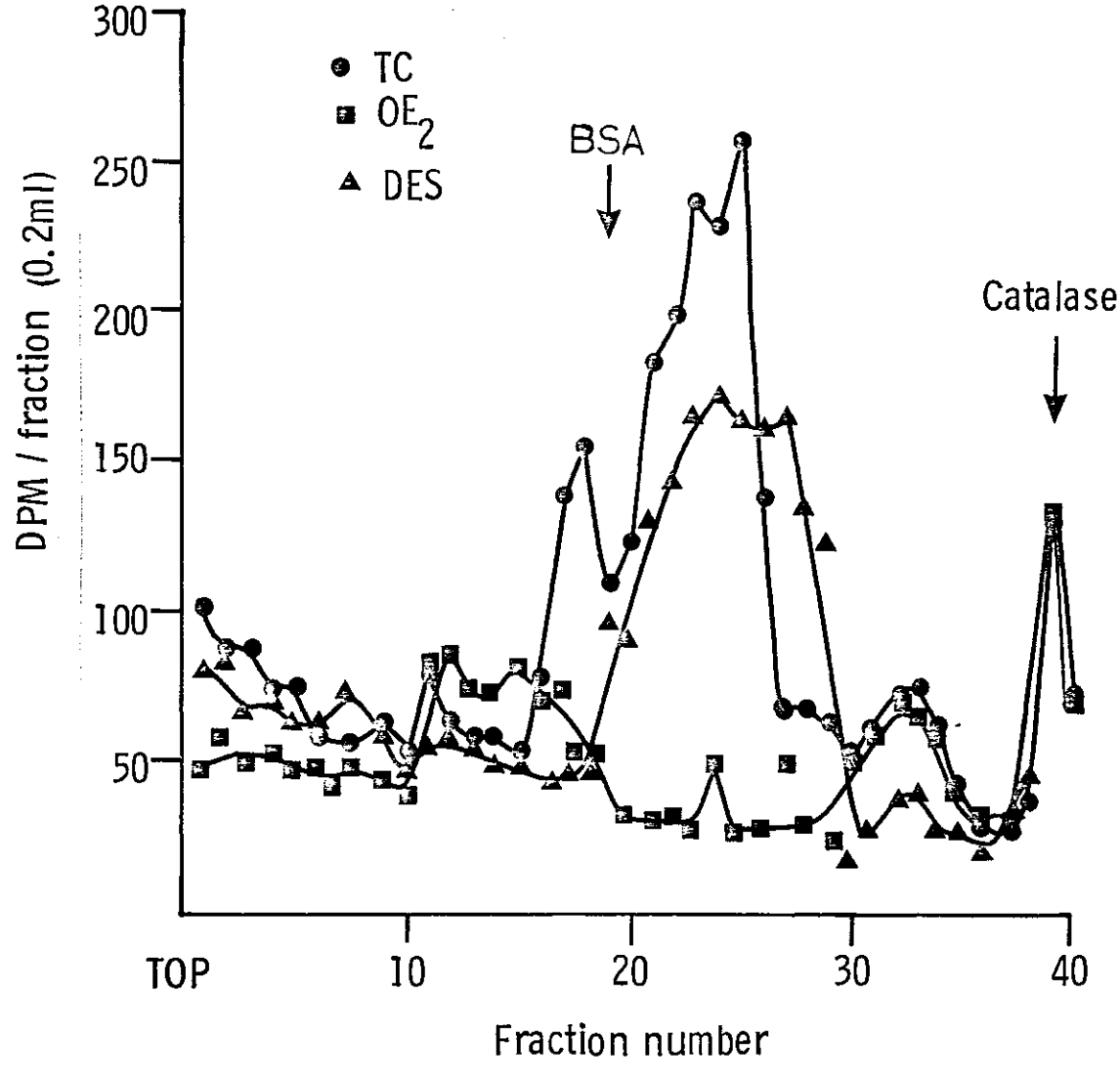


Fig.3.28. Analysis of granulosa cell cytosol oestrogen binding species by sucrose density centrifugation under high salt conditions. DCC-treated cytosol was incubated with 10nM [^{3}H]-OE_2 in the absence (TC) or presence of 1μM DES_3or OE_2 for 18 hr at 4°C. Macromolecular bound [^{3}H]-OE_2 was separated from free steroid using Sephadex LH-20 chromatography. Aliquots(0.2ml) were layered on 5-30% sucrose gradients containing 0.4M KCl and centrifuged at 300,000 x g for 16.5 hr at 2°C. BSA and catalase (69,000,4.6S;230,000,11.3S) were run on separate gradients concurrently with the cytosol as reference molecular weight markers. Forty 0.1ml fractions were collected for radioactivity measurement but the absorbance of the reference proteins were determined directly during fractionation of the gradients.

The species not suppressed by DES was, by definition taken to be the second oestrogen binding species. In order to estimate the size of this species in the absence of the receptor, granulosa cell cytosol was depleted of receptor by injecting 28 day old rats with 50µg DES (i.p.) 1 hr prior to sacrifice. The cytosol, pretreated with DCC, was incubated with 20nM [^{3}H]-oestradiol in the presence or absence of 2µM OE_2 (A preliminary check revealed that DES-suppressible sites were depleted from the cytosol). Macromolecular bound [^{3}H]-oestradiol was separated from free steroid by Sephadex LH-20 chromatography and 0.2 ml samples were centrifuged at 300,000 x g for 16.5 hr at 2°C on a 5 - 30% sucrose density gradients containing no KCl. Only one peak of radioactivity was observed at less than 1S. Neither the 10.3S nor the 4-5S species were observed (Fig 3.29A). It was concluded therefore that the oestrogen receptor had disappeared from the cytosol.

3.21.3 Post-labelling of Oestrogen Binding Species from SDG

As the second oestrogen binding species was found to be a lower affinity binder, it was considered that the extended period of centrifugation (16.5 hr) at 300,000 x g could cause extensive dissociation of [^{3}H]-oestradiol and lead to underestimation of the size of SOB. Cytosol, depleted of SOB, was pretreated with DCC and incubated with 20nM [^{3}H]-oestradiol in the presence or absence of 2µM OE_2 for 18 hr at 4°C. After sephadex LH-20 chromatography to separate

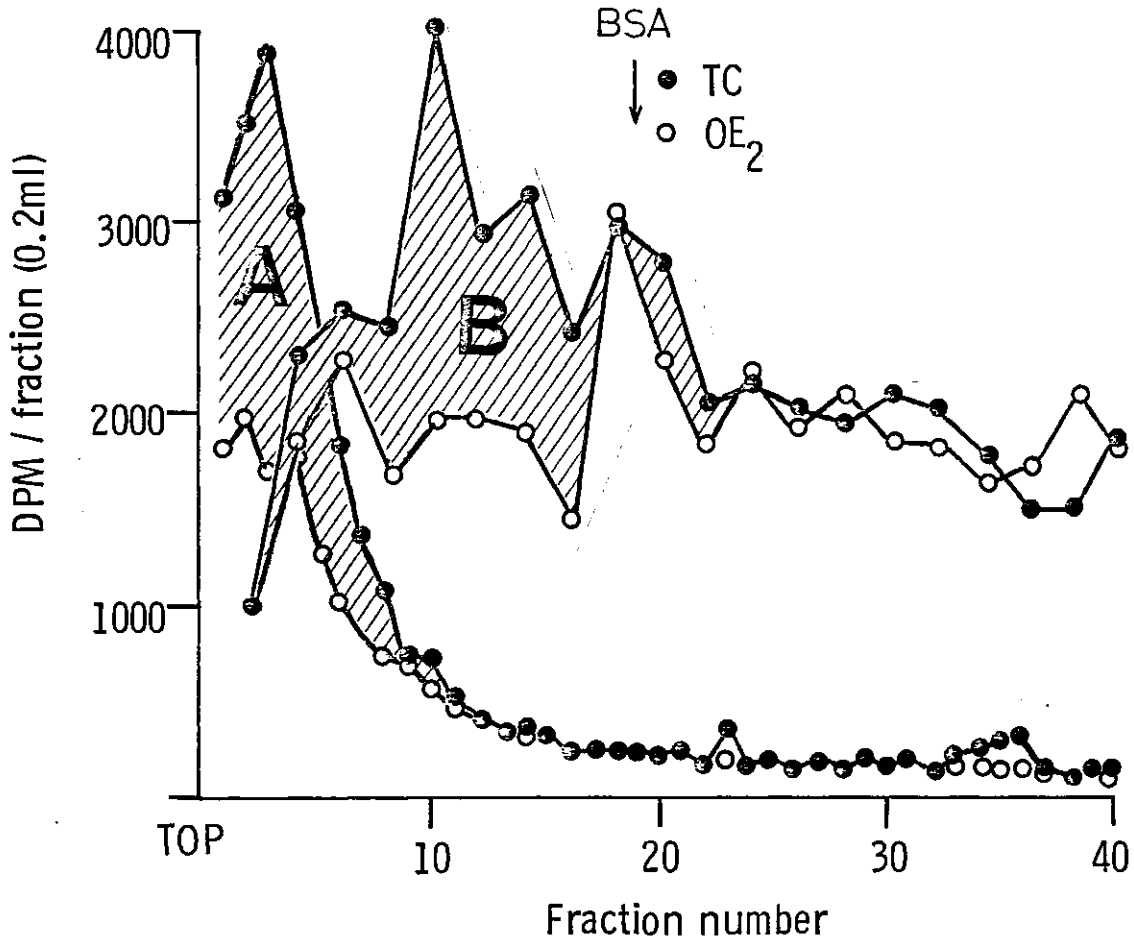


Fig.3.29. Post-labelling of granulosa cell Second Oestrogen Binding Species(SOB) as analysed on sucrose density gradients. Oestrogen receptor-depleted cytosol was prepared from granulosa cells isolated from rats injected with 50 µg oestradiol 2 hr prior to sacrifice(see Fig.3.24.). Pretreated with DCC, the cytosol was incubated with 20 nM [^{3}H]-OE_2 in the absence(TC) or presence of 2 uM OE_2 for 18 hr at 4°C. Chromatographed on Sephadex LH-20, 0.2 ml aliquots were layered on 5-30% sucrose gradients containing no KCl and centrifuged at 300,000 x g for 16 hr at 2°C. Forty 0.1 ml fractions were collected and counted for radioactivity (A) or 20 0.2 ml fractions were collected, treated with DCC and relabelled with 20 nM [^{3}H]-OE_2 in the absence or presence of OE_2 (2µM) for 18 hr at 4°C. Macromolecular bound [^{3}H]-OE_2 was separated from free steroid by Sephadex LH-20 chromatography (B). BSA(69,000) was used as a reference protein.

macromolecular bound from free [^{3}H]-oestradiol, 0.2 ml samples of cytosol were centrifuged as usual, on 5 - 30% sucrose density gradients at 300,000 x g for 16.5 hr at 2°C. After centrifugation, the gradients were fractionated into 0.2 ml aliquots and each was relabelled with 20nM [^{3}H]-oestradiol in the presence or absence of 2μM OE_2 for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol in each fraction was then separated from free steroids on Sephadex LH-20, to estimate [^{3}H]-oestradiol binding. Fig 3.29B illustrates the recovery of [^{3}H]-oestradiol. The radioactive peak was observed at 2.2S (M.W. 23,000). This confirmed the fact that [^{3}H]-oestradiol dissociation occurred during centrifugation.

3.22 Sucrose Density Gradient Centrifugation of Immature Rat Uterine Cytosol

Immature rat uterine cytosol was prepared and incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated on Sephadex LH-20 and 0.2 ml samples were centrifuged at 300,000 x g for 16.5 hr at 2°C. In gradients without KCl, the receptor sedimented as a single peak at 10S (M.W. 193,000) using catalase (M.W. 230,000; 11.3S) as a standard molecular weight marker. The cytosol oestrogen receptor prepared in 0.4M KCl sedimented in a sucrose gradient containing 0.4M KCl, as a 4.2S species (M.W. 60,000) (Fig 3.30).

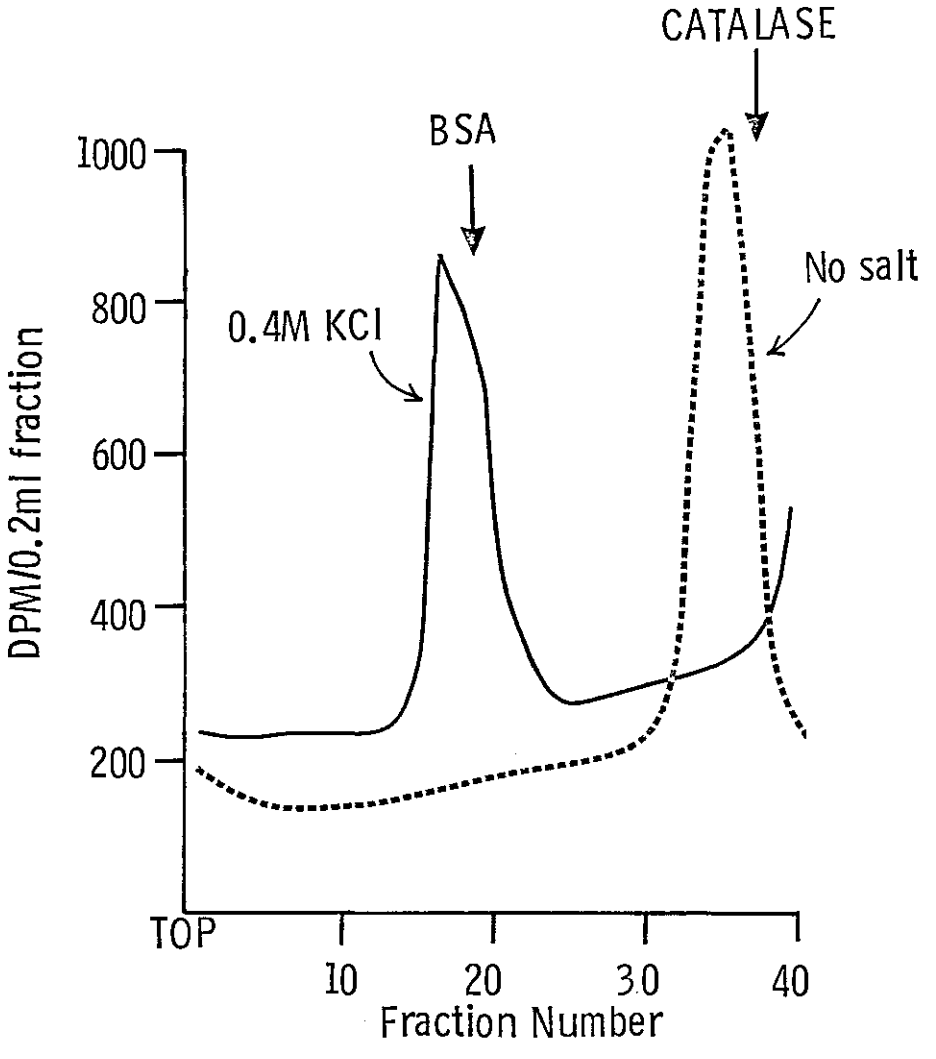


Fig.3.30. Analysis of immature rat uterine cytosol receptor by sucrose density gradient centrifugation.
Cytosol was prepared either in buffer containing no salt or in one containing 0.4M KCl and 0.2 ml aliquots labelled with with 10 nM [^{3}H]-oestradiol with DES as competitor. Macromolecular bound [^{3}H]-oestradiol was separated by Sephadex LH-20 gel filtration and 0.2 ml aliquots of the break-through volume centrifuged on 5-30% sucrose gradients either containing no salt or with 0.4M KCl for 16.5 hr at 300,000 x g at 2°C. The gradients were fractionated into 40 fractions and radioactivity counted. BSA(69,000) and catalase(230,000) were used as reference molecular weight markers.

3.23.1 Gel Filtration of Granulosa Cell Cytosol Oestrogen Binding Species on Sephacryl S-200 Superfine

On Sephacryl S-200 macromolecules with molecular weight greater than 250,000 are excluded from the gel bed. Preliminary runs indicated that the granulosa cell cytosol oestrogen binding components, pre-incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES or OE_2 for 18 hr at 4°C, gave two peaks of [^{3}H]-oestradiol binding when eluted with TED buffer containing no KCl (Fig 3.31). One peak was observed in the void volume and this was suppressible by both DES and OE_2. This macromolecular species therefore has a molecular weight greater or equal to 250,000 and a Stoke's radius greater or equal to 52 Å. The second peak was broad and was suppressed by OE_2. DES however, only partially suppressed that part of the peak which corresponded to a molecular weight of 70,000 daltons, Stoke's radius 37 Å but not the portion corresponding to 82,000 daltons (M.W.) and a Stoke's radius of 40 Å. Comparison of the radioactivity in the eluates with the amount loaded onto the Sephacryl gel showed that 45% of radioactivity associated with the receptor and 74% with the second oestrogen binding species was lost during the 3.5 hr chromatographic separation at 4°C.

3.23.2 The Effect of 0.4M KCl on Sephacryl S-200 Chromatography

The granulosa cell cytosol, pretreated with DCC, was brought to 0.4M KCl concentration, incubated with 10nM [^{3}H]-oestradiol in the presence or absence of

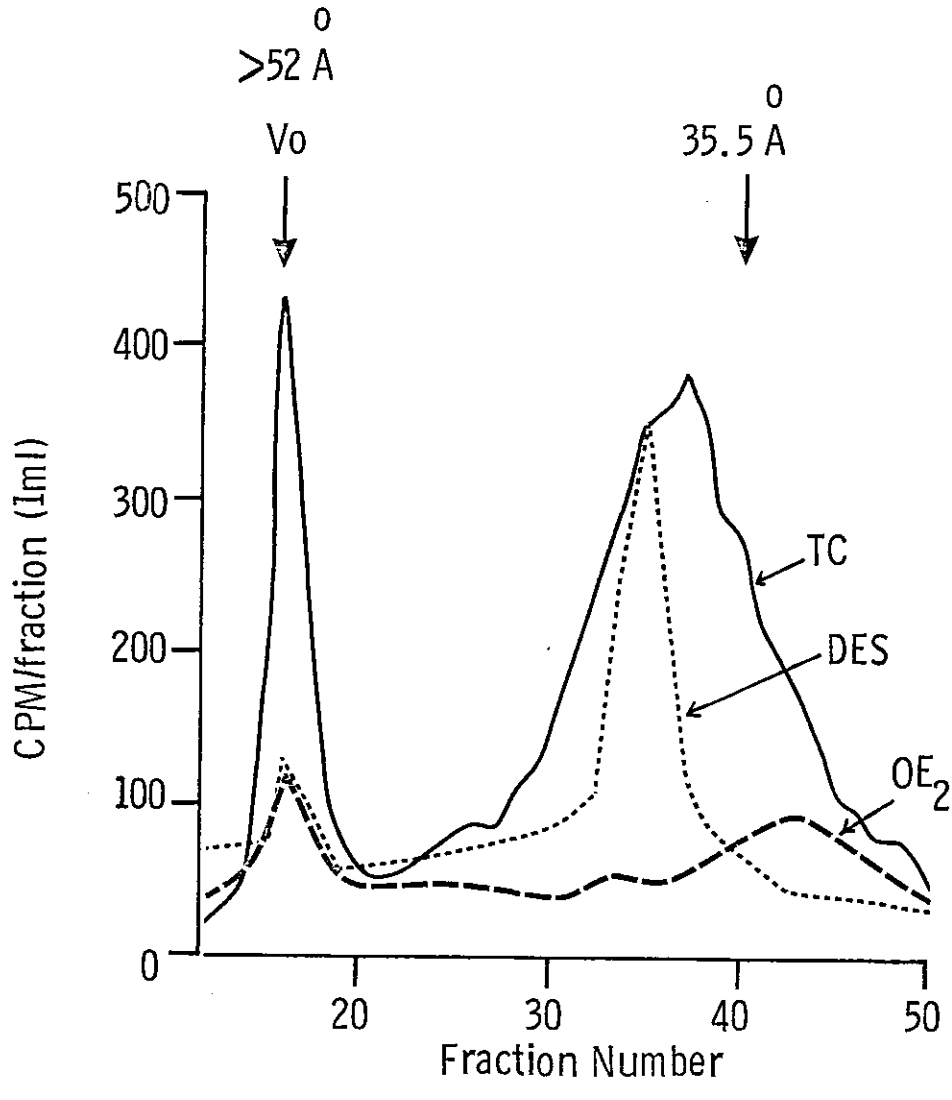


Fig.3.31. Analysis of granulosa cell cytosol oestrogen binding species on Sephacryl S-200. Cytosol was prelabelled with 10 nM [^{3}H]-oestradiol without(TC) or with 1 µM DES or OE_2 for 18 hr at 4°C. Samples(1.2ml) of Sephadex LH-20 chromatographed cytosol were fractionated on Sephacryl S-200 columns(30 x 1.5cm) under 40cm water prssure. Fifty 1 ml fractions were collected over 3 hr and molecular sizes of the species estimated as described in the materials and methods. The void volume was determined using Blue Dextran 2000(M.W. 2 x 10^6) and the columns calibrated using BSA,aldolase; gel exclusion size = 250,000.

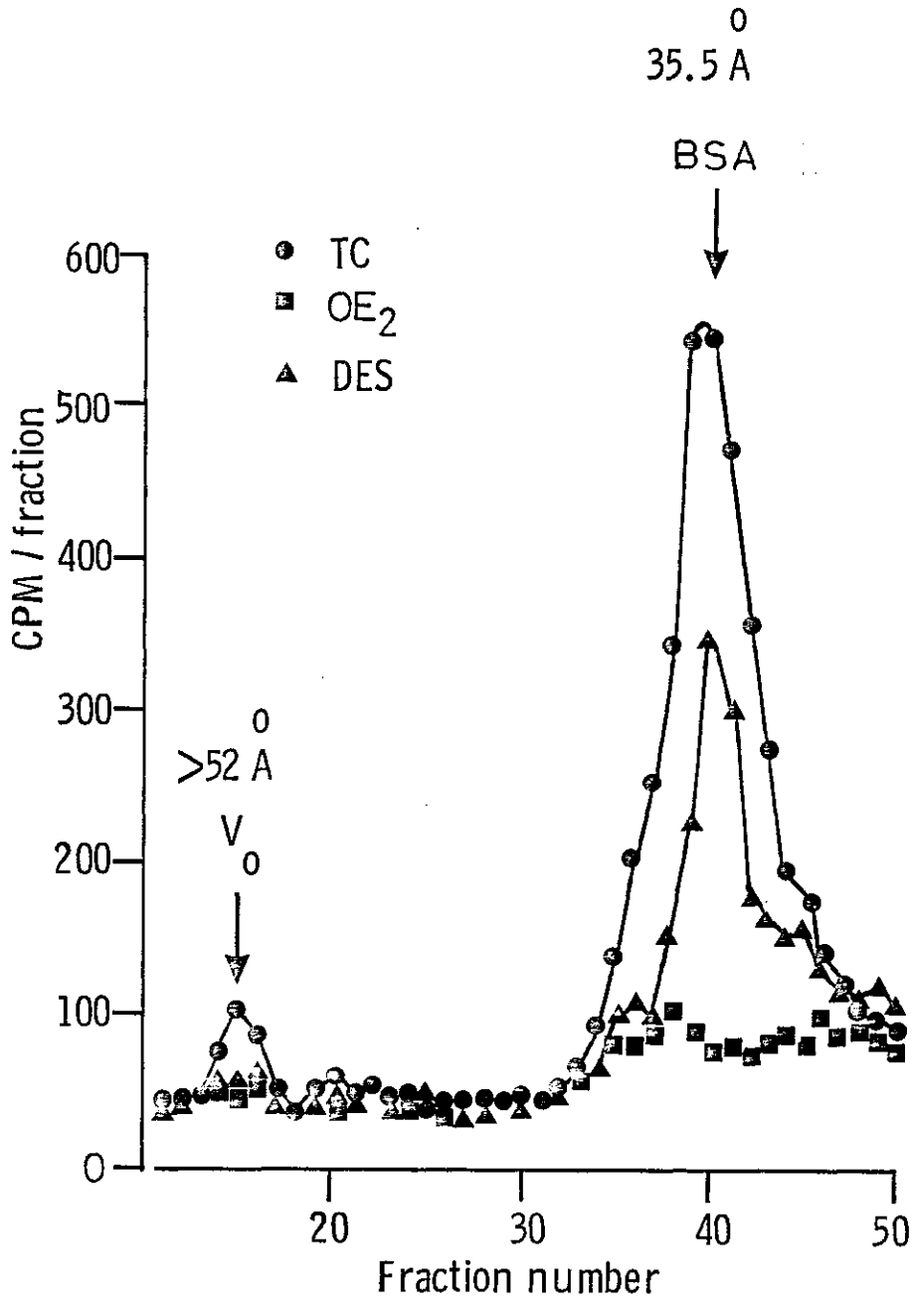


Fig.3.32. Analysis of granulosa cell cytosol oestrogen binding species under high salt conditions on Sephacryl S-200. Cytosol was prepared in 0.4M KCl and labelled with 10nM [^{3}H]-oestradiol in the absence(TC) or presence of 1μM DES or OE_2 for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated from unbound steroid by Sephadex-LH-20 chromatography, was fractionated on Sephacryl S-200 gel columns(30 x 1.5cm) using degassed TED buffer containing 0.4M KCl. Fifty 1 ml fractions were collected over a period of 3 hr. Column void volume was determined using Blue Dextran 2000 and gel exclusion size = 250,000.BSA(69,000) was used as a reference molecular weight marker.

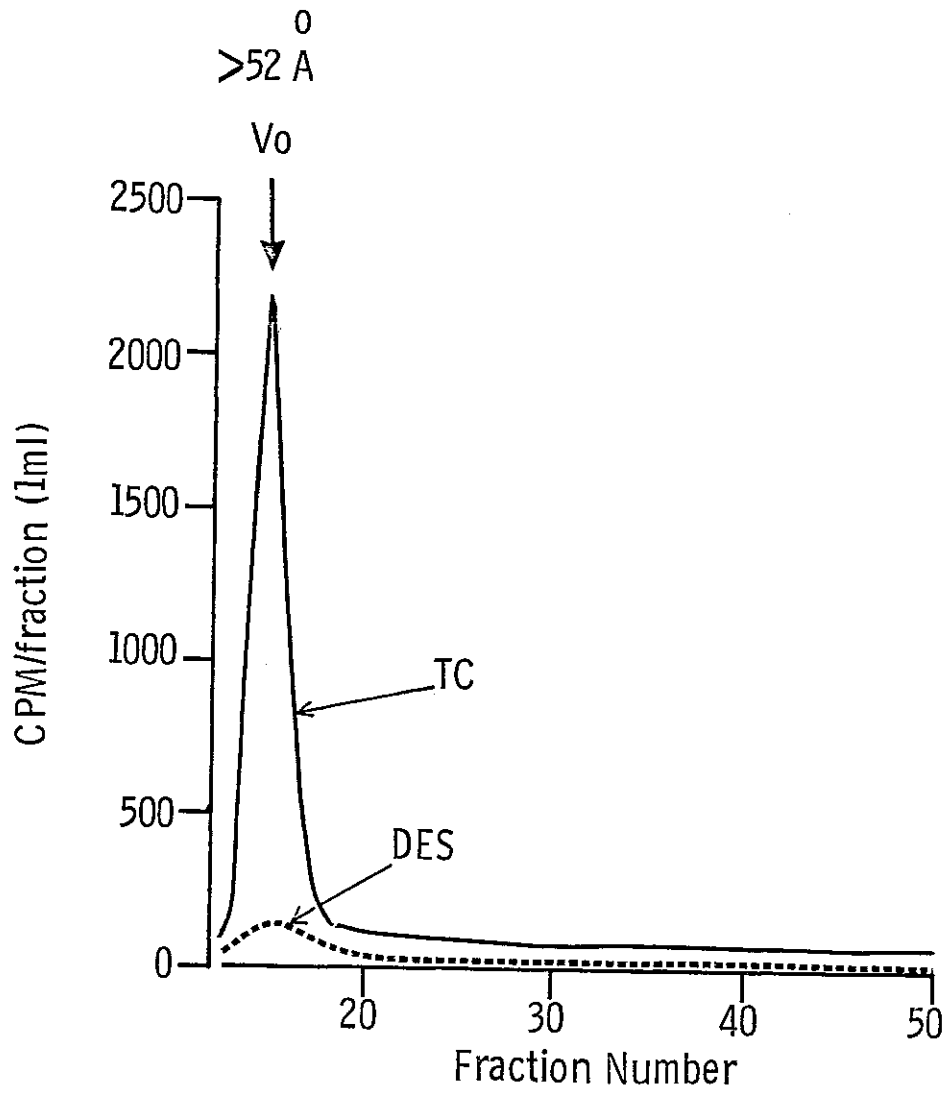


Fig.3.33. Analysis of immature rat uterine cytosol oestrogen receptor on Sephacryl S-200. Cytosol was prelabelled with 10nM ^{3}H-oestradiol in the absence (TC) or presence of 1uM DES for 18 hr at 4°C. After gel filtration on Sephadex LH-20, 1.2 ml of the break-through volume was fractionated on Sephacryl S-200 with TED buffer containing no KCl, collecting 50 1 ml fractions over 3 hr. Gel exclusion size = 250,000. Column void volume was determined using Blue Dextran 2000.

1μM DES or OE_2 for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol separated from free steroid was chromatographed using TED buffer containing 0.4M KCl. The void volume peak observed in elution with TED buffer containing no KCl (Fig 3.31) disappeared and all the oestrogen binding components eluted in the 35 A (M.W. 69,000) region, all of which was suppressible by OE_2 and only partially by DES (Fig 3.32). It appeared that both the oestrogen receptor and the second oestrogen binding species coeluted under the high salt conditions.

3.23.4 Elution of Immature Rat Uterine Oestrogen Receptor on Sephacryl S-200 Superfine

Immature rat uterine cytosol oestrogen receptor was incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated on Sephadex LH-20 and eluted on Sephacryl S-200 with TED buffer. All the oestrogen receptor eluted in the void volume (M.W., $\geq$ 250,000, Stoke's radius $\geq$ 52 Å) (Fig 3.33).

3.24 Ammonium Sulphate Fractionation of Granulosa Cell Cytosol Oestrogen Binding Components

The oestrogen receptor and the second oestrogen binding species of granulosa cell cytosol could be differentially precipitated at 30, 50 and 70% ammonium sulphate saturation in the presence of 2 mg/ml BSA (Table 6). The ammonium sulphate fractionated precipitates were resuspended in TED buffer and labelled

with 10nM [^{3}H]-oestradiol for 18 hr at 4°C. Because OE_2 (but not DES) binds to the carrier protein (BSA) these competitors were not included in incubations. Fractionation of the macromolecular bound [^{3}H]-oestradiol on Sephacryl S-200 showed that the radioactivity in the void volume increased with increasing concentration of ammonium sulphate (Fig 3.34). As can be seen from Table 6 increased precipitation of SOB occurred with increasing concentration of ammonium sulphate from 30 to 70% saturation suggesting that the second oestrogen binding species could form aggregates big enough to elute in the void volume.

3.25 Post-labelling of Sephacryl S-200 Fractionated Granulosa Cell Cytosol Oestrogen Binding Components

To confirm that the oestrogen receptor could form aggregates in the unfractionated cytosol and thereby co-elute in the void volume on Sephacryl S-200, granulosa cell cytosol was prepared in 5nM cold oestradiol and chromatographed on Sephacryl S-200.Fractions Fractions 14 to 20 (void volume) were collected, treated with DCC to remove endogenous steroid and 0.2 ml fractions incubated with 10nM [^{3}H]-oestradiol with or without a 100 fold excess of DES or OE_2. The results indicated that indeed some SOB co-eluted with the oestrogen receptor in the void volume with molecular weight equal to or greater than 250,000 (Stoke's radius equal to or greater than 52 Å) (Fig 3.35).

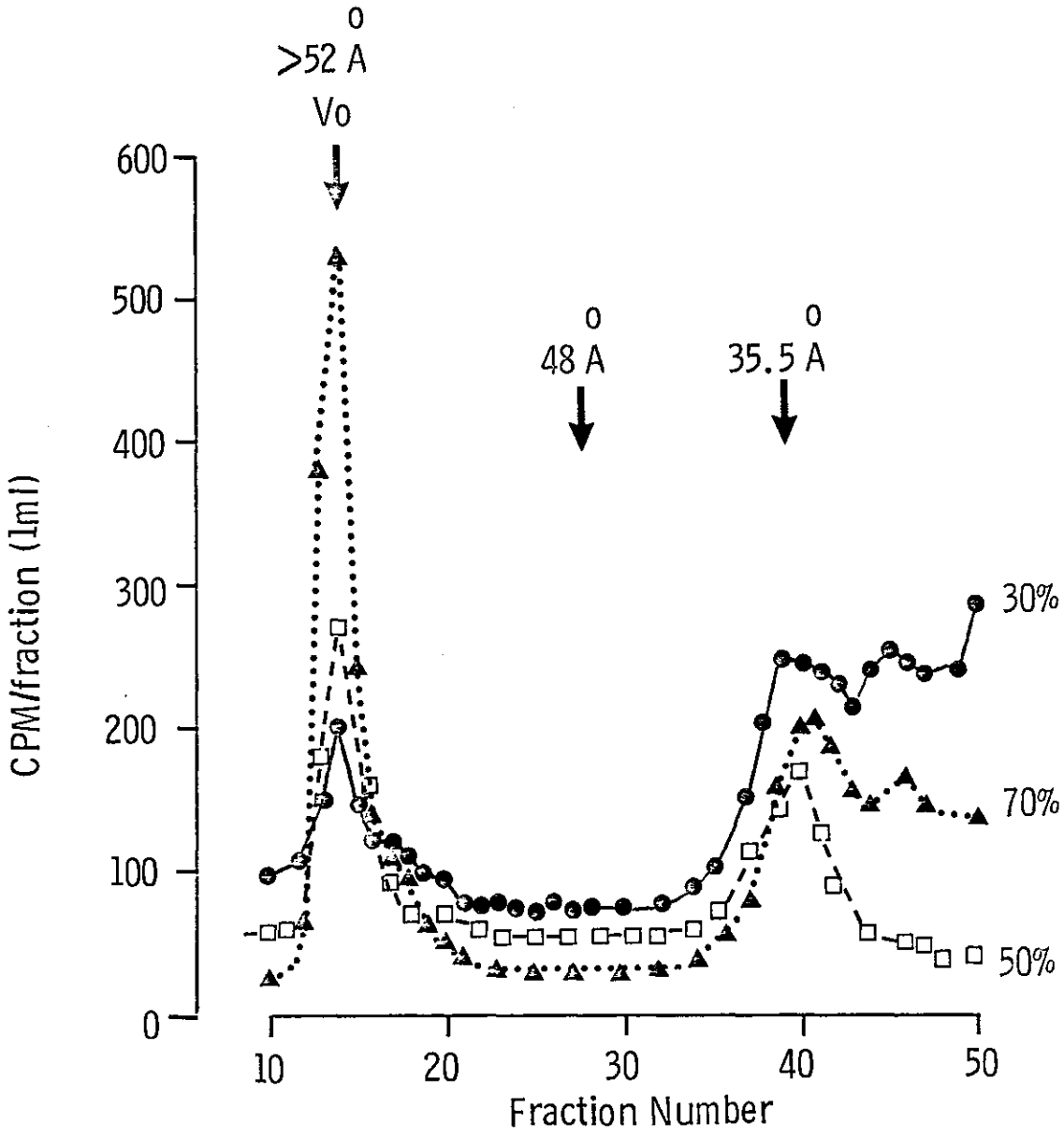


Fig.3.34. Elution of ammonium sulphate precipitated granulosa cell cytosol oestrogen binding species on Sephacryl S-200. Cytosol was augmented with 2mg/ml carrier protein(BSA) and precipitated out at 30, 50 and 70% ammonium sulphate saturation. The precipitates were sedimented by centrifugation at 20,000 x g for 30 min at 4°C, reconstituted in TED buffer and labelled with 10nM [^{3}H]-oestradiol for 18 hr at 4°C. Unbound steroid was removed on Sephadex LH-20 and the break-through volume (1.2ml) fractionated on Sephacryl S-200 at 4°C. Fifty 1 ml fractions were collected and collected for radioactivity. Gel exclsion size = 250,000 and the column void volume was determined using Blue dextran 2000.

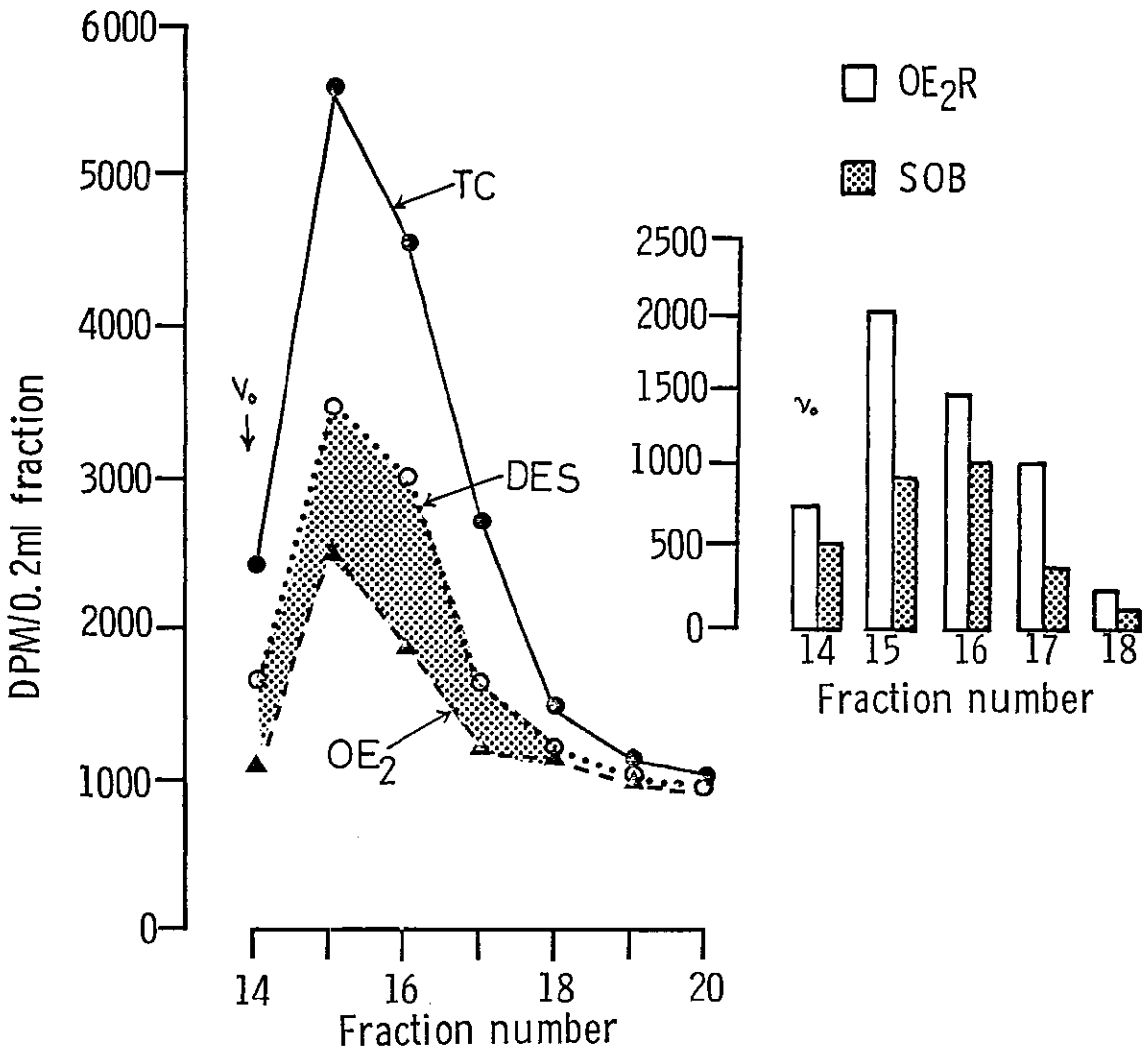


Fig.3.35. Post-labelling analysis of granulosa cell cytosol oestrogen binding species on Sephacryl S-200. Cytosol was prepared in TED buffer containing 5nM cold OE_2 and fractionated on Sephacryl S-200 under 40 cm of water pressure. Fractions 14 to 20 were collected, treated with DCC and 0.2ml aliquots incubated with 10nM [^{3}H]-oestradiol in the absence (TC) or the presence of 1μM DES or OE_2. Free unbound steroid was removed using Sephadex LH-20 chromatography. SOE = OE_2-DES-suppressible sites (OE_2-R). Column void volume was determined using Blue Dextran 2000 and the gel exclusion size = 250,000.

3.26 Sephacryl S-300 Chromatography for estimation of Molecular size of the Granulosa Cell Cytosol Oestrogen Binding Components

Nishizawa et al.(1981) have suggested that the rat uterus contained a factor which inactivated the oestrogen receptor by inducing molecular aggregation. These aggregates eluted on Sephadex G-200 in a form corresponding to 5 to 20S. The inability of granulosa cell cytosol oestrogen receptor to bind to oligo (dT)-cellulose and the observation that SOB and the oestrogen receptor co-eluted in the void volume on Sephacryl S-200 (exclusion limit 250,000) after incubation under conditions which promoted activation, indicated that probably molecular aggregation might occur in vitro. To estimate the actual sizes of the aggregates, Sephacryl S-300 was employed. Only macromolecules with molecular weight greater than 1.5×10^6 daltons are excluded from Sephacryl S-300 gel columns. The standard curves used for the estimation of the molecular sizes of the macromolecules are shown in Fig 3.36. Unexpectedly the oestrogen binding species eluted in the void volume for cytosols incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM OE_2 or DES at 4°C for 18 hr and eluted in TED buffer containing no KCl (Fig 3.37). Thus SOB and the oestrogen receptor formed aggregates and eluted with molecular weight equal to greater than 1.5×10^6 daltons. As the ionic strength of the cytosol and the eluting buffer was increased the elution of the receptor and the second oestrogen

binding species along the gel column was restricted such that by 0.4M KCl a broad peak of radioactivity stretching from 35 Å to 12 Å was observed (with a peak at 23 Å, M.W. 28-30,000) which was suppressible by a 100 fold excess of both OE_2 and DES. The void volume peak however, was suppressible by only OE_2. Thus 0.4M KCl could disaggregate the oestrogen receptor complex but not the SOB (Fig 3.38). These results demonstrated that the granulosa cell cytosol oestrogen binding species are able to form aggregates.

3.27 Elution of Mature Rat Uterine Cytosol Receptor on Sephacryl S-300 Superfine

Mature rat uterine cytosol receptor was incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES at 4°C for 18 hr and fractionated on Sephacryl S-300. The receptor was found to elute as 54 and 37 Å peaks (M.W. 230,000 and 69,000) in TED buffer containing no KCl (Fig 3.39). These values are consistent with those published by King & Mainwaring (1974) and further demonstrate that under identical conditions, the granulosa cell cytosol oestrogen binding components' are peculiar in their ability to form aggregates *in vitro*.

3.28 Characterisation of the Granulosa Cell Nuclear Oestrogen Receptor

The inability of the oestrogen receptor to accumulate in the granulosa cell nuclei was found to be peculiar meriting further characterisation of the nuclear oestrogen receptor.

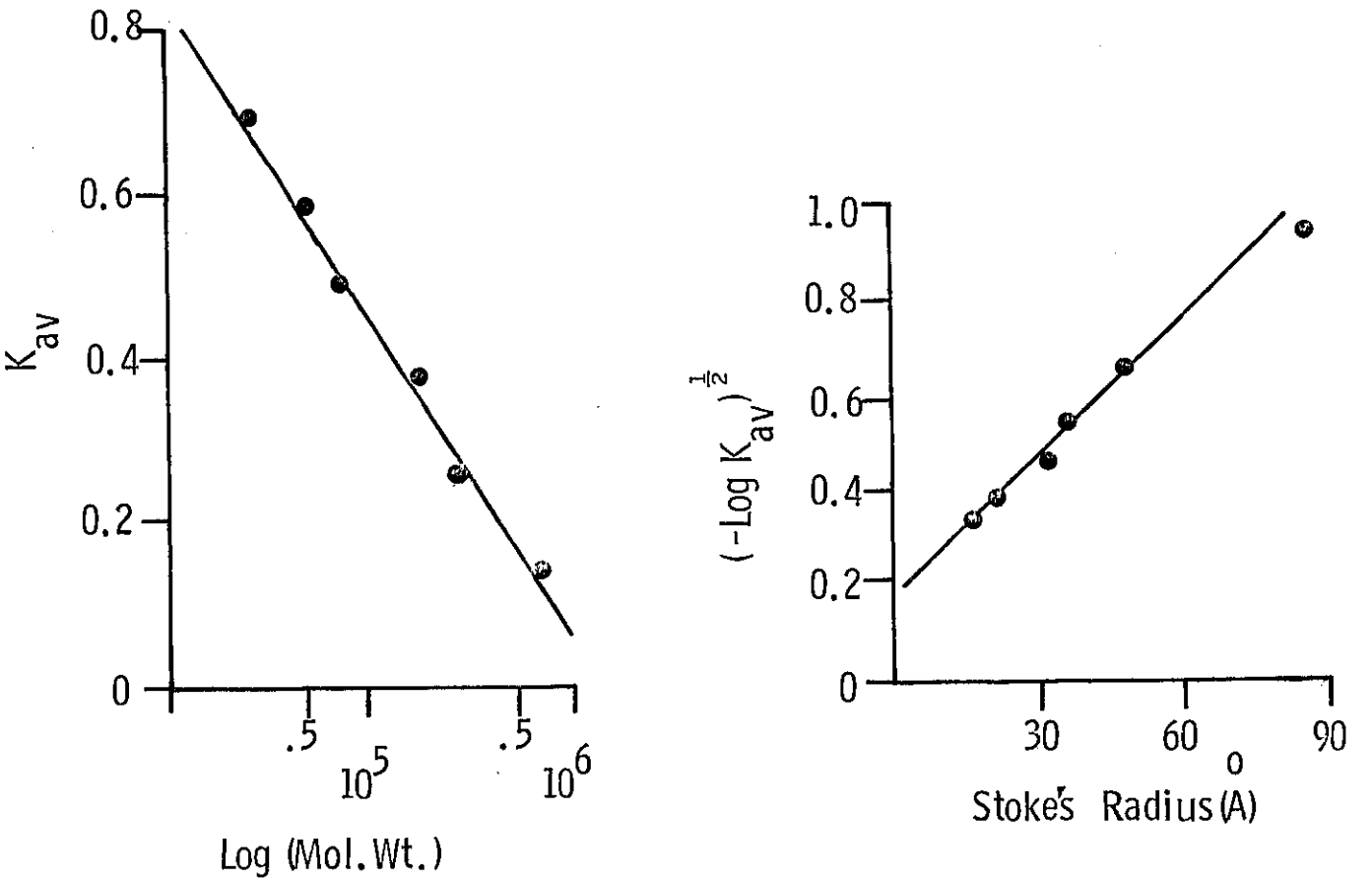


Fig.3.36. Standard curves for calibration of Sephacryl S-300 gel columns. Columns were packed as described in the materials and methods. Samples (0.5-1.0ml;1-5mg/ml) of reference proteins ferritin, thyroglobulin,catalase aldolase,BSA,ovalbumin,chymotrypsinogen A and ribonuclease A were fractionaed on a 60 x 0.9cm column under 180cm water pressure using degassed TED buffer. Forty 1 ml fractions were collected and absorbance measured on a spectrophoto meter at 280nm. K_{av} for each of the proteins was calculated as described in section 2.20.2 and used for constructing standard curves A and B.

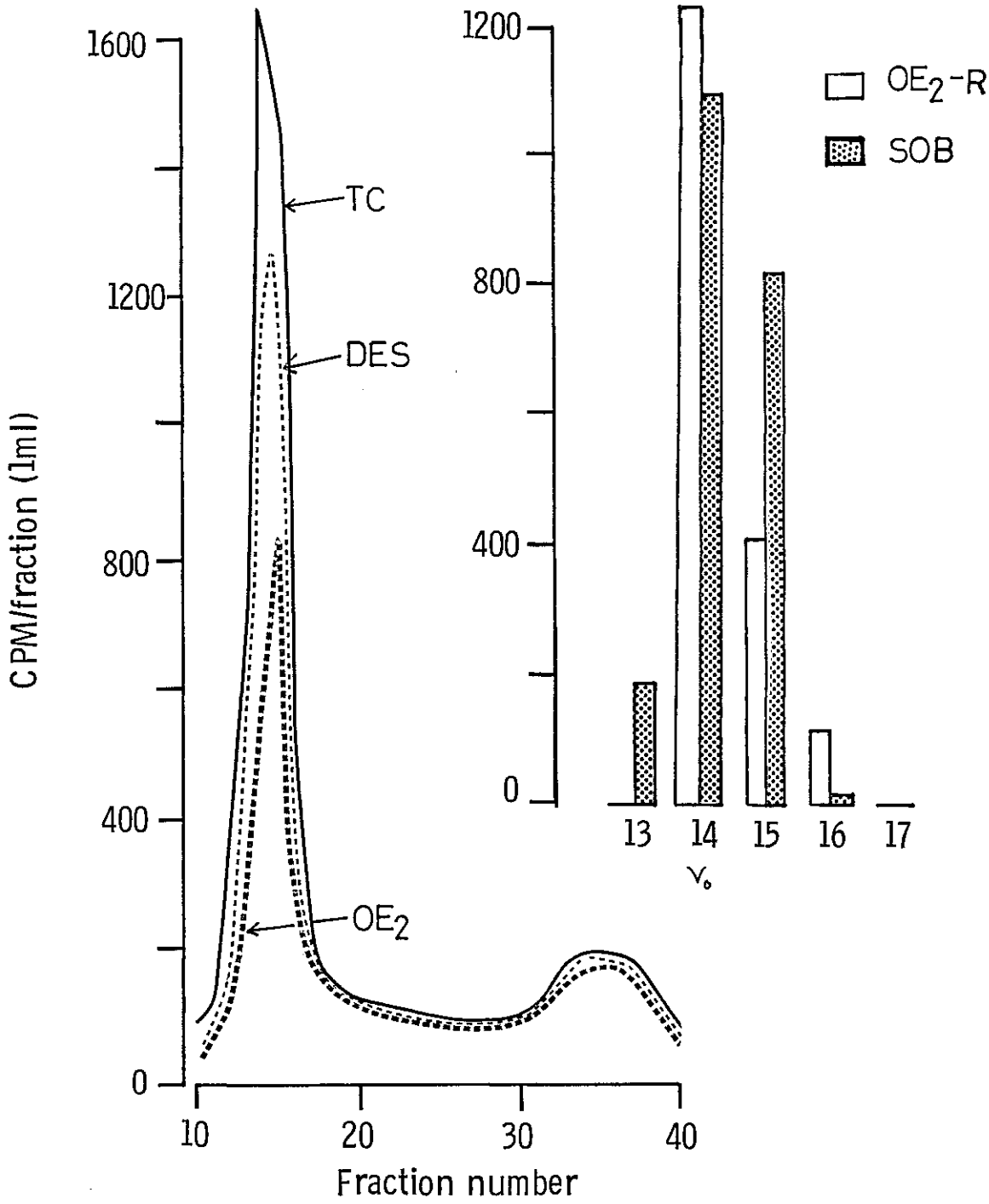


Fig.3.37. Analysis of granulosa cell cytosol oestrogen binding species on Sephacryl S-300 in no salt. DCC-treated cytosol was labelled with 10nM [^{3}H]-OE_2 in the absence(TC) or in the presence of 1μM DES or OE_2 at 4°C for 18 hr. Samples(0.5ml) of Sephadex LH-20 chromatographed cytosol were fractionated on Sephacryl S-300 using TED buffer containing no KCl under 180 cm water pressure. Forty 1 ml fractions were collected over 2.5-3 hr.Column void volume was determined using Blue Dextran 2000. Gel exclusion size = 1.5×10^6.

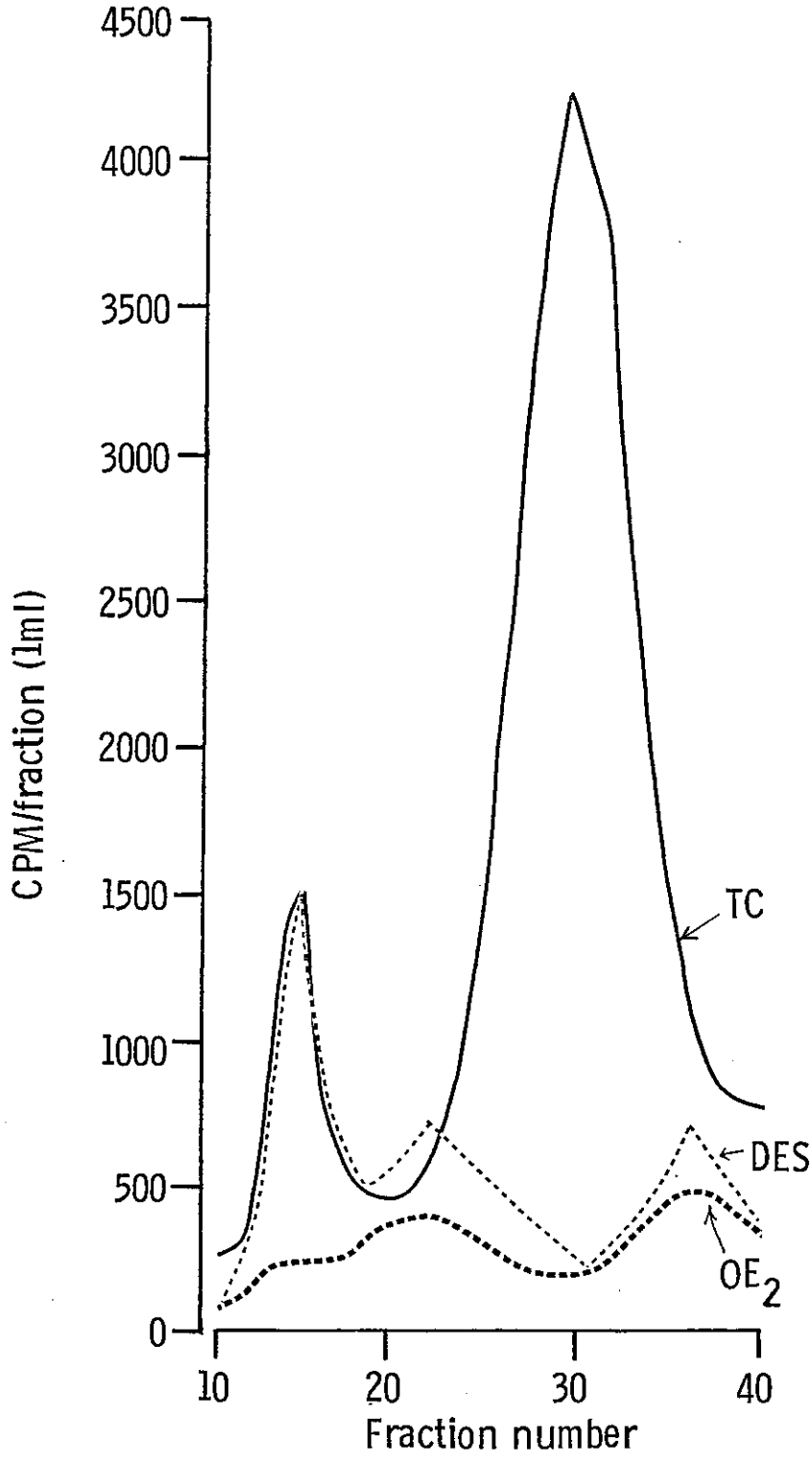


Fig.3.38. Analysis of granulosa cell cytosol oestrogen binding species on Sephacryl S-300 under high salt conditions. DCC-treated cytosol was labelled with 10nM [^{3}H]-OE_2 in the absence (TC) or presence of 1uM DES or OE_2 for 18 hr at 4°C. Samples (0.5 ml) of Sephadex LH-20 chromatographed cytosol were fractionated on Sephacryl S-300 using TED buffer containing 0.4M KCl under 180 cm of water pressure. Forty 1 ml fractions were collected over 2.5-3 hr. Gel exclusion size = 1.5 x 10^6.

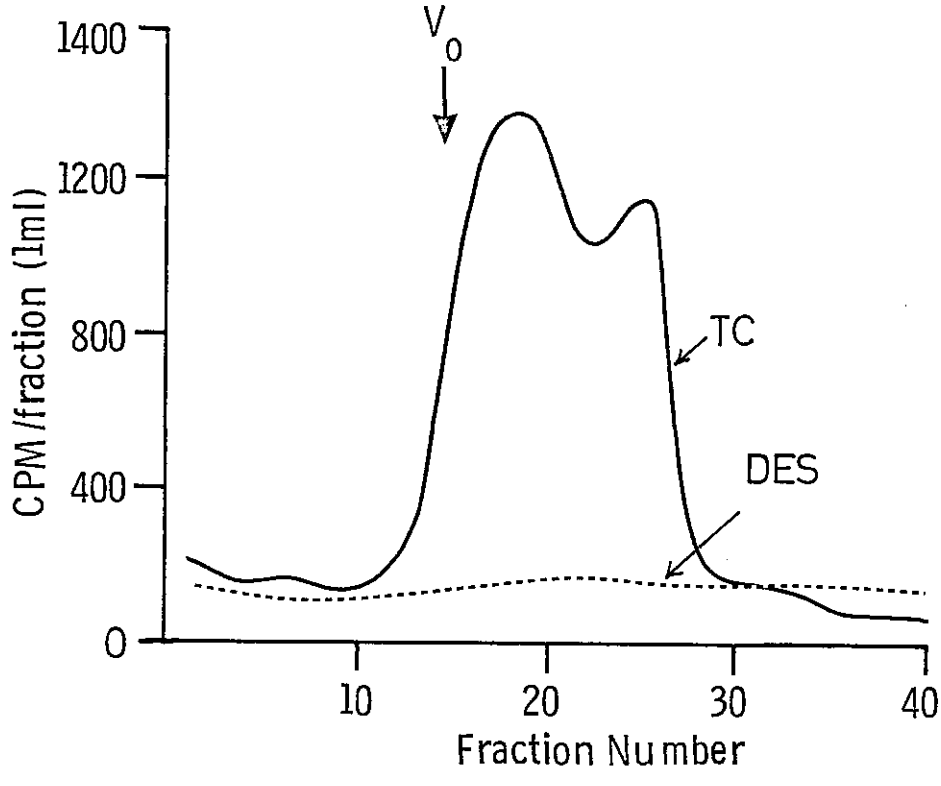


Fig.3.39. Analysis of mature rat uterine cytosol oestrogen receptor on Sephacryl S-300 in buffer containing no salt. Cytosol was labelled with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES for 18 hr at 4°C. Sephadex LH-20 chromatographed samples(1.2ml) were fractionated on Sephacryl S-300 with degassed TED buffer under 180 cm of water pressure at 4°C.Forty 1 ml fractions were collected and radioactivity counted. Gel exclusion size = 1.5 x 10^6 and the column void volume was determined using Blue Dextran 2000(m.w. 2 x 10^6).

3.28.1 Time course of Labelling with [^{3}H]-oestradiol

Granulosa cell nuclear suspensions were incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES. As exchange of endogenous steroids for [^{3}H]-oestradiol on receptor sites takes place slowly at 0 - 4°C (Clark et al.,1982) total exchangeable receptor content at 0 to 4°C was estimated after 18 hr. Nuclear oestrogen receptor concentration was also estimated following incubation with [^{3}H]-oestradiol at 4, 25 or 37°C for 1 hr. It was observed that increasing the temperature of incubation enhanced the rate of exchange so that maximum receptor content was measured at 37°C (Fig 3.40). No significant difference was found between the receptor measured at 37°C for 1 hr and 4°C for 18 hr. Therefore, for routine assays total nuclear oestrogen receptor content was estimated at 37°C with 1 hr incubations.

3.28.2 Occupied and 'Unoccupied' Nuclear Binding Sites

Incubations performed at 4°C for 1 hr are thought to measure unoccupied receptor sites although alternatively they may be measuring exchange of [^{3}H]-oestradiol with low affinity ligand on the receptor. These 4°C sites amounted to 70% of the total (unoccupied plus exchangeable) nuclear oestrogen receptor sites measured at 37°C (Fig 3.40) in granulosa cell nuclei. Statistical analysis (Student's t - test) showed that the population of "unoccupied" receptor sites' was significantly different from the total receptor sites available.

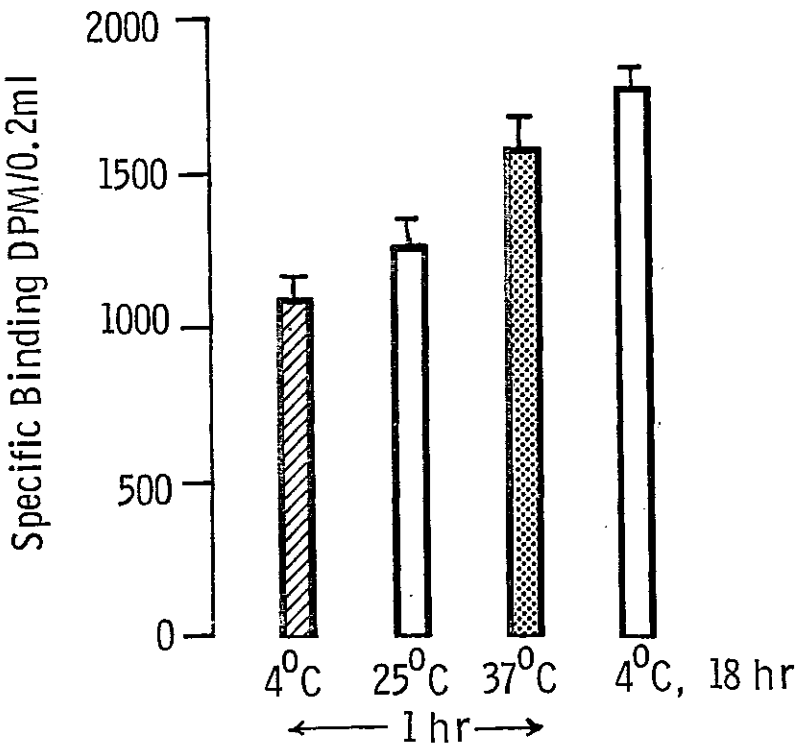


Fig.3.40. Estimation of occupied and unoccupied nuclear oestrogen receptor in the granulosa cell. Triplicate aliquots of nuclear fractions (0.2 ml) were incubated with 10nM [^{3}H]-oestrdiol with or without 1μM DES at 4, 25 and 37°C for 1 hr or for 18 hr at 4°C. Specific binding was assessed as described in the materials and methods. The experiments were repeated three times (values = mean ± S.E.M.). Each experimental group contained 15-20 rats, aged 26-28 days. Total receptor [▩] was significantly different from the unoccupied sites [▨] at $p<0.001$.

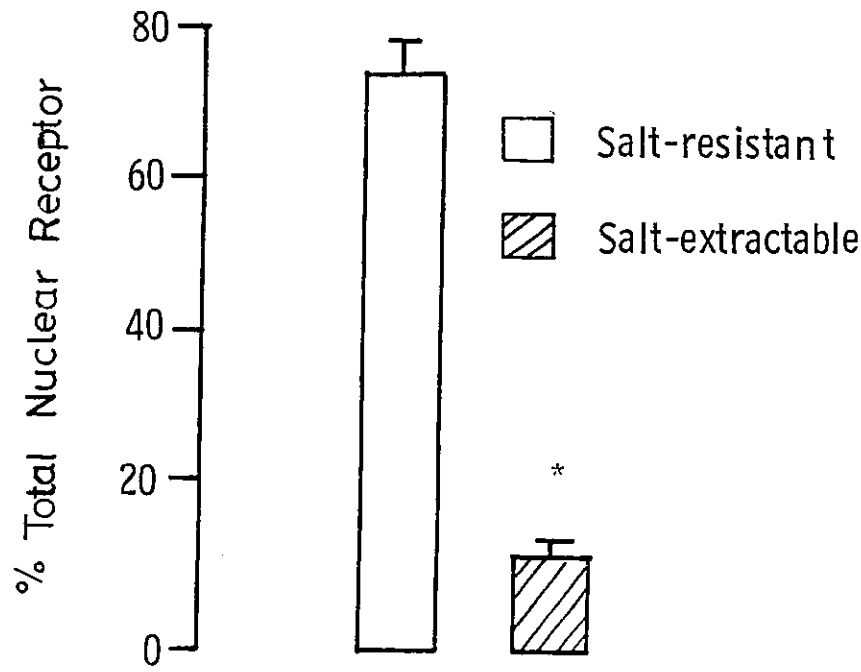


Fig.3.41. Estimation of granulosa cell salt-resistant nuclear oestrogen receptor. Granulosa cell nuclear fraction was divided into two portions: Total oestrogen receptor was assyed in one portion at exchange conditions(37°C,1 hr) as described in the materials and methods. Oestrogen receptor was extracted from the other portion with 0.4M KCl/TED buffer at 4°C for 1 hr. The salt- resistant receptor was assayed in the residual nuclear pellet at 37°C for 1 hr and the salt-extracted receptor assayed at 4°C for 18 hr with 10nM [^{3}H]-OE_2 (with DES as competitor) in all cases. Determinations were conducted in triplicate and the experiments repeated three times(values = mean ± S.D.). Each experiment contained 15 rats,aged 28 days. (*) receptor concentration was significantly different at $p<0.001$ from total receptor.

3.28.3 Salt Extraction of Nuclear Oestrogen Receptor

Granulosa cell nuclear oestrogen receptor was extracted from the nuclei using 0.4M KCl exposure for 1 hr at 4°C. The extracted receptor was then incubated with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES 18 hr at 4°C. It was observed that only 10% of the measurable total nuclear oestrogen receptor (1 hr, 37°C) content could be extracted (Fig 3.41). Incubation of the residual nuclear pellet after KCl extraction with 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES at 37°C for 1 hr, showed that 75% of the nuclear oestrogen receptor was still measurable and therefore resistant to KCl-extraction. Statistical analysis showed that the residual nuclear receptor content was significantly different from the total nuclear oestrogen receptor content ($p< 0.05$). The proportion of salt-resistant receptor in the granulosa cell nuclei is therefore higher than that reported for the rat uterine nuclear oestrogen receptor (10 to 20%) (Clark & Peck, 1976).

3.29 Ontogeny of Oestrogen Receptor in the Granulosa Cell

Granulosa cells proliferate in response to oestrogen stimulation (Richards, 1980). Therefore the oestrogen receptor content per granulosa cell must reflect both the change in oestrogen receptor sites and the modifying influence of cell division. These granulosa cells are unlike brain cells (which do not proliferate in response to oestrogenic stimulation) where the

changing oestrogen receptor content can be determined with certainty. The granulosa cells are however similar to the uterine cells which undergo a limited amount of cell proliferation but a pronounced hypertrophy. To study the ontogeny of the granulosa cell oestrogen receptor system, rats aged from 8 days to 42 days were used. The receptor data has been presented as receptor concentration per mg DNA, per cell, per mg cytosol protein or as receptor sites per cell.

3.29.1 Estimation of Oestrogen Receptor Sites Per Cell

To calculate the concentration of oestrogen receptor sites per cell, each granulosa cell has been assumed to contain a haploid genome, each receptor has only one steroid binding site and all granulosa cells irrespective of the age of the animal from which they were isolated, contain 8 pg DNA.

3.29.2 Estimation of Granulosa Cell Content per Ovary during Postnatal Development of the Rat

Cell number was computed by determining the DNA content of granulosa cell nuclear suspensions assuming that each granulosa cell contained 8pg DNA (section 3.3). Ovaries removed from rats aged 8 days were found to contain 1.6×10^5 granulosa cells. A significantly higher number could be isolated as the animals grew older: $2.3 \pm 0.4 \times 10^5$, $7.8 \pm 1.7 \times 10^5$, $1.44 \pm 0.29 \times 10^6$, $1.90 \pm 0.16 \times 10^6$ and $2.59 \pm 0.24 \times 10^6$ were isolated on days 12, 15, 18, 19 and 20

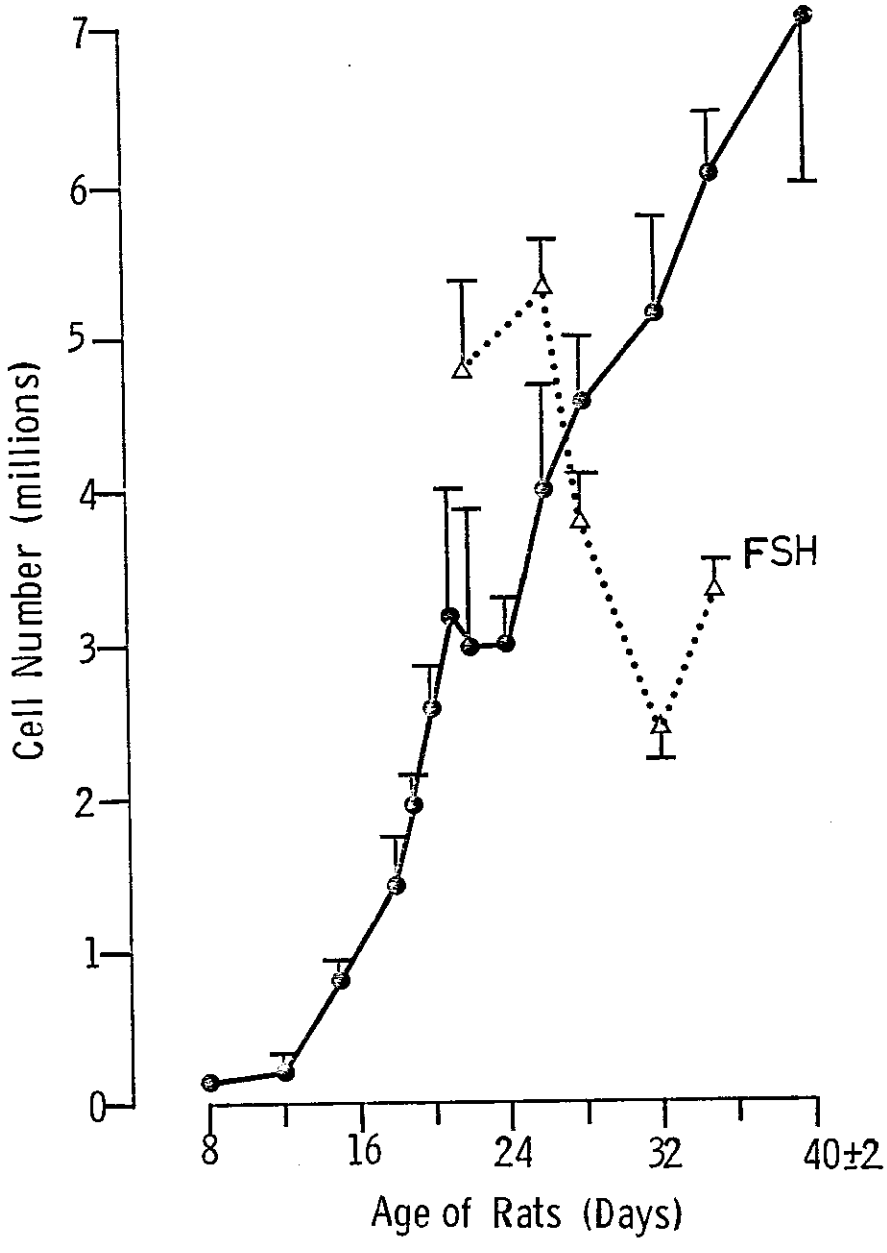


Fig.3.42. Granulosa cell proliferation during postnatal development of the ovary. Granulosa cells were isolated from ovaries removed fromrats aged 8 days to the day of vaginal opening(day 40 ± 2). Some rats aged 20 days and over were injected with 5 I.U. FSH/LH mixture(70:30%) intraperitoneally for two consecutve days prior to sacrifice. DNA content of nuclear fractions (prepared for receptor analysis) was determined using the method of Burton(1956) and the granulosa cell number estimated assuming that each cell contained 8pg DNA(see Fig.3.1.). Determinations were conducted in duplicate and experiments repeated four times(values = mean ± S.D.). Each experimented group contained 8 rats except for those aged 8 and 12 days which contained 14 rats/group.

respectively. Significant differences could be found for the granulosa cell content of ovaries removed from rats aged between 20 and 32 days (Fig 3.42). On day 35, 6.05 ± 0.40 x 10^6 cells were isolated a number which was not significantly different from the granulosa cell content of ovaries removed from rats on the morning of vaginal patency (40 ± 2 days). The general tendency however, was towards an increase in cell number with increasing age of the animals.

3.30 Nucleo-Cytoplasmic Distribution of Granulosa Cell Oestrogen Receptor during Postnatal Development

Granulosa cells were removed from the animals (sacrificed by cervical dislocation if older than 12 days, or decapitation if 8 or 12 days old) between 10.00 a.m. and 12.00 midday. A time interval of approximately 3 hr is required to isolate granulosa cells from 20 to 25 rats. After the preparation of cytosol and nuclear suspensions, samples were kept for determination of cytosol protein and DNA content respectively. Cytosol oestrogen receptor content was estimated using 10nM [^{3}H]-oestradiol in the presence or absence of 1μM DES with the cytosol (pretreated with DCC) being incubated for 18 hr at 4°C. Macromolecular bound [^{3}H]-oestradiol was separated from free steroid using Sephadex LH-20 chromatography. Nuclear oestrogen receptor content was estimated using 10nM [^{3}H]-oestradiol with or without 1μM DES and incubation of the nuclear suspension at 37°C for 1 hr.

3.30.1 Cytosol Oestrogen Receptor Content during Postnatal Development

3.30.1a Sites per cell or Femtomoles per mg DNA

About 35,000 fmol/mg DNA (approximately 170,000 sites/cell) of cytosol oestrogen receptor were measured in granulosa cells isolated from rats aged 8 days. Because of homogeneity of the granulosa cell population, the changes in receptor concentration were similar when the receptor content was expressed as sites per cell or as fmol per mg DNA. As the animals grew older the receptor concentration rapidly declined (Fig 3.43). On day 15, the cytosol receptor content was 15,579 ± 3788 fmol/mg DNA (75,074 ± 18,269 sites/cell) (n = 4). No significant differences could be found between the receptor content in the granulosa cell cytosol on day 18 (5704 ± 1123 fmol/mg DNA; 29,766 ± 5403 sites/cell) and on day 19 (4352 ± 1450 fmol/mg DNA; 20,928 ± 6917 sites/cell). On day 20 a significant rise of receptor concentration was observed (8732 ± 2005 fmol/mg DNA; 40,512 ± 7664 sites/cell) ($p < 0.001$). A significant decline ($p < 0.001$) took place on day 21 2797 ± 690 fmol/mg DNA; 14,078 ± 2504) to a level which was maintained on day 22. On day 24, the cytosol receptor content was 1438 ± 267 fmol/mg DNA (6925 ± 1289 sites/cell) which was significantly less than the receptor concentration on day 22 ($p < 0.01$). No significant difference was found between cytosol receptor concentrations on days 24 and 26. Between days 28 and 35, the cytosol receptor

content declined from 2245 ± 94 fmol/mg DNA (10,822 ± 440 sites/cell) to 785 ± 155 fmol/mg DNA (3788 ± 750 sites/cell). The cytosol receptor concentration in granulosa cells isolated from rats on the morning of vaginal opening was 1477 ± 74 fmol/mg DNA (7120 356 sites/cell) representing a significant increase ($p < 0.001$) over the receptor concentration observed on day 35.

3.30.1b Cytosol Receptor Content (fmol/mg Cytosol Protein)

Granulosa cell cytosol receptor concentration on day 8 was found to be 17,600 ± 9433 fmol/mg cytosol protein ($n = 4$) which was significantly higher ($p < 0.025$) than the concentration on day 12 (6590 ± 728 fmol/mg cytosol protein). From day 15 to day 18, the receptor concentrations were significantly reduced from 5559 ± 2085 to 2116 ± 366 fmol/mg cytosol protein ($p < 0.005$).

As shown in Fig 3.44, the level of cytosol receptor concentration was maintained on day 19 but the receptor concentration increased significantly on day 20 (4301 ± 742 fmol/mg cytosol protein) over the granulosa cell cytosol oestrogen receptor content observed on day 19 ($p < 0.005$). On day 21, the cytosol receptor concentration fell to 1785 ± 524 fmol/mg cytosol protein, a level which remained unaltered through to the morning of vaginal patency.

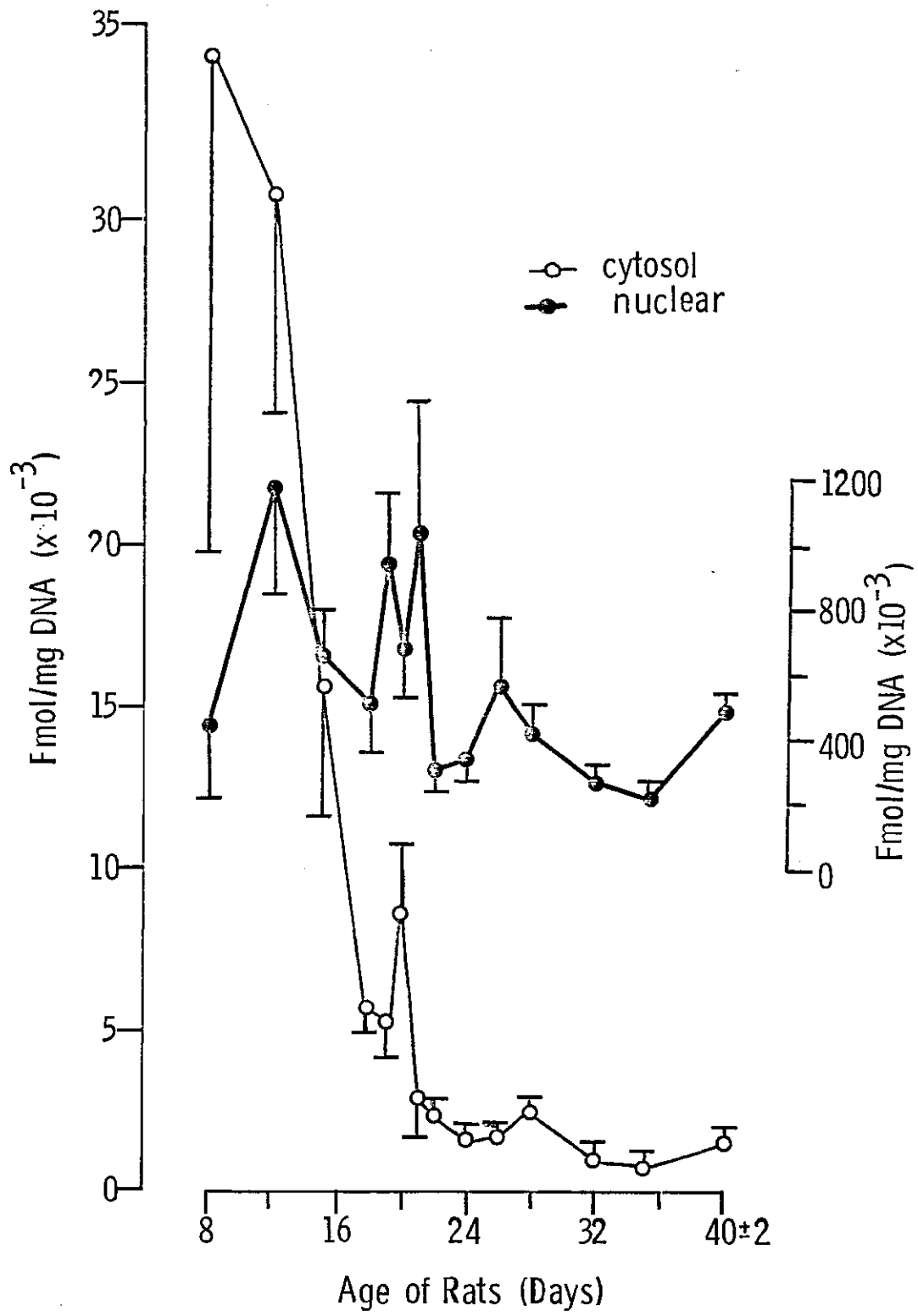
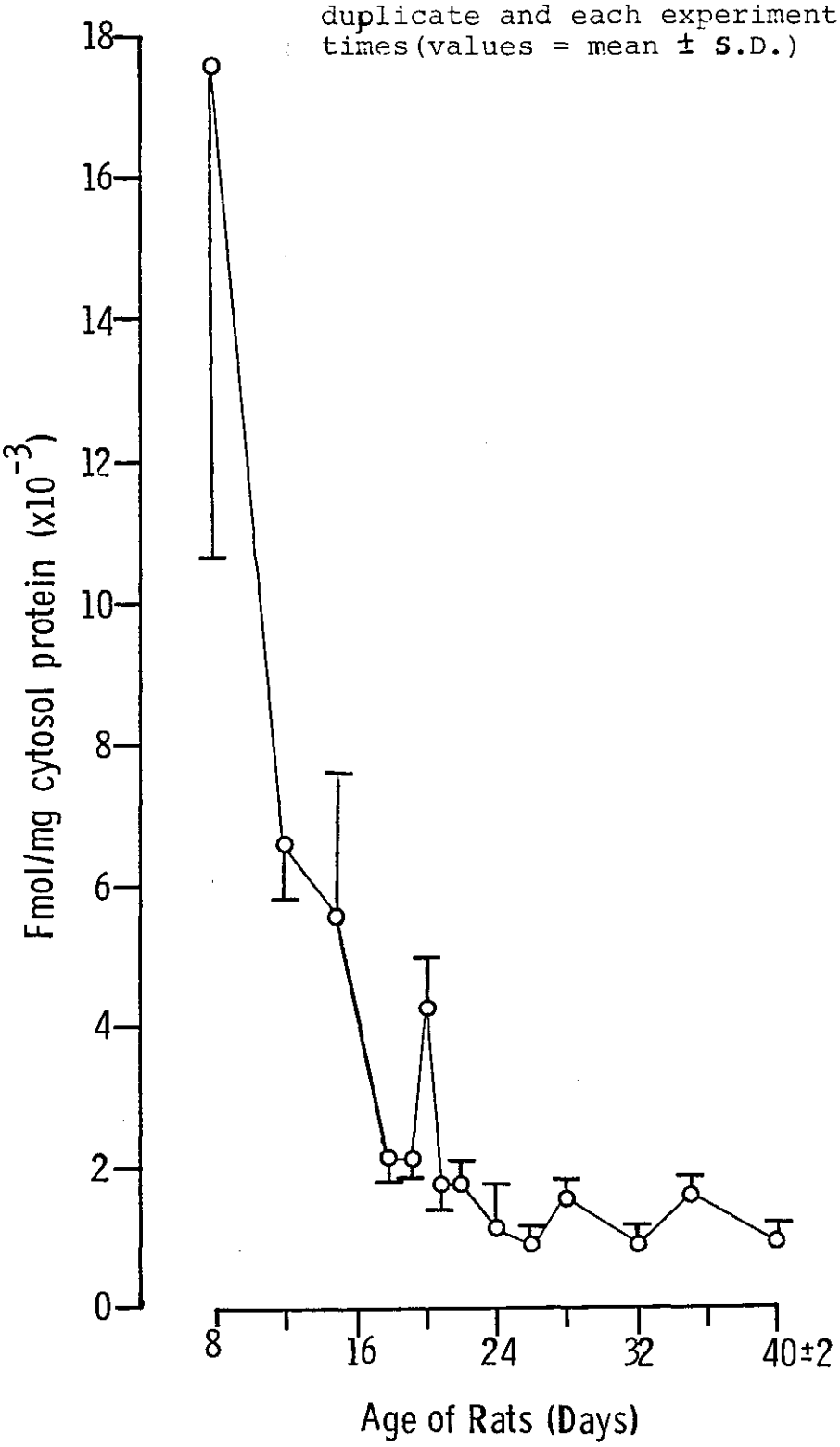


Fig.3.43. Nucleo-cytoplasmic distribution of oestrogen receptor in granulosa cells during postnatal development of the ovary. Oestrogen receptor was assayed in both the cytosol and the nuclear fraction of the granulosa cell for each group of rats as described in the materials and methods. Each experiment was repeated four times (values = mean ± S.D.)

Fig.3.44. Granulosa cell cytosol oestrogen receptor per mg cytosol protein during postnatal development of the ovary. Cytosol receptor was assayed as described in the materials and methods. Cytosol protein concentration was determined using the method of Lowry et al. (1951). Each experiment was conducted in duplicate and each experiment repeated four times (values = mean ± S.D.)



3.30.2 Nuclear Oestrogen Receptor Content during Postnatal Development

The concentration of nuclear oestrogen receptor in granulosa cells increased significantly between days 8 and 12 when 422 ± 196 fmol/mg DNA (2034 ± 940 sites/cell) and 1163 ± 292 fmol/mg DNA (5398 ± 1468 sites/cell) were measured respectively ($p < 0.001$, $n = 4$). As shown in Fig 3.43, the nuclear receptor concentration fell on days 15 ($p < 0.05$) and 18. Between days 19 and 20 the receptor concentration was maintained at 674 ± 153 fmol/mg DNA (3249 ± 737 sites/cell). On day 21, the nuclear receptor increased to 1120 ± 480 fmol/mg DNA (5398 ± 2314 sites/cell) and thereafter the receptor level declined with age until on day 35, only 195 ± 60 fmol/mg DNA (939 ± 290 sites/cell) was measured. The nuclear receptor concentration in granulosa cells isolated from rats on the morning of vaginal patency significantly increased ($p < 0.005$) over the day 35 values to 475 ± 18 fmol/mg DNA (2288 ± 88 sites/cell).

3.30.3 Total Receptor Sites per Ovary

To determine to what extent the receptor content in the granulosa cell might be influenced by granulosa cell proliferation, the receptor data has also been expressed as total cytosol or nuclear receptor sites per ovary. Expression of data in this manner would establish if changes in receptor sites per ovary follow the same trend as receptor sites per cell. For comparative purposes therefore, it was assumed

that the same proportion of cells were isolated from each ovary irrespective of the age of the animal from which the ovaries were removed.

3.30.3a Total Cytosol Receptor sites per Ovary

The total cytosol oestrogen receptor per ovary increased significantly from 2.71 ± 1.45 x 10^{10} sites/ovary (n = 7) on day 8 to a peak of 1.04 ± 0.16 x 10^{11} on day 20. On day 21, the cytosol receptor concentration fell drastically to a level not significantly different from that measured on day 8 (i.e. 4.61 ± 1.72 x 10^{10} sites/ovary). The receptor level continued to fall up to day 24. From day 26 through day 28, the receptor concentration rose significantly ($p< 0.001$) to 4.97 ± 0.51 x 10^{10} sites/ovary from 3.09 ± 0.30 x 10^{10} sites/ovary. This was followed by another significant fall ($p< 0.001$) on days 32 and 35 (2.73 ± 0.30 x ^{10} and 2.29 ± 0.45 x 10^{10} sites/ovary respectively). On the morning of vaginal patency, the oestrogen receptor concentration found in the cytoplasmic compartment of the ovary rose to 4.99 ± 1.37 x 10^{10} sites/ovary, which represents a significant rise ($p< 0.01$) over the day 35 concentration (Fig 3.45).

3.30.3b Total Nuclear Receptor Sites per Ovary

The oestrogen receptor concentration in the nuclear compartment of the ovary increased from 5.64 ± 3.3 x 10^{8} sites (n = 7) on day 8 to 1.70 ± 0.37 x 10^{10} sites/ovary (n = 4) on day 21 (Fig 3.45). Equally obvious

is the fact that the nuclear receptor concentration fell drastically on day 22 to 3.95 ± 1.11 x 10^9 sites/ovary, a level which remained unaltered on day 24. On day 26, the nuclear receptor concentration increased significantly ($p < 0.025$) but decreased slowly with age until 5.80 ± 2.2 x 10^9 sites/ovary were measured on day 35. A significant increase of the oestrogen receptor concentration was found in the nuclear compartment of the ovary on the morning of vaginal opening when 1.6 ± 0.5 x 10^{10} sites/ovary were measured ($p < 0.025$) (Fig 3.45).

Comparison of the receptor data expressed as sites per cell with that expressed as sites per ovary (Figs 3.43 to 45) shows that different conclusions may be drawn. While the cytosol oestrogen receptor sites per cell appeared to decline with increasing age, the total oestrogen receptor content of the cytoplasmic compartment of the ovary remained relatively constant.

The nuclear receptor data (Figs. 3.43 & 3.45) shows that the nuclear receptor sites/cell decreased with age but the oestrogen receptor content of the nuclear compartment of the ovary appeared to increase with age.

3.30.3c Total Oestrogen Receptor Content of the Ovary (nuclear + cytosol) during Postnatal Development

Since there appeared to be relocalization of the

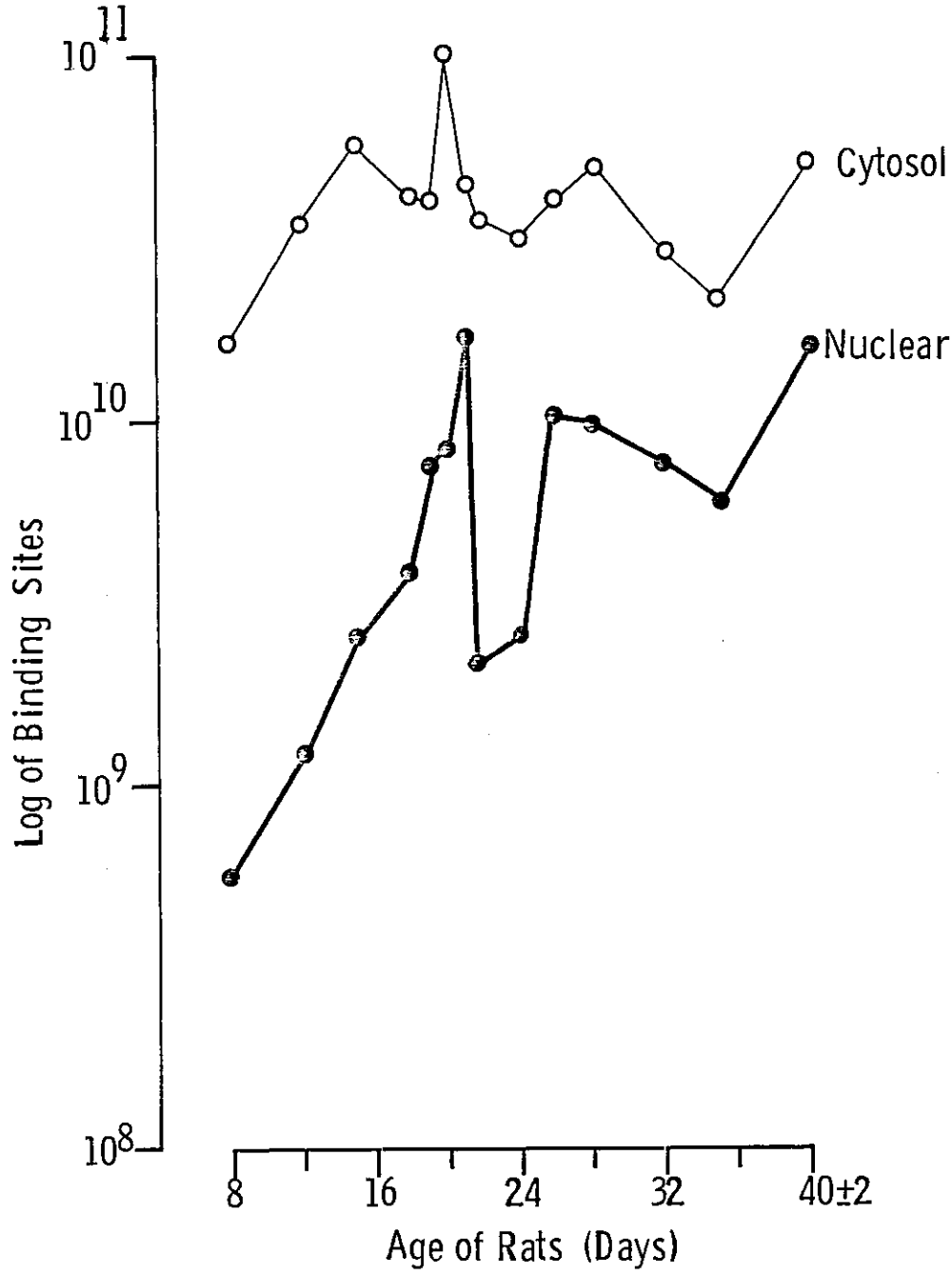


Fig.3.45. Oestrogen receptor concentration in the nuclear and cytosolic compartments of the ovary during postnatal development. The oestrogen receptor concentration in granulosa cells isolated per ovary was computed assuming that the same proportion of cells were removed from the ovay irrespective of age. See Figs.3.42 and 43.

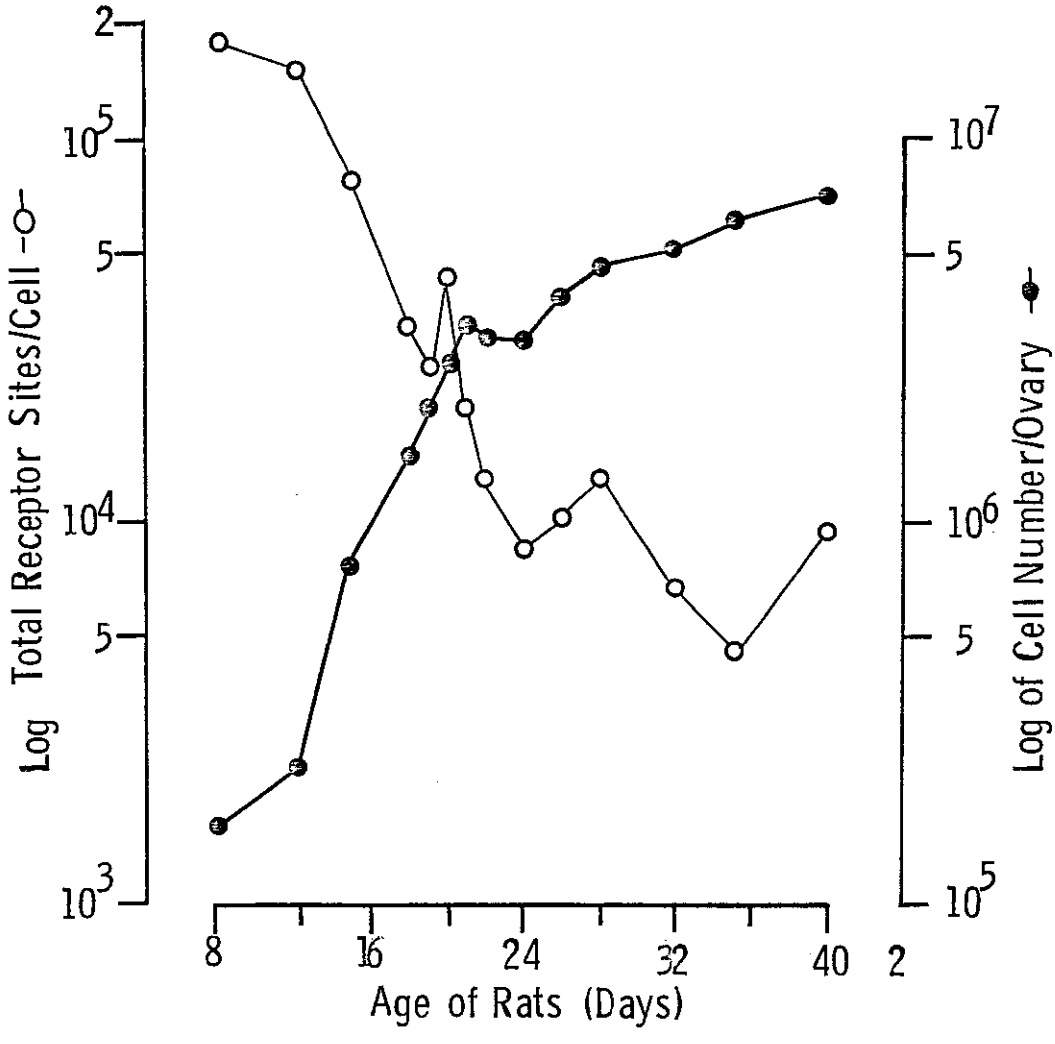


Fig.3.46. Relationship of total oestrogen receptor sites(nuclear + cytosolic) per granulosa cell and the number of granulosa cells per ovary during postnatal development. Receptor concentration and granulosa cell number were estimated as described in the materials and methods.

oestrogen receptor within the nuclear and cytosolic compartments of the cell with age (Fig 3.45) the total receptor sites in the cells (nuclear + cytosol) were determined to see if there was any change in total receptor concentration with age. This data was then compared with the changing number of granulosa cells per ovary (Fig 3.42). The total receptor sites in the granulosa cell declined with age while the cell number increased with age. A product of these two parameters gave a measure of the total receptor content (sites) per ovary which can be seen to show very little alteration with age (Fig 3.46). Thus it appeared that the oestrogen receptor content of the ovary may undergo re-distribution among dividing granulosa cells during postnatal development of the ovary.

3.31 Effect of Gonadotrophins (FSH/LH) on Intracellular Distribution of Oestrogen Receptor in the Granulosa Cell

Richards (1975) demonstrated that in 25 day old intact rats, Pregnant Mares Serum Gonadotrophin (PMSG) or FSH, given for two days stimulated follicular growth and endogenous oestrogen production. The granulosa cell nuclear oestrogen receptor content however, was observed to undergo a significant reduction as a result of the gonadotrophin injection. Goldenberg et al.,(1972) showed that oestrogen enhanced ovarian uptake of FSH and LH. The mechanism by which oestrogen might act on granulosa cells to increase their responsiveness to FSH/LH might involve

the granulosa cell FSH and LH receptors (Richards et al., 1976). Granulosa cell FSH receptor concentration remains constant during follicular development but LH receptor concentration increases with age (Uilenbroek, 1982). To determine how the gonadotrophins might act through the varying proportions of FSH/LH receptors to modify oestrogen receptor distribution in both the cytosol and the nuclei, 5 I.U. of an FSH/LH mixture (70 : 30%) was administered intraperitoneally for 2 consecutive days to rats aged 20, 24, 26, 30 and 33 days old. The rats were then sacrificed on the morning of the third day and oestrogen receptor content determined in both the nuclear and cytosolic fractions of the granulosa cells.

3.31.1 Effect of FSH/LH on Granulosa Cell Proliferation

Granulosa cell number was computed by determining the DNA content of a known volume of the nuclear suspension, assuming that each granulosa cell contained 8 pg DNA.

For rats injected on days 20 and 24 and sacrificed on days 22 and 26, $4.77 \pm 0.65 \times 10^6$ and $5.33 \pm 0.28 \times 10^6$ cells/ovary respectively, were isolated (Fig 3.41). Comparison with untreated rats aged 22 and 26 showed that FSH/LH significantly increased the number of granulosa cells per ovary ($p < 0.02$). However, fewer granulosa cells were isolated when the rats were injected on days 26, 30 and 33 and sacrificed on days 28, 32 and 35, respectively. The number of cells

isolated were 3.80 ± 0.28 x 10^6 which was not significantly different from the number isolated from 28 day old untreated rats; on days 32 and 35, 2.44 ± 0.08 x 10^6 and 3.33 ± 0.28 x 10^6 granulosa cells were isolated which, compared with untreated rats aged 32 and 35 days respectively, indicated that FSH/LH significantly reduced the granulosa cell yield ($p < 0.001$) (Fig 3.41). These results indicated that the granulosa cells might have different sensitivities to the gonadotrophin in an age dependent manner which may probably result from different proportions of FSH/LH receptors.

3.31.2 Effect of FSH/LH on Cytosol Oestrogen Receptor Content

In the FSH/LH treated animals, the cytosol oestrogen receptor concentrations were 1592 ± 108, 2818 ± 134, 7528 ± 193, 9593 ± 602 and 7879 ± 849 sites/cell for animals aged 22, 26, 28, 32 and 35 days respectively. Comparison with untreated animals of identical ages showed that the gonadotrophins significantly reduced the oestrogen receptor content on days 22, 26 and 28 ($p < 0.001$) but increased the receptor content on days 32 and 35 at the same level of significance (Table 10).

3.31.3 Effect of FSH/LH on Nuclear Oestrogen Receptor Content

The nuclear oestrogen receptor measured in rats treated with 5 I.U. FSH/LH for two consecutive days before sacrifice were 5775 ± 620, 2422 ± 85, 2506 ± 533, 3273 ± 266 and 2047 ± 240 sites/cell for animals

aged 22, 26, 28, 32 and 35 days, respectively. Comparison with the nuclear oestrogen receptor content of untreated rats aged 22 showed that FSH/LH increased the receptor concentration significantly ($p < 0.001$) (Table 10). FSH/LH produced no significant alteration of the nuclear receptor content when administered to rats aged 24 and 26 days. The gonadotrophins, however, increased the nuclear receptor concentration when 30 and 33 day old rats were injected ($p < 0.001$).

From Table 10, it can be seen that from day 20 to 21 the cytosol receptor sites/cell declined by about 65% and the nuclear receptor sites/cell increased by the same percentage. The actual cytosol receptor loss (about 26,000 sites/cell) could not be accounted for by uptake into the nucleus which increased by only 2000 sites/cell. When the 20-day olds were treated with FSH/LH and sacrificed on day 22, the cytosol receptor content reduced from about 12,000 sites to 1600 sites/cell (86% loss). The nuclear oestrogen receptor content increased 3-fold as a result of the gonadotrophin stimulation.

These results suggest that the gonadotrophins could control the intracellular movement of the oestrogen receptor in the granulosa cell possibly by stimulating endogenous steroidogenesis: LH would induce increased thecal production of testosterone, which under FSH stimulation, would be aromatized to oestradiol in the granulosa cell.

TABLE 10. Effect of FSH/LH on Granulosa Cell Oestrogen Receptor Content

Age of Rats (Days)	Oestrogen Receptor Concentration (Sites/Cell)					
	Untreated Rats (Control)		FSH/LH Treated Rats		Students 't' test	
	Cytosol	Nuclear	Cytosol	Nuclear	Cytosol	Nuclear
20	40,512 ± 7664	3249 ± 737	-	-	-	-
21	14,078 ± 2504	5398 ± 2314	-	-	-	-
22	11,720 ± 2149	1339 ± 200	1592 ± 108	5775 ± 620	$p < 0.001$	$p < 0.001$
26	7,898 ± 907	2695 ± 1031	2818 ± 134	2422 ± 85	$P < 0.001$	N.S.
28	10,822 ± 440	1958 ± 517	7528 ± 193	2506 ± 53	$p < 0.001$	N.S.
32	5,389 ± 1070	1212 ± 139	9593 ± 602	3273 ± 226	$p < 0.001$	$p < 0.001$
35	3,788 ± 750	939 ± 290	7899 ± 849	2047 ± 240	$P < 0.001$	$p < 0.001$

Rats aged 20, 24, 26, 30 and 33 days were injected with 5 I.U. of an FSH/LH mixture (70:30%) for two consecutive days before sacrifice. Cytosol and nuclear factions were prepared as usual (See Materials and Methods). Duplicate DCC-treated cytosol (0.2ml) and triplicate nuclear suspensions (0.2ml) were incubated with 10nM $[^3H]$-oestradiol in the presence or absence of 1μM DES for 18 hr at 4^oC and 1 hr at 37^oC, respectively. Specific cytosol and nuclear $[^3H]$-oestradiol binding was determined as described in the Materials and Methods. All determinations were repeated four times and each experimental group contained 8 rats.

CHAPTER FOUR

DISCUSSION

4.1. Ovarian Follicular Development

The two primary roles ascribed to the ovarian follicle are the production of gametes and the synthesis of steroids. The organisation of the follicular unit was apparent on day 8 postpartum with the appearance of granulosa cells. The origin of the granulosa cells is however still being disputed. Histological studies of the ovary of 8 day old rats revealed (Plates 1a & b) that follicles were being formed both in the cortical and medullary regions of the ovary. The granulosa cells (1 - 4 rows) were found to enclose primary oocytes which were also dispersed in the matrix of the ovary. It is possible that the granulosa cells had arisen through differentiation of some stromal cells (as suggested by Peters & Levy, 1964) and to a lesser extent from the rete ovarii (Byskov et al., 1977). The localisation of the primordial follicles made it rather unlikely that the granulosa cells could have arisen solely from the surface epithelial cells which surround the oocytes at birth (Zuckerman & Baker, 1977). The resulting follicles, each containing a primary oocyte, are thought to form a pool of small non-proliferating follicles which remain as such until an unknown signal triggers off their growth. The initial formation of the follicle apparently occurs independently of any pituitary gonadotrophin stimulation (Williams, 1940; Pencharz, 1940; Peters et al., 1975). The enclosed primary oocytes are also non-proliferating and are arrested in the diplotene stage of the first mieotic

prophase (dictyate stage of maturation) and they remain in this state until they either degenerate (as 99% of them do) or are stimulated to resume division at puberty. The inhibitory factor which arrests cell division however did not prevent growth (increase in size) of the oocyte during postnatal development (compare Plates 4 and 5).

Once the signal for the growth is received, cohorts of follicles continually leave the non-proliferating pool so that a section through the ovary at any age reveals follicles at all stages of growth. The ovary undergoes tremendous enlargement, (Plates 1, 2 and 3) due principally to increases in the number and the enlargement of some of the follicles being formed (as a result of increased mitosis of granulosa cells). The size of the granulosa cells (diameter 12-15μm) (Plates 4 and 5) however does not appear to change when examined at the light microscope level (Channing & Ledwitz-Rigby, 1975). After the formation of the primordial follicles, growth appears to become dependent on both intra- and extra ovarian control mechanisms which ensure that the requirements of the ovary are met.

Granulosa cells have been shown to be the predominant site of oestrogen synthesis (Falck, 1959; Armstrong & Dorrington, 1975; Dorrington, 1979). Indeed, oestrogen synthesis is the hallmark of the developing follicle. Oestrogen-induced granulosa cell proliferation and

consequent ovarian weight gain can be demonstrated in hypophysectomized rats (Williams, 1940; Pencharz, 1940; Payne, 1955; Goldenberg et al., 1972). Bradbury (1961) also observed a stimulation of granulosa cell mitosis in vivo after locally implanting oestrogen into the ovary. These results suggest that oestrogens might serve as one of the major intraovarian factors controlling follicular maturation.

The experiments of Greep et al., (1942) highlighted the involvement of the gonadotrophins FSH and LH on ovarian oestrogen production and consequently on follicular maturation. Goldenberg et al., (1972) demonstrated that FSH was effective in stimulating granulosa cell maturation and antral formation and its mild mitotic activity on granlosa cells may be due to an accelerated oestrogen production as FSH induces aromatizing enzyme activity (Dorrington et al., 1975; Armstrong & Papkoff, 1976; Hillier et al., 1982a). This probably explains why when administered sequentially, FSH and oestrogen act synergistically to produce a marked enhancement of granulosa cells proliferation (Goldenberg et al., 1972). LH also acts as an extra-ovarian factor controlling follicular maturation by stimulating the ovarian interstitial compartment, especially the theca cells, to secrete androgens that serve as substrate for aromatization to oestrogen in the granulosa cell (Moon et al., 1976; Armstrong & Dorrington, 1975). In vitro studies of whole mouse ovaries have shown that these two gonadotrophins (FSH and LH) act synergistically to

produce oestrogen (Ryle et al ,1974). Of the two, LH was found to be the least effective in inducing oestrogen production.

The cooperative effect of the intra- and extra-ovarian factors has led to the 'two-cell, two gonadrotrophin and two-steroid' theory of ovarian follicular development (Baird, 1977). Studies with isolated tissue preparations from several mammalian species indicate that the predominant steroids secreted in response to LH stimulation are testosterone and androstenedione (Makris & Ryan, 1975; Erickson & Ryan, 1976; Fortune & Armstrong, 1978). Direct biochemical data also indicate that granulosa cells can convert testosterone and androstenedione to oestradiol (Hillier et al, 1980) but cannot synthesize androgens from pregnenolone or progesterone (Moon et al 1975, 1978; Moor, 1977). These results laid the foundation for the view that androgens produced in the theca cell compartment must be "transported" into the granulosa cell compartment which possesses the aromatase enzyme required for oestrogen biosynthesis, a process which is performed under FSH stimulation (Armstrong & Papkoff, 1976; Erickson & Hsueh, 1978; Moon et al 1978). Apparently, granulosa cells of those follicles exposed to low concentrations of FSH fail to develop an active aromatizing enzyme system (McNatty & Baird, 1978; Hillier & Zwart, 1982a).

4.2. Granulosa Cell Proliferation during Follicular Development

Since the granulosa cell is the predominant site of ovarian oestrogen biosynthesis and cell division is

commonly accepted as the end product of oestrogen action in these cells, it is to be expected that an increasing level of endogenous oestrogen would induce granulosa cell proliferation. Indeed, increasing numbers of granulosa cells per ovary were isolated from rats aged from 8 days to those killed on the morning of vaginal patency (Fig. 3.42). Histological studies revealed that over 90% of the granulosa cells could be removed using the procedure described, which demonstrates that the efficiency of the method is as good as any described in the literature for isolation of granulosa cells from the rodent ovary (Zeleznik et al., 1974; Rao et al., 1978).

Oestrogen production in the rat ovary is reported to begin 8-10 days after birth, after the assemblage of the necessary apparatus, i.e. granulosa cells, theca cells and cholesterol (Price, 1947; Falck, 1953, 1959; Presl et al., 1968). Available data on circulating gonadotrophin and steroid levels in the rat between birth and the first pro-oestrus (Fig. 4.1, derived from Döhler and Wuttke, 1975) shows that very high levels of total circulating oestradiol (200-400pg/ml) are present at birth but these fall to 60-80pg/ml on day 5. The next rise in oestradiol concentration ($\simeq$ 200pg/ml) occurs on day 9 and this level is sustained until day 23. On the morning of vaginal opening (day 39-40), another rise in oestradiol concentration was observed. It has been suggested that these increases in oestradiol are the product of the high levels

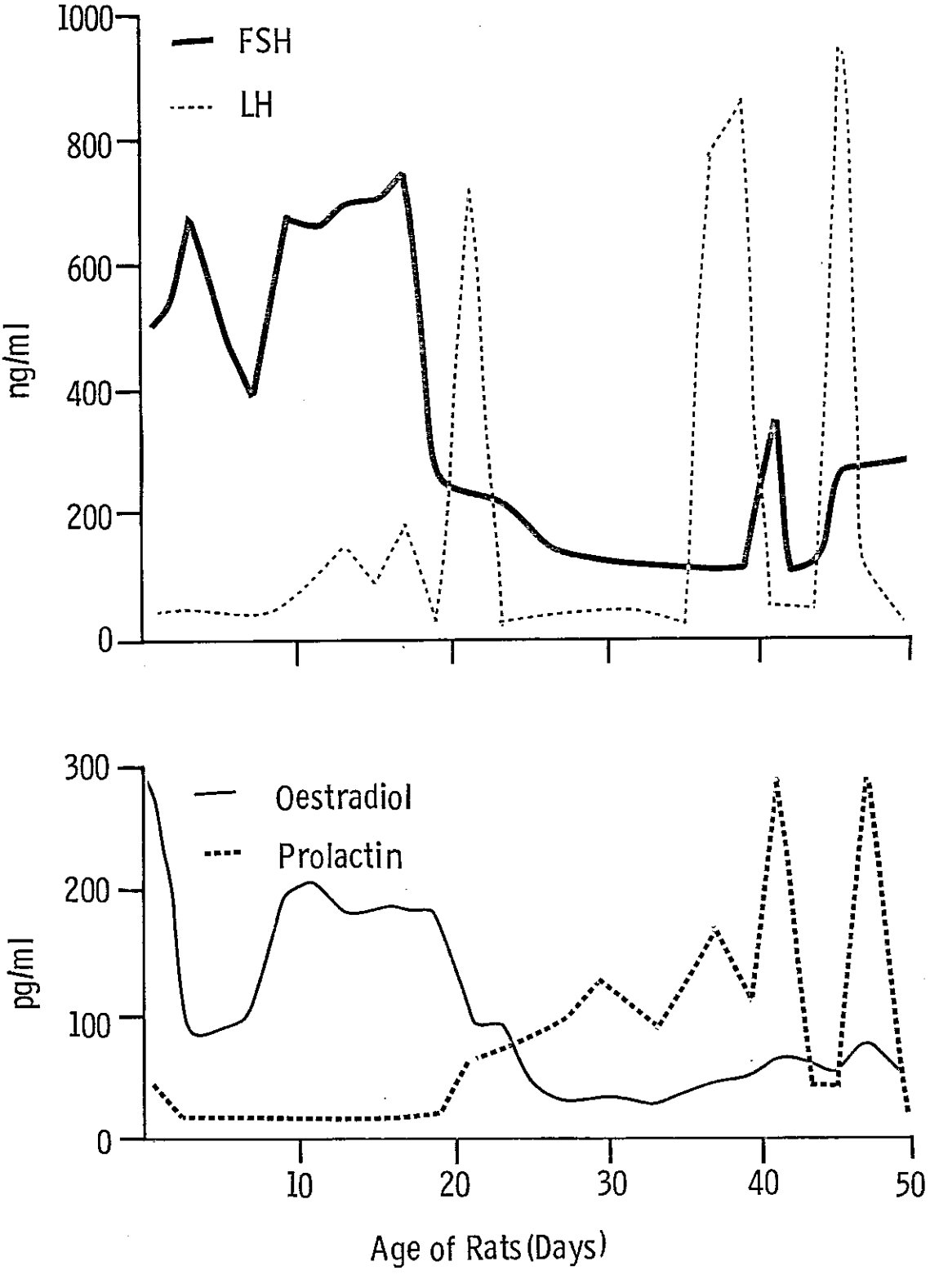


Fig.4.1. Circulating levels of FSH,LH, Prolactin and Oestradiol in the rat during postnatal development (derived from Döhler & Wuttke,1975)

of FSH and LH. At birth approximately 500ng/ml of FSH is present and this high level is maintained up to day 17 when 800-1000ng/ml are present. High levels of LH are observed between days 11 and 17 (80-200ng/ml). Because no granulosa cells are present in the ovary prior to day 8, the high circulating levels of oestradiol in the new born rat must have come from the maternal circulation. The rise in oestradiol concentration after day 9 may have arisen from synthesis in the ovary and in turn may have provoked the increase in granulosa cell number on day 12 compared to the day 8 yield. Significant increases in cell number were observed with increasing age after day 12 until day 4o ± 2. Between day 22 and 32 the FSH level was low (less than 200ng/ml), LH levels were also very low (less than 50ng/ml) but prolactin levels had risen tremendously. This might explain the low level of oestradiol (less than 40pg/ml) found at this time as Dorrington & Gore-Langton (1981) have shown, with cultured granulosa cells, that prolactin inhibited oestrogen biosynthesis. In this respect prolactin may be considered to exert an antimitotic effect on granulosa cells. The increased granulosa cell yields between day 35 and the day of vaginal opening might be due to an over-riding mitogenic effect of oestrogen, the concentration of which increases in the general circulation (Dohler & Wuttke, 1975).

To determine the role of FSH and LH during postnatal development of the ovary, 10 I.U. of an FSH/LH mixture (70:30%) was administered over two days to rats aged

from 20 days onwards. This dose regimen allows the ovaries to be exposed to FSH/LH over a 48 hour period when circulating levels of FSH *in vivo* drop sharply from about 800ng/ml on day 17 to 250ng/ml on day 19, a level which is maintained until day 47 (Fig. 4.1).

In rats injected on days 20 and 24, FSH/LH increased granulosa cell proliferation which was apparent when the granulosa cells were isolated on days 22 and 26 (compared to uninjected rats of identical ages). This was probably due to an increase in ovarian oestrogen biosynthesis under FSH/LH stimulation resulting in mitosis (Fig. 3.42). In rats aged 26 days and over, the gonadotrophin administration caused a reduction of the granulosa cell yield which was possibly a reflection of an inhibition of oestrogen biosynthesis.

Studies in rats (Richards & Midgley, Jr. 1976; Rajaniemi *et al.*, 1977) have shown that granulosa cells possess FSH receptors and acquire LH receptors later under oestrogen induction. LH binding to the granulosa cells then results in their differentiation into luteal cells (accompanied by LH and FSH receptor loss) which in the presence of prolactin and LH will secrete progesterone. Carson *et al.*, (1978) have shown that granulosa cells from large ovine follicles possess fewer FSH binding sites than those from small and medium sized follicles and showed morphological atresia. Moor *et al.*, (1978) have suggested that it is a decrease in oestrogen biosynthesis which predisposes the granulosa cells to atresia and as FSH is required for oestrogen biosynthesis by granulosa

cells (Dorrington et al, 1975) the loss of FSH binding sites from granulosa cells might militate against oestrogen production. This probably explains why FSH/LH injection to rats older than 26 days resulted in a reduction in granulosa cell number. It is likely that the FSH/LH injection may have led to selection of some follicles for ovulation thereby committing a substantial number of other follicles to undergo atresia. Another possibility exists which might explain the antimitotic effect of FSH/LH after day 26. The gonadotrophin stimulation may have led to great increases in local oestrogen concentration and this may then have triggered off a negative feedback reaction leading to an accumulation of androgens and possibly the anti-mitotic effect. Since prolactin levels too are very high during this period, this would enhance inhibition of oestrogen synthesis and the antiproliferative activity in the granulosa cells. This situation is probably consistent with the report of Rao et al., (1978) in which oestrogen-primed hypophysectomized 28 day old rats when injected once with 2μg FSH showed granulosa cell proliferative activity but an additional day of injection reduced granulosa cell proliferation. These workers also found that although oestrogen alone, when given for 2 days to 28 day old rats, increased granulosa cell proliferation, 4 days of treatment reduced granulosa cell number. Thus each hormone exhibited a limited capacity to evoke a proliferative response in the granulosa cell probably because the cells are continually undergoing degeneration.

Experiments in the uterine epithelium of the ovariectomized mouse show that the presence of oestrogen stimulates cell proliferation by shortening the cell cycle, presumably by stimulating biochemical events which take place in the prereplicative (G_1) and the DNA synthetic (S) phases (Epifanova, 1966; Das, 1972; Stormshak et al., 1978). Administration of chronic levels of oestrogen however produces an antimitotic effect presumably due to the accumulation of some product (chalones) which limits the ability of the uterine cell to respond to the oestrogen (Bullough, 1946,1973). Sustained oestrogen also suppresses DNA polymerase and DNA synthesis (Stormshak et al., 1976) and probably the synthesis of induced protein (IP) (Katzenellenbogen, 1975). The end-product of this antimitotic effect of oestrogen appears to be a blockage of the target cell's capacity to pass from the resting or pre-synthetic phase into the S phase of the cell cycle (Stormshak et al., 1976). The anti-proliferative effect of FSH/LH on the granulosa cells, which presumably is mediated via oestrogen production in the ovary, therefore is in agreement with oestrogen action in other target tissues.

4.3 The Phenomenon of Atresia during Follicular Development

Although many follicles advance beyond the primordial stage it is considered that the predominant fate of a follicle is to degenerate (Weir & Rowlands, 1977). The physiological and biochemical events associated with follicular atresia are still not well understood and therefore it has been difficult to evolve a unifying concept about the phenomenon.

Arai (1920) determined that between birth and day 20, approximately 1000 follicles degenerate each day in the rat. Granulosa cells isolated from morphologically atretic follicles are characterised by a reduction or absence of [^{3}H] -thymidine incorporation (suggesting an inhibition of DNA synthesis and cessation of mitotic activity (Byskov, 1979)) pycnotic nuclei and acid phosphatase activity (Greenwald, 1974) and failure to exclude trypan blue dye *in vitro* (Channing *et al.*, 1978). Since this degeneration process occurs concomitantly with follicular growth, it is conceivable that a fair proportion of the cells isolated could come from atretic follicles and be at various stages of cell necrosis. Many studies have implicated ovarian androgens as the sole inducers of atresia (Payne & Runser, 1958; Pencharz, 1940; Louvet *et al.*, 1975; Richards *et al.*, 1976; Saiduddin & Zassenhaus, 1978). Since ovarian androgens are precursors for oestrogen biosynthesis in the ovary, it would appear that a delicate control over factors which determine androgen accumulation in the ovary is imperative during postnatal follicular development. The novel oestrogen-androgen binding species, termed the second-oestrogen binding (SOB) species of the granulosa cell might serve to control the androgen-oestrogen balance in the cell and therefore participate in follicular degeneration/growth.

Administration of FSH/LH would inevitably affect the androgen/oestrogen balance in the ovary. On day 21, the serum LH level is about 4 to 5 times higher than the

serum FSH level. Therefore injecting a 70:30% FSH/LH mixture (total 10 I.U.) at this time might reverse the normal gonadotrophin-induced changes taking place at this time by possibly reducing androgen production but enhancing oestrogen biosynthesis. This is reflected in increased cell number on days 22 and 24. The reduced cell number observed when the animals were injected after day 24 might probably be due to a proposed intrafollicular negative feedback effect: overstimulation of the aromatase activity would lead to enhanced oestrogen production. High levels of oestrogen will then switch off androgen production and therefore only limited amounts of androgen substrate are available for oestrogen biosynthesis. Such a mechanism has already been suggested for the porcine ovary by Leung et al., (1979a,b) in which high levels of oestradiol inhibited LH-induced ovarian androgen levels both in vivo and in vitro. It has therefore been suggested that not only does the theca interact with the granulosa cell through the production of androgen but also oestrogen production in turn controls the production of androgen substrates.

4.4. Characterisation of the Ovarian Oestrogen Receptor System

Oestradiol -17β is retained against a concentration gradient within organs such as the uterus because of the properties of a highly specialised receptor system (Jensen & Jacobson, 1962; Stone, 1963). Autoradiographic and quantitative biochemical binding studies have shown that, in addition to synthesising oestrogen, the ovary also possesses oestrogen receptors (Stumpf, 1969; Saiduddin & Zassenhaus, 1977,1978). Because oestrogen

is highly mitogenic towards granulosa cells and it is synthesised in the granulosa cell (Moor, 1977; Fortune & Armstrong, 1977,1978; Anderson et al.,1978) it is possible that there is a regulatory mechanism to prevent the ovary from being under constant oestrogen stimulation and undergoing perpetual hyperplasia.

Over 90% of the immature rat ovarian cytosol oestrogen receptor, measured using DES as competitor, was found to be occupied with endogenous steroids (Fig. 3.8). Saturation analysis and Scatchard plots of oestrogen binding in day 28 rat ovarian cytosol, pretreated with DCC to remove endogenous steroid, revealed a single class of oestrogen binding species which was saturated at 4nM [^{3}H] -oestradiol. The apparent dissociation constant was 0.39nM and the binding capacity was 350fmol/mg cytosol protein (Fig. 3.2). When DES was employed as competitor in the [^{3}H] -oestradiol exchange assay, a single class of oestrogen binding sites was observed in the ovarian nuclear preparation. Saturation was observed at 8nM [^{3}H] -oestradiol and Scatchard analysis showed that the apparent equilibrium dissociation constant was 0.35nM and the binding capacity, 208fmol/mg DNA (Fig. 3.3). These values are in agreement with those in other oestrogen target tissues. However, the literature on the ovarian oestrogen receptor system is scanty. The values for dissociation constants agree with the studies of Saiduddin & Zassenhaus (1977) who also showed, using several biochemical characteristics, that the oestrogen receptor systems of the immature rat ovary and uterus

were identical. The autoradiographic studies of Stumpf (1969) actually showed that [^{3}H] -oestradiol was located predominantly in the granulosa cell compartment of the ovary. Because the ovary is composed of heterogeneous tissue types and not all possess receptor binding sites, expression of the receptor binding capacity per mg DNA, per mg tissue cytosol protein or per ovarian cell is not indicative of receptor concentration amongst particular cell types. Comparison of the oestrogen receptor content of the granulosa cell fraction of the ovary with that of the whole ovary (Table 1), in conjunction with histological studies and receptor analysis of the residual ovarian tissues (Plate 8, Table 1), indicates that the ovarian oestrogen receptor is located exclusively in the granulosa cell. In fact, only 6% of the total ovarian oestrogen receptor was found in the residual ovarian tissue and this was possibly due to contamination from unremoved granulosa cells rather than from receptor present in the surface epithelial cells, as suggested by Hamilton et al (1982).

The granulosa cell system offered a very pure homogeneous cell preparation in which the sole contamination came from oocytes (Plates 4 and 5). Saturation analysis of the granulosa cell nuclear oestrogen receptor using a range of [^{3}H] -oestradiol concentrations (up to 10nM) and DES as competitor in an exchange assay at 37°C revealed only a single class of oestrogen binding sites which were saturated at 5nM [^{3}H]-oestradiol (Fig.3.4) (cf 8nM for the nuclei of the whole ovarian tissue).

Scatchard analysis revealed an equilibrium dissociation constant (Kd) and a binding capacity which was in agreement with published reports (Kd.,1.64 ± 0.92 nM; n = 440 ± 93fmol/mg DNA) for the granulosa cell (Richards, 1977) and for other oestrogen target tissues (King & Mainwaring, 1974; Buller & O'Malley, 1976; Clark & Peck, 1979). These values were also not significantly different from those measured in the whole ovarian nuclei and identical results were obtained when oestradiol was used as competitor in saturation analysis rather than DES.

The use of a granulosa cell preparation resulted in the cytosol having a low protein concentration ($\simeq$ 200µg/ml) such that the DCC techniques of separating free steroid from the macromolecular bound [^{3}H] -oestradiol could not be employed. Sephadex LH-20 chromatography proved successful and reproducible for quantification of specific oestrogen binding in the cytosol.

Treatment of the cytosol with DCC to remove endogenous steroid was mandatory prior to assay and when a range of [^{3}H] -oestradiol concentrations (up to 10nM) was used to perform saturation analysis, with DES as competitor, only one class of oestrogen binding sites was observed (Fig. 3.5). This showed saturation at 2nM [^{3}H] -oestradiol which was half the concentration required to saturate the same class of binding sites in the whole ovarian cytosol. The Kds were however similar (0.58nM). The concentration of binding sites appeared to be about 10-fold higher ($\simeq$ 2780/fmol/mg cytosol protein) compared to the

ovarian cytosol and this was no doubt due to the homogeneous population of receptor-containing granulosa cells.

Competition studies were performed using a saturating concentration of [^{3}H] -oestradiol in the presence of a 100-fold excess of various ligands, to determine the specificity of the oestrogen receptor (Tables 2 and 3). Oestradiol and DES were equally effective in displacing [^{3}H] -oestradiol from the nuclear oestrogen receptor. Oestriol and oestrone competed for 50 to 70% respectively, of the [^{3}H] -oestradiol binding while progesterone, cortisol and testosterone all competed for about 20% of the binding in the granulosa cell nuclei. The androgen metabolite DHT did not compete for any oestrogen binding sites in the granulosa cell nuclei.

In the cytosol, oestradiol was found to be more effective than DES in displacing [^{3}H] -oestradiol from the oestrogen binding sites. This was unexpected as these steroids were equally effective in binding to the oestrogen receptor in the nuclei, as has been reported for the cytosol and nuclear receptor of other oestrogen target tissues (King & Mainwaring, 1974; Buller & O'Malley, 1976). At equivalent concentrations, DES was able to displace only 36% of the [^{3}H] -oestradiol binding displaced by cold oestradiol. Because both DES and oestradiol are reported to have the same coefficient of binding to the oestrogen receptor (Hospital et al., 1975) it appeared that the [^{3}H] -oestradiol binding in excess of that displaceable by DES might be due to the presence of

another oestrogen binding species.

Oestrone and oestriol displaced 68 and 27% respectively. of the [^{3}H] -oestradiol binding while the androgens, testosterone, DHT and R1881 competed for 33, 53 and 67% respectively of the cytosol oestrogen binding sites.

These findings were again unexpected because these androgens did not compete significantly for the nuclear oestrogen binding sites. Moreover, studies in the rat uterine oestrogen receptor system suggest that androgens only competed for the oestrogen receptor when administered in high doses (Rochefort & Garcia, 1976; Schmidt et al., 1976; Garcia & Rochefort, 1976). Van Doorn et al (1982) also demonstrated that the androgen Adiol bound to the cytoplasmic oestrogen receptor with a rather high affinity in the immature rat uterus. Clark et al.(1978) also showed that testosterone and progesterone were ineffective in inhibiting oestrogen receptor binding of oestradiol in the rat uterus. In the dog prostate cytosol, cortisol, progesterone, testosterone and 5α-dihydrotestosterone were incapable of displacing [^{3}H] -oestradiol (Dubé et al., 1979). In virtually all target tissues studied, any binding by oestradiol-17β in excess of that displaceable by DES and other hormones has been ascribed to another oestrogen binding species (Dubois, 1980; Dickson et al., 1978), Dubé et al.,1979; Taylor & Smith, 1982). In the granulosa cell cytosol, an apparent additive displacement of [^{3}H] -oestradiol was observed when DHT and DES were used in combination as competitors. This implied that DHT and DES displaced [^{3}H] -oestradiol from at least

two different binding sites — DES denoting the oestrogen receptor binding and DHT competing for another site distinct from the oestrogen receptor. This additive property was not observed however, when OE_2 and DHT were used together, an indication that presumably both steroids displaced [^{3}H] -oestradiol from the same binding species (Tables 2 and 3). The employment of a synthetic androgen, R1881, which is a specific ligand for the androgen receptor (Dubé et al., 1980) established that the species that bound [^{3}H] -oestradiol which could be displaced by DHT was not the oestrogen or androgen receptors.

The binding of [^{3}H] -oestradiol in excess of that displaceable by DES, to an entity termed the second oestrogen binding species (SOB), might be similar to that of an oestrogen-androgen binding species described for some oestrogen target tissues. The sex-steroid binding protein found in the male, but not female, rat liver cytosol (Dickson et al., 1978; Singletary et al., 1982; Miroshnichenko et al., 1983) like SOB did not bind DES, but bound both oestradiol and [^{3}H] -5α-dihydro-testosterone at the same site. The species found inthe chick oviduct cytosol (Taylor & Smith, 1982) also did not bind DES but did bind progesterone, R5020 and the other androgens testosterone and DHT. Dubois (1980) also observed a similar protein in the rat prostate. In addition to oestrogens and androgens, the second oestrogen binding species of the granulosa cells bound the anti-oestrogens nafoxidine, clomiphene citrate and

tamoxifen. Sudo et al.(1983) recently described a distinct anti-oestrogen binding protein of the rat ovary which however did not bind either oestradiol or DES, thus making it quite distinct from the SOB described here.

A similarity between SOB and other sex-steroid binding proteins apart from their ligand binding specificity is the fact that they are present only in the cytosol of their respective tissues. The Kds reported for these sex-steroid binding proteins are all in the region of 30 to 50nM. Therefore a wide range of [^{3}H] -oestradiol concentrations (up to 40nM) were used to determine the Kd of SOB, employing oestradiol as competitor.

Saturation analysis revealed a biphasic curve with saturation at 4nM for one oestrogen binding species, probably the oestrogen receptor proper. A concentration of greater than 30nM [^{3}H] -oestradiol was required to saturate the second class of oestrogen binding sites, presumably the SOB, whose Kd was found to be lower (45nM) than that of the receptor. The binding capacities observed using OE_2 as competitor were 5.0pmol and 16.60pmol/mg cytosol protein for the higher and lower affinity binders, respectively.

The higher affinity binder, which represents the classical oestrogen receptor appeared to have a greater binding capacity than that measured using DES as competitor probably because of interference in measurement from SOB, the high capacity binder. Theoretically only the receptor

should preferentially bind [^{3}H] -oestradiol at low concentrations. The value of the Kd for the lower affinity binder, was similar to those measured for the non-DES sex-steroid binding proteins of the male liver cytosol, chick oviduct cytosol and the dog prostate cytosol (Dickson et al., 1978; Dubois, 1980; Taylor & Smith, 1982).

4.5 Distinction between the Granulosa Cell Cytosol Oestrogen Receptor and the Second Oestrogen Binding Species (SOB)

In order to irrevocably demonstrate the presence at the novel second oestrogen binding species (SOB) in the granulosa cell cytosol, attempts were made to identify distinguishing features between the classical oestrogen receptor and SOB. (see Table 11)

It became apparent early in the studies that the DCC technique for removing unbound steroid from macromolecular bound [^{3}H] -oestradiol could not be employed for determination of specific oestrogen binding. This was presumably because of the low protein concentration of the granulosa cell cytosol (Powell et al., 1981). The inability of Saiduddin & Zassenhaus (1977) to observe differential [^{3}H] -oestradiol displacement by DES and OE_2 in whole ovarian cytosol at a protein concentration of 2mg/ml could be attributed to the fact that the DCC technique was employed. As the SOB has an affinity for oestradiol which is approximately 80-100 times lower than that of the classical oestrogen receptor, it is conceivable that the charcoal would strip the ligand completely from the SOB. This view is reinforced by the observation

that DCC treatment of the cytosol prior to incubation with radioligand exposed over 90% of the available oestrogen binding sites (Fig 3.8), by removing the endogenous steroids occupying these sites. Inspite of the fact that measurements were made under conditions which were optimal for measuring the oestrogen receptor, the novel SOB could be reproducibly measured using Sephadex LH-20 chromatography. When 10nM [^{3}H] -oestradiol was used for routine assays (which is approximately 5 times the concentration of ligand required to saturate the oestrogen receptor) 60-65% of the radiolabel was observed to be associated with the SOB when a Sephadex LH-20 chromatographic separation was employed. Moreover, the same concentration of SOB was measured using 10nM [^{3}H] -oestradiol concentration when the concentration of DES and OE_2 was varied from a 100-fold to a 1000-fold excess with regard to the radioligand (Fig. 3.6).

Spheroidal hydroxylapatite (SHAP) chromatography offered one way of distinguishing between the receptor and SOB. Although no significant differences were observed in the oestrogen receptor content when either the SHAP or Sephadex LH-20 separation techniques were used (Table 4) 60% of SOB binding was lost during SHAP adsorption chromatography. The binding characteristics of SHAP have not yet been fully analysed but available evidence suggests that it may act as an adsorption matrix as well as possessing ion exchange properties (BDH Technical Booklet on Spheroidal Hydroxylapatite). It is therefore possible that SOB and the oestrogen receptor have

different charges and/or different adsorption properties for the 60% calcium phosphate matrix of SHAP. The SOB may either have been washed out prior to labelling with [^{3}H] -oestradiol or may have bound so firmly to the gels that it was resistant to elution with O.1M NaOH.

By augmenting the granulosa cell cytosol protein concentration with a carrier protein (BSA) it was possible to show a differential ammonium sulphate precipitation of the oestrogen receptor and the SOB. An increasing yield of the SOB was obtained with increasing concentration of the ammonium sulphate from 30 to 70% saturation. At 30% saturation only 37% of the SOB was precipitated compared to complete recovery of the oestrogen receptor Table 5). Taylor & Smith (1982) also observed that at 30% ammonium sulphate, no detectable Z activity (the novel chick oviduct sex-steroid non-DES binding species) could be recovered compared to 23-39% yield of the oestrogen and progestin receptors. Dickson et al (1978) also could not precipitate the 'unusual sex-steroid binding protein' of the male liver cytosol with 30% ammonium sulphate.

These findings indicate that the SOB (as well as the other sex-steroid binding species) might have a different solubility to the oestrogen receptor and therefore be differentially 'salted out' of solution.

The hormone binding specificity of the SOB, with regard to oestrogen and androgen, suggested that it might be similar to the plasma-borne testosterone-oestradiol binding globulin (TeBG) (for review of TeBG see Anderson,

1974). Studies in patients with polycystic ovarian syndrome by Vigersky & Loriaux (1976) showed that the cyst fluids contained an androgen-oestrogen binding protein which was identical to TeBG. To find out if this androgen-oestrogen binding protein was similar to SOB and therefore was produced by the granulosa cells, a solid phase method for the measurement of the binding capacity of TeBG was employed (Nisula et al., 1978). Preferential binding of the SOB to the carbohydrate specific adsorbent, Concanavalin-A would have also enabled proper characterisation of the oestrogen receptor to be carried out in the residual cytosol. Unexpectedly both SOB and the oestrogen receptor bound to Con-A Sepharose. The binding of the oestrogen receptor to Con-A was confirmed using immature rat uterine cytosol labelled with 10nM [^{3}H] -oestradiol (Table 6).

These results suggested that both the oestrogen receptor and SOB might be glycoproteins or may possess carbohydrate residues. There is no direct evidence in the literature to suggest that the oestrogen receptor is a glycoprotein. Since glycoproteins are components of cell surfaces, this finding is consistent with the report of Pietras & Szego (1977) suggesting that oestradiol-17β specific and saturable binding sites were present on the outer surfaces of endometrial cells.

Uriel et al. (1976) presented evidence to suggest that alpha-fetoprotein, a serum glycoprotein was responsible,

wholely or in part, for the oestrogen binding properties of the immature rat uterine cytosol. Using various biochemical techniques, including immunoadsorption to specific AFP antibodies, sucrose density centrifugation and charcoal adsorption technique, this suggestion has been refuted (Radanyi et al., 1977; LaBarbera & Linkie, 1978; Toft & Tomasi, 1978).

The similarity between the oestrogen receptor and AFP which is at the centre of the disagreements in the literature might be due to the fact that they are both glycoproteins, something which the results from the present study (i.e. binding to the glycoprotein specific Concanavalin-A) suggests. The novel SOB might also be mistaken for AFP but for the fact that it binds androgens which AFP does not (Versee & Barel, 1978). Employing immunological methods for localizing AFP in the ovary, Castelli et al.(1982) found that AFP was present only in the secondary follicles and its distribution was limited to the liquor folliculi and the zona pellucida but never in the granulosa cells. Therefore SOB, prepared from properly washed granulosa cells could not be AFP.

The follicular fluid fraction, pre-treated with DCC, contained only 12% of the SOB activity found in the granulosa cell cytosol, suggesting that 1) the SOB might be synthesised in the granulosa cell and 2) the SOB activity in the follicular fluid fraction might have been a contamination from the granulosa cell.

Sato et al. (1981) reported the presence of an oestrogen binding species in the mouse Leydig cell tumour cytosol which apparently did not bind DES in vitro, until the cytosol was exposed to a chaotropic salt such as NaSCN. It was suggested that the DES binding site on this oestrogen binding species was concealed through protein-protein aggregation and disruption of the aggregation with 0.4M NaSCN unmasked the binding sites. Because chaotropic salt treatment did not alter the [^{3}H] -oestradiol binding capacity of either the oestrogen receptor or SOB of the granulosa cell cytosol (Table 7) it did not appear that SOB was a precursor for the oestrogen receptor (with masked DES binding sites) or identical to the Leydig cell oestrogen binding species. Neither was the SOB formed in vitro as a result of the phenomenon of macromolecular aggregation.

The ovary is known to contain many proteolytic enzymes which might participate in follicular rupture at ovulation. Among the proetolytic enzymes identified is plasminogen activator which is synthesised by the granulosa cells (Strickland & Beers, 1976; Wang & Leung, 1983). Sherman et al.(1980) reported that plasminogen activators may be responsible for cleavage of the oestrogen receptor into mero-receptors (sedimentation constant 3S and Stoke's radius 24Å). To examine whether SOB was a product of endogenous proteolysis during in vitro cytosol preparation, the granulosa cell cytosol was exposed to 100µg/ml of

protease inhibitors; phenylmethylsulphonyl-fluoride (PMSF) reduced the oestrogen receptor and SOB content by 55 and 50% respectively. It is possible that PMSF inhibits serine and thiol proteases whose action in the cytosol might normally lead to either promotion of receptor-ligand interaction or to the degradation of other enzymes which normally might cause a reduction of the receptor/SOB's oestrogen binding ability. Bacitracin however, increased the oestrogen receptor binding by 86% without altering the concentration of the SOB (Table II). This protease inhibitor is thought to be a specific inhibitor for hydrolytic enzymes such as leucine aminopeptidase (Makinen, 1972). Such enzymes appear to have no effect on SOB but may be involved in regulation of receptor-ligand binding.

Exposure of the granulosa cell cytosol to trypsin increased the measured concentrations of oestrogen receptor and SOB by 176 and 93% respectively (Table 7) which is consistent with the reports of Agarwal & Paillard (1979) in rat male liver cytosol and Petterson et al.(1982) in breast cancer cytosol. Petterson et al.(1982) have suggested that this augmentation in oestrogen binding by trypsin might be due to reactivation of previously inactivated oestrogen binding sites. In a previous study (Lövgren et al. 1979) it had been observed that the proteolytic fragment 4.3S bound twice as much hormone as the 9S peak and Petterson et al.(1982) found 79 - 100% increase as a result of trypsin treatment, which is in agreement with the present finding. Since

the action of trypsin is thought to involve cleavage of specific amino acid sequences especially Arg-Gly, Glu-Arg (Kenny, 1977), these amino acids might be present in both SOB and oestrogen receptor and might be involved with masking of the steroid binding sites.

From these findings there is no indication that SOB could have been formed in vitro as a product of proteolysis, in which case increased amounts of SOB would have been produced at the expense of the oestrogen receptor in the presence of the protease.

The sensitivity of the interaction of oestrogen receptor and its ligand to thermal activation is well documented and the general consensus is that the occupied oestrogen receptor is completely denatured at 50-65°C after incubation for 30min (Puca et al., 1971; King & Mainwaring, 1974; Myatt et al., 1982). In the absence of oestrogen, the oestrogen receptor exhibits extreme thermal lability even after incubation at 37°C for 30min (Chen & Feldman, 1978). When granulosa cell cytosol, prelabelled with 10nM [^{3}H] -oestradiol for 18 hr at 4°C, was further incubated at 37°C for 30 min, 40% of the control binding to both the SOB and the receptor was lost (Fig. 3.14). Heating at 55°C for 30min revealed that the oestrogen receptor- [^{3}H] -oestradiol complex was more labile than the SOB- [^{3}H] -oestradiol complex. Incubations at 65°C for 30min resulted in complete irreversible degradation of the oestrogen receptor complex (Fig. 3.15) but approximately 30% of the control binding of [^{3}H] -oestradiol to SOB

remained. Thus on the basis of their sensitivity to increasing temperature, SOB and the oestrogen receptor could be distinguished.

Chen & Feldman (1978) have previously employed this procedure to distinguish between the rat uterine oestrogen receptor and AFP in which AFP was more stable at 37°C. The fact that Castelli et al.(1982) could not immunologically localise AFP in the granulosa cells diminishes the possibility that SOB could be AFP. The anti-oestrogen binding protein of the ovary described by Sudo et al.(1983), though thermostable up to 100°C, could not be identical to SOB because it does not bind oestrogen.

Macromolecular aggregation involving either thiol groups or the steroid binding site of receptor would affect [^{3}H] -oestradiol binding and activation of the receptor (King & Mainwaring, 1974; Ikeda, 1982; Moncharmont et al., 1982). Because no significant alteration of the [^{3}H] -oestradiol binding was observed in the presence of 20mM sodium molybdate (Table 7) which would prevent receptor aggregation (Moncharmont et al., 1982) it did not appear that aggregation of the SOB and the oestrogen receptor would involve masking of either the thiol or the steroid binding site. In 10mM molybdate, Petterson et al (1982) observed increased binding in the breast cancer cytosol and Sherman et al. (1980) found that the decreased oestrogen binding produced by the protease inhibitor leupeptin could be restored by simultaneous inclusion of molybdate in

the buffer. These results indicated that the molybdate action involved reactivation of inactivated binding sites probably by interaction with thiol groups (Chong & Lippman, 1982; Ikeda, 1982).

4.6 Kinetic Analysis of the Interaction of [^{3}H] -oestradiol with Oestrogen Receptor and SOB

Determination of the association rate constants of the two oestrogen binding species revealed that at 0°C the SOB associated with [^{3}H] -oestradiol 2- to 3-fold faster than did the oestrogen receptor. At 25°C SOB bound 3- to 4-fold faster than the receptor. By elevating the temperature to 25°C the receptor- [^{3}H] -oestradiol association rate constant increased only 1- to 2-fold while that for the SOB increased 2- to 3-fold (Figs. 3.16 and 3.17). The association rate constant for the oestrogen receptor (K_{+1}, 1.2 x 10^6 moles min^{-1} at 4°C, 2.05 x 10^6 moles min^{-1} at 25°C) is consistent with that reported for the pituitary and hypothalamic oestrogen receptors (Clark et al., 1982). The ability of SOB to associate with [^{3}H] -oestradiol more rapidly than the oestrogen receptor might be due to its high binding capacity but the mechanism involved is obscur.

Measurement of the rates of association of [^{3}H] -oestradiol with the SOB and the oestrogen receptor therefore afforded another distinguishing feature between SOB and the oestrogen receptor of the rat granulosa cell cytosol. The rapid rate of association of the SOB with oestradiol was also consistent with that published

for the sex-steroid binding protein of the male rat liver cytosol (Dickson et al, 1978). Indeed the rate was so rapid that these workers thought that a gel filtration separation technique was too slow to be employed for its determination. Instead, the DCC method was employed to separate free steroid from the macromolecular-bound [^{3}H] -oestradiol.

The rate at which [^{3}H] -oestradiol dissociates from both of the granulosa cell cytosol oestrogen binding species was determined at 25°C and 0-4°C. A 1000-fold excess of DES was used to determine the dissociation of the radioligand from the oestrogen binding sites and a 1000-fold excess of oestradiol was used to cause dissociation of the radioligand off both the oestrogen receptor and the SOB. As the Kd of the SOB- [^{3}H] -oestradiol interaction was about a 100-fold higher than that of the receptor- [^{3}H] -oestradiol, it was conceivable that dissociation would be comparatively rapid as Dickson et al.,(1978) had observed for the 'unusual sex-steroid binding protein' of the male rat liver cytosol.

The dissociation of [^{3}H] -oestradiol at 25°C was biphasic and there were no significant differences between the rate constants when the dissociation was initiated either with DES or with oE_2. The rate constant for the rapidly dissociating component (K_{-1}) was 0.23-0.30 min^{-1} and that for the slow dissociating component (K_{-2}) was 3-4 x 10^{-3} min^{-1}. These observations

imply that the dissociation of [^{3}H] -oestradiol induced by either ligand was from the same oestrogen binding species in both cases, i.e. the oestrogen receptor. This would suggest then that [^{3}H] -oestradiol had already dissociated from the SOB at a rate too fast to observe when using Sephadex LH-20 chromatographic separation. In the presence of SOB (OE_2-induced dissociation) however, the magnitude of the rapid dissociating component of the cytosol was reduced by 50%. Weichman & Notides (1977,1980) have suggested that the dissociation of an oestrogen from the receptor could be used as an index for its biological activity. The rapid dissociating component has been assumed to represent the dissociation of the oestrogen from the non-activated state of the receptor and the slower dissociating component is thought to represent the activated state of the receptor. On this basis the granulosa cell cytosol should contain a high concentration of activated oestrogen receptor. This conclusion is examined later in the light of the results of oligo (dT)-cellulose binding studies. Incubation of the cytosol at 25°C for 30min to activate the oestrogen receptor reduced the magnitude of the rapid dissociating component to less than 25% of the total cytosol receptor content (Fig. 3.18B) which is consistent with the results of Weichman & Notides (1977,1980) and Mauck et al.(1982). However warming the reaction mixture (25°C,30min) and then inducing dissociation with oestradiol (SOB & oestrogen receptor)

did not alter the magnitude of the rapid dissociating form indicating that activation of the receptor had been impeded.

Comparison of these kinetic parameters with those of the immature rat uterine cytosol oestrogen receptor (Fig. 3.20) showed that only the rate constant of the rapid dissociating component (K_{-1}) was dissimilar (0.12 and 0.30 min^{-1} for the uterine and granulosa cell cytosol respectively). The K_{-1} for the immature rat uterine cytosol oestrogen receptor (Fig 3.22) is identical with that of the calf and mature rat uterine oestrogen receptor (Weichman & Notides, 1977, 1980; Mauck *et al.*, 1982; Myatt *et al.*, 1982).

As the SOB is a low affinity binder the elevated temperature used in dissociation rate studies could have enhanced the rapid dissociation of [^{3}H]-oestradiol from it. Therefore the dissociation kinetic studies were performed at 0-4°C in order to determine if a unique dissociation curve could be ascribed to SOB. The dissociation of [^{3}H]-oestradiol from both OE_2 binding sites (SOB + oestrogen receptor) and DES binding sites (the oestrogen receptor) remained identical. Radioactivity was lost rapidly within the first 3 min presumably due to the addition of excess steroid (DES or OE_2). This was followed by a phase of slow dissociation with a rate constant identical to K_{-2} at 25°C ($4 - 5 \times 10^{-3}$ min^{-1}) and which lasted for up to 45 min for the DES-displaceable sites and 60 min, for the OE_2-

displacebale sites (Fig. 3.21). After this the complexes became highly unstable, dissociating rapidly with a rate constant of 0.09 - 0.12 min^{-1} so that by 90 min, all the [^{3}H] -oestradiol had completely dissociated from the DES-displaceable sites but the OE_2-displaceable sites took up to 120 min to dissociate completely.

The fact that pre-labelling the granulosa cell cytosol with high concentrations of [^{3}H] -oestradiol (>10nM) somehow abolished this phenomenon, producing only a slow monophasic [^{3}H] -oestradiol dissociation, is possibly an indication that the presence of high ligand concentration may have inhibited a cold labile moiety thus stabilising the oestrogen binding species. It has been established that in vitro the oestrogen receptor system is an oestrogen-dependent monomer-dimer reaction which exhibits a positive cooperative binding interaction (Notides et al., 1981) in which increasing levels of ligand binding enhance the binding capacity of the receptor. It has been suggested that cold lability of certain enzymes at 0-4°C in ligand-dependent oligomerising systems might be a regulatory mechanism with dissociation being an end product (Wood & Dunne, 1975). Such a regulatory mechanism may explain the mechanism of [^{3}H] -oestradiol dissociation from the oestrogen binding species at 0-4°C (Fig. 3.21). The dissociation pattern appears to be the direct opposite of the positive cooperative process (but not a negative binding process) i.e. there is an initial slow dissociation but after a certain concentration of the ligand had

dissociated this provokes the remaining ligand to come off rapidly. It is also possible that the rapidly dissociating phase may bave been enhanced by denaturation of some cold labile enzymes which at elevated temperature could stabilise the oestrogen binding species *in vitro*. the fact that this phenomenon could be observed in both the granulosa cell and the immature rat uterine cytosols probably indicates that such putative cold labile enzyme(s) which affect the oestrogen receptor stability are present in both target tissues.

Dickson *et al.*(1978) found that the 'unusual sex-steroid binding protein' of the male rat liver cytosol also dissociated very rapidly and made the suggestion that because it took about 10min to separate free steroid from the macromolecular bound [^{3}H] -oestradiol by gel filtration this technique could contribute to the extent of dissociation. Therefore for cytosols with high levels of protein, the DCC technique, requiring only a 2min exposure was preferred. As shown in Fig. 3.9, [^{3}H] -oestradiol dissocaition does indeed take place upon extended exposure of the radiolabelled cytosol oestrogen binding species to Sephadex LH-20, albeit from only non-DES binding sites, probably the SOB. This might explain the inability to demonstrate a distinct dissociation curve that could be ascribed to [^{3}H] -oestradiol dissociation from SOB at either 25 or 0-4°C. Because the dissociation curves are identical irrespective of whether DES- or OE_2-displaceable sites were studied

TABLE 11. Distinguishing Characteristics between the Classical Oestrogen Receptor & the Second Oestrogen Binding Species (SOB) of the Rat Granulosa Cell Cytosol.

	PROPERTY		OESTROGEN RECEPTOR	SOB
1.	Measurement of Specific Binding			
	(1) DCC		negative	negative
	(2) Sephadex LH-20		positive (++)	positive (++)
	(3) SHAP		positive (++)	positive (++)
2.	Thermal Stability ($65^{\circ}C$, 30min)		Labile	Stable
3.	Ammonium Sulphate Fractionation			
	(+ 2mg/ml BSA) @	30%	100%	37%
		50%	100%	53%
		70%	100%	136%
4.	Hormone Binding Specificity			
	(i) DES		Yes	No
	(ii) Oestradiol-17β		Yes	Yes
	(iii) DHT		No	Yes
	(iv) Progesterone		No	Yes
	(v) Anti-oestrogens		Yes	Yes
5.	Con-A Sepharose Binding		Yes	Yes
6.	Effect of Protease/Protease Inhibitors/ phosphatase inhibitor			
	(i) Trypsin		Increase	Increase
	(ii) PMSF		Decrease	Decrease
	(iii) Bacitracin		Increase	No effect
	(iv) $NaMoO_4$		No effect	No effect
7.	Translocation with exogenous OE_2	In vitro	negative	negative
		In vivo	negative	negative
8.	Oligo (dT)Cellulose Binding		negative	negative
9.	Equilibrium Dissociation Constants (Kd)		≃ 0.4nM	≃ 40nM
10.	Cellular localisation		Cytosol + Nucleus	Cytosol only

TABLE 11 contd.

PROPERTY		OESTROGEN RECEPTOR	SOB
11. Kinetic Rates:			
(i) Association	4°C	k_{+1} 1.2×10^{6} moles min^{-1}	2.87×10^{6} moles/min^{-1}
	25°C	k_{+1} 2.05×10^{6} moles min^{-1}	7.50×10^{6} moles/min^{-1}
(ii) Dissociation	4°C	k_{-1} 0.12 min^{-1}	Not measurable
		k_{-2} 5.79×10^{-3} min^{-1}	"
	25°C	k_{-1} 0.3 min^{-1}	"
		k_{-2} 3.73×10^{-3} min^{-1}	

this might indicate that only [^{3}H] -oestradiol dissociation from the oestrogen receptor proper can be accurately measured.

4.7 Intracellular Movement of Oestrogen Receptor

Attempts to cause translocation of the oestrogen receptor in the granulosa cell (i.e. depletion from the cytosol and a corresponding accumulation in the nucleus) were unsuccessful both in vitro and in vivo.

Incubation of isolated granulosa cells with 10nM cold DES at 37°C for 1 hr in vitro produced no singificant alteration of the nuclear or cytosol receptor concentration. Unexpectedly, in vitro incubation with 10nM cold oestradiol caused over a 100% increase in the cytosol receptor content and over a 200% decrease of the nuclear receptor content (Fig. 3.22). This observation suggests that the oestrogens do indeed gain entry into the granulosa cells and that at the concentrations used, the machinery responsible for the induction of "new" oestrogen receptors may have been sensitive for the natural oestrogen, oestradiol-17β but not the non-steroidal synthetic oestrogen, DES. It does not seem likely that the increase in oestrogen receptor content in 1 hr, induced by oestradiol could have been due to de novo receptor synthesis for, receptor replenishment in the immature rat uterus requires up to 15 hr (Cidlowski & Muldoon, 1974, 1978) and about 20 hr in the mouse uterus (Auricchio et al., 1982).

The increase in the oestrogen receptor content could have

been brought about by three possibilities:

i. oestradiol may have caused unmasking or 'activation' of oestrogen-sensitive putative pre-receptors.

ii. oestradiol may have enhanced receptor recycling as part of the receptor replenishment process. Cidlowski & Muldoon (1974,1978) have shown that the rate of this process in the uterus depends on the oestrogen status of the animal and since oestrogen is synthesised in the granulosa cell, its high local concentration might enhance such a process. These workers also established that receptor which appeared in the rapid phase of early replenishment are capable of binding newly introduced oestrogen immediately.

iii. oestradiol may have caused direct stimulation of translation of mRNA, which may have been present in the cytoplasm but in an 'inactive' state, leading to receptor synthesis.

Studies on oestrogen action in the uterus suggest that oestrogen effects on protein synthesis is secondary to its effect on synthesis of mRNA (Noteboom & Gorski, 1963; Hamilton, 1963; Ui & Mueller, 1974). More recently it has been shown that during the first hour of uterine response to oestradiol, there is an increased rate of protein synthesis which could be ascribed to an increase in peptide elongation of nascent peptides rather than the synthesis of new peptides. At concentrations which inhibit uterine RNA synthesis, Actinomycin D could not inhibit the peptide elongation process (Whelly & Barker, 1974, 1982). It seems

probable that the rapid receptor turn-over observed with oestradiol injection could be due to a similar mechanism. The high local concentration of oestrogen in the granulosa cell would promote such a process as Whelly & Barker (1982) found that increasing concentrations of oestradiol (from 0.1 to 10µg/animal) increased the rate of the peptide elongation. The fact that the process in the granulosa cell is probably in an 'inactive' state prior to oestradiol administration might be due to the presence of an inhibitory factor, which Whelly & Barker (1982) also identified in the uterus.

Intraperitoneal injection of 28 day old rats with various doses of cold DES or oestradiol to induce in vivo oestrogen receptor translocation was also unsuccessful. As it was likely that alpha-fetoprotein (AFP) might have bound the oestradiol thereby reducing the level of the oestrogen reaching the ovary 50µg of DES was injected instead. This would ensure no reduction in SOB concentration either, as it does not bind DES. As expected, no significant alteration of the level of the SOB was observed up to 120min after injection of the oestrogen. Neither was there an increase in the nuclear oestrogen receptor content. The cytosol oestrogen receptor concentration however, showed over a 100% increase 45min after the injection and thereafter decreased significantly down to about 20% of the receptor content of control uninjected animals at 120min (Fig.3.23). This decrease in cytosol receptor content could be due to receptor processing which takes place subsequent to

translocation but the inability to find a corresponding increase in nuclear oestrogen receptor suggested that translocation might not have taken place. The DES-induced receptor loss may also have been due to proteolysis, which could be a mechanism by which the granulosa cells might be protected from hyperstimulation of oestrogen or a simple receptor inactivation phenomenon in which the receptor loses its steroid binding site.

For the sake of comparison, the experiments were conducted with the natural oestrogen 17β- oestradiol. Complete depletion of the cytosol oestrogen receptor was observed 120min after the administration of 50μg oestradiol. No significant alteration of SOB was observed during the 120min following injection of oestradiol. A 20% increase of nuclear oestrogen receptor was however observed 60min after the steroid injection (Fig. 3.24) but this did not reflect a corresponding cytosol receptor loss.

When 50μg testosterone was injected into the animals about 80% loss of cytosol oestrogen receptor was observed at 60min, but the loss was only 20% by 120min. As no increase in nuclear oestrogen receptor content was observed over the 120min, these changes again could not be directly attributed to a translocation phenomenon. An increased level of SOB was measured as a result of the androgen injection. A possible explanation of this is that SOB induction in the granulosa cell might be androgen dependent as observed

for the sex-steroid binding protein of the rat male liver cytosol (Eagon et al., 1980; Dickson et al., 1978).

These experiments revealed no apparent translocation in the granulosa cell even when 50µg of natural or synthetic oestrogens was injected. Saiduddin & Zassenhaus (1977) had previously shown that no significant translocation of oestrogen receptor could be observed following injection of 10µg oestradiol in intact immature rats. As this dose of oestradiol was capable of causing complete translocation of rat uterine oestrogen receptor (Cidlowski & Muldoon, 1974, 1978; Saiduddin & Zassenhaus, 1977) the failure of translocation in the ovary cannot be attributed to the presence of AFP in the peripheral circulation. Moreover, Castelli et al (1982) have shown that intra-ovarian AFP was found in only secondary follicles and was limited to only the zona pellucida of the oocytes.

The absence of translocation could be due to the presence of some intraovarian factors such as SOB in the granulosa cell cytoplasm or to the nature of the nuclear oestrogen receptor system. As the SOB has a high oestrogen binding capacity it could reduce the concentration of oestrogen and androgen in the granulosa cell cytoplasm available for binding to the cytosol oestrogen receptor.

The ability of androgens to bind to SOB might be of physiological significance in preventing accumulation of free androgens in the granulosa cells. This is

illustrated by the report of Sutton et al.(1981) in which although massive levels of serum testosterone was observed in a patient, this did not produce virilization or alteration of the menstrual cycle. This was presumably because very high levels of circulating TeBG were also present. Androgens have been implicated in the phenomenon of follicular atresia (Payne & Runser, 1958; Louvet et al 1975) by possibly reducing the oestrogen receptor content of the ovary (Saiduddin & Zassenhaus, 1978; Richards et al.,1976).

From studies on human antral follicles, it has been suggested that those incapable of further growth (undergoing atresia) contained less than 50% of their full complement of granulosa cells and therefore contained low concentration of oestradiol (McNatty et al., 1979). It is conceivable that with inadequate granulosa cells present to aromatize thecal androgens, follicular degeneration due to androgen accumulation is inevitable. It is also possible that follicular degeneration might be due to equally inadequate concentrations of SOB capable of binding androgen and rendering it biologically inactive. As high androgen concentration are capable of tranlocating uterine oestrogen receptor (Schmidt et al., 1976; Rochefort & Garcia, 1976) it is possible that high local androgen concentrations in the granulosa cell could cause the reduction in oestrogen receptor level which accompanies atresia. It is uncertain whether the oestrogen

receptor content is low because the ovary is atretic or the ovary becomes atretic because of a decline in the oestrogen receptor content through which oestrogen could act. What is certain however, is that androgen injection does cause reduction of cytosol oestrogen receptor content of the granulosa cell (Fig. 3.24) and of both the cytosol and nuclear oestrogen receptor of the whole ovary (Saiduddin & Zassenhaus, 1978).

The inability to observe nuclear oestrogen receptor accumulation warranted examination of the ability of the granulosa cell cytosol oestrogen receptor to bind to the synthetic nuclear acceptor matrix oligo (dT)-cellulose and of the characteristics of the nuclear oestrogen receptor.

Thrower et al. (1976) and Myatt et al. (1982) have shown that the proportion of the rat uterine oestrogen receptor which bound to oligo (dT)-cellulose corresponded to the proportion that was in the activated state, with apparent molecular weight 100,000-120,000 ($\simeq$ 5S). It was found that granulosa cell cytosol oestrogen receptor complexes could not bind to oligo (dT)-cellulose which suggested that no activated receptor was present (Table II). However, a slow dissociating form was observed suggesting that probably the cytosol receptor is activated but not transformed to an oligo (dT)-cellulose binding state. Since these two processes have been shown to be distinct (Buchi & Villee, 1976) and it was not possible to produce either the 5S receptor on sucrose density

gradients or its corresponding form on Sephacryl S-200 gel filtration it can be concluded that the inability of granulosa cell receptor to bind to oligo (dT)-cellulose is probably due to its inability to form the 5S dimer. This might be due to the absence of the putative 'activating factor' (Myatt et al., 1979; Thrower et al., 1976; Yamamoto & Alberts, 1974). This might also probably explain why oestrogen receptor translocation is difficult to demonstrate in the rat granulosa cells with exogenous hormones.

The inability of cytosol receptor to migrate into the nucleus might also be due to the fact that its full complement of acceptor sites are occupied because of the high local concentration of oestrogen in the granulosa cell. There is still controversy about the nature of that proportion of nuclear receptor sites which are measured during incubation at $4^{o}C$ for 1 hr and are referred to as 'unoccupied' sites. It is unclear whether at $4^{o}C$ one is simply exchanging $[^{3}H]$ -estradiol with some low affinity ligands occupying the binding sites or whether these sites are actually unoccupied by ligand. Since oestradiol injection can induce an increase in the level of unoccupied nuclear receptor in the hypothalamus and the pituitary (Clark et al., 1982) it appears that they are under hormonal control and form a constitutive part of the nuclear receptor binding sites. In the granulosa cell nuclei, the 'unoccupied' receptor sites amounted to 70% of the total (unoccupied + exchangeable) nuclear receptor

sites (Fig. 3.39B) which is consistent with the level found in the rat hypothalamus and pituitary (Clark et al., 1982; White & Lim, 1980) and the rat uterus at pro-oestrus (Thrower et al., 1981). In all these tissues, positive translocation of oestrogen receptor has been demonstrated. It does not therefore appear that the inability to demonstrate translocation in the granulosa cell could be attributed to any inherent difference in the proportion of unoccupied oestrogen receptors between granulosa cells and other target tissues.

Two forms of the nuclear receptor have been identified on the basis of their relative extractability with 0.4M KCl (Clark & Peck, 1976; Baudendistel & Ruh, 1976; Ruh & Baudendistel, 1977; Clark & Peck, 1979). The salt-resistant fraction of the uterine nuclear oestrogen receptor amounts to 10-20% of the total receptor (2-3,000 sites/cell) (Clark & Peck, 1976) and this proportion of nuclear acceptor sites are thought to exhibit long term nuclear stay and are possibly responsible for stimulating biochemical events which produce rat uterine growth (Anderson et al, 1975). Since in the granulosa cell, the salt-resistant fraction was 75% of the total nuclear receptor sites ($\simeq$ 2,000 sites/cell) and translocation could not be demonstrated in these cells (as in uterus with 10-20% salt-resistant sites) it is possible that the inability to demonstrate nuclear accumulation of the oestrogen receptor may be related in part, to the proportionally high salt-resistant nuclear receptors which may be occupying all the long-

term nuclear acceptor sites. It is possible that another mechanism might be present to signal when nuclear occupancy might be feasible, possibly through the activation/transformation process of the cytosol oestrogen receptor or the concentration of SOB within the granulosa cell.

4.8 Molecular Forms of the Granulosa Cell Oestrogen Binding Species

The techniques of sucrose density gradient centrifugation and gel chromatography were employed to determine the molecular weights (M), and Stoke's radius (a) of the oestrogen binding species in the granulosa cell. From this data, the fr ctional ratios (f/fo) of the various molecular forms of the species were estimated using the formula

$$f/fo = \frac{a}{\left[\frac{3\bar{v}M}{4\pi N}\right]^{1/3}}$$ (Siegel & Monty, 1966)

Where $\bar{v}$ = partial specific volume, assumed to be 0.725 cm^3/g for most proteins (Martin and Ames, 1966) and N = Avogadro's number. The results are summarised in Table 12.

The nuclear oestrogen receptor, extracted with 0.4M KCl at 0^oC, ran on sucrose density gradients containing 0.4M KCl with a sedimentation constant of 4.6S (60-70,000 mol. wt.) and its molecular weight was estimated by gel filtration to be 69,000 with a Stoke's radius of 35Å. The frictional ratio (f/fo) or the macromolecule was found to be 1.29 and therefore the

TABLE 12. Molecular Parameters of Oestrogen Receptors and SOB from Gel Chromatography and Sucrose Gradient Analysis

GRANULOSA CELL OESTROGEN BINDING SPECIES

Property	NUCLEAR	CYTOSOL				OE_2-R	Rat Uterine Cytosol		
		NO SALT		0.4M KCl		Depleted	Immature	Immature	Mature
		OE_2-R	SOB	OE_2-R	SOB	No Salt	No Salt	High Salt	No Salt
Sedimentation Velocity (S)	4.6	10.3	2-3	4.4	5.8	2.2	10	4.2	-
Molecular wt. ($x10^{-3}$) SDG	69	2.2	30-45	64	110	23-30	193	60	
Sephacryl S-200	69	250;70	82	69	69	69	250	-	-
Sephacryl S-300	-	$1.5x10^6$	$1.5x10^6$	23-70	23-70	-	-	-	230;69
Stokes Radius (Å)	35	52;37	40	12-35	12-37	35	52	-	54;37
Frictional ratio (f/fo)	1.29	?	1.40	1.35	1.12	1.29-1.70	?	-	1.37

macromolecule was assymetric. These values are different from those reported in the literature for rat/calf uterine nuclear oestrogen receptor (cf 5S, 110-140,000 mol. wet. ; f/fo = 1.60) which suggests that the granulosa cell nuclear oestrogen receptor is smaller and less assymetric. This finding is however consistent with the reports of Siiteri et al.(1973) and Linkie (1977) who could isolate the '4S' oestrogen receptor (presumably with molecular weight 60-70,000) from the nuclei of the uterus, pituitary and hypothalamus. These workers also observed that the 5S transformed receptor was produced at the expense of the 4S in the nucleus. The absence of transformed receptor (5S, mol. wt. 110-140,000) in the granulosa cell nuclei might account for the inability of the cytosol receptor to translocate into the nucleus and its inability to bind to oligo (dT)-cellulose.

In buffers containing no salt the cytosol oestrogen receptor had a sedimentation constant of 10.3S and an estimated molecular weight of 212,000 daltons on sucrose density gradients (SDG). The precise molecular Stoke's radius could not be assigned to the macromolecule by gel filtration. It was found to be either equal to greater than 52Å when chromatographed on Sephacryl S-300 indicating that possibly the receptor had aggregated to give forms with molecular weights greater than 1.5×10^6 daltons. On SDG, this aggregation phenomenon was not apparent probably because of the high centrifugal force exerted on the macromolecule over the

16.5 hr period. Under this condition, [^{3}H] -oestradiol dissociation from the macromolecule also occurs (Fig. 3.29) which is consistent with the report of Clark et al.(1978). Stancel et al.(1973a) have also shown that appropriate dilution of the immature rat uterine cytosol could cause a reduction of the sedimentation constant of the receptor complexes from 10S to 7S. Examination of the literature shows that the protein concentration of cytosol used for SDG vary between 2 and 8mg/ml and therefore might introduce differences in sedimentation constant of the receptor.

The in vitro aggregation phenomenon observed on gel filtration (which requires only 3 hr for elution) might be similar to that described by Nishizawa et al.(1981) where rat uterine oestrogen receptor aggregates were eluted on Sephadex G-200 in a form corresponding to about 20S (molecular weight greater than 600,000 daltons). As this aggregation phenomenon inhibited receptor activation, this might explain why the granulosa cell cytosol oestrogen receptor did not bind to oligo (dT)-cellulose. This would suggest that the factors which may promote receptor aggregation by identifying activated receptor (Nishizawa et al.,1981) are present in the granulosa cell cytosol. The frictional ratio of the granulosa cell cytosol oestrogen receptor could not be estimated because of this aggregation phenomenon.

When immature rat uterine cytosol was chromatographed under identical conditions on Sephacryl S-200, the

molecular weight was greater or equal to 250,000 and the Stoke's radius greater or equal to 52Å. On SDG the molecular weight was found to be about 10S.

When prepared in 0.4M KCl, the granulosa cell cytosol receptor sedimented as a 4.4S species on sucrose density gradients containing 0.4M KCl, the apparent molecular weight was 64-69,000 daltons and the Stoke's radius 35Å. The frictional ratio (f/fo) of the receptor was estimated to be 1.35 and these values are in good agreement with those reported in the literature (Jensen & DeSombre, 1972; King & Mainwaring, 1974; Notides, 1978) and suggests that oestrogen receptors, in their basic monomeric form, are probably identical irrespective of the target tissue from which they are extracted. The fact that the cytosol receptor in 0.4M KCl is a similar size to the 0.4M KCl extracted nuclear receptor (i.e. 4S) is an indication that the cytosol receptor does not undergo transformation upon translocation in vivo. Interestingly, the heating of cytosol, which was prepared in buffer containing no salt, under conditions which would normally promote receptor activation (25°C for 30min) did not affect the chromatographic elution profile or the sedimentation constant of the receptor. This suggests, in conjunction with the results of oligo (dT)-cellulose binding species, that factor(s) necessary for converting receptor to an oligo (dT)-cellulose binding form might be absent in the cytosol ("Activating Factor"?) and indicates that receptor activation might be a prerequisite for receptor transformation as suggested by Buchi & Villee (1976).

It is possible however, that the action of the 'activating factors' may have been inhibited by factors which promote receptor aggregation in the cytosol (Nishizawa et al., 1981).

In the cytosol containing no salt, the SOB sedimented as a 2-3S (30-45,000 mol wt.) species on SDG. Because of its low affinity for oestradiol, extensive dissociation of the radioligand from the SOB occurred during the 16.5 hr at 2°C as confirmed by postlabelling fractionated gradients with [^{3}H] -oestradiol (Fig. 3.31). On Sephacryl S-200 however, it was apparent that SOB eluted predominantly as an 82,000 dalton species with Stoke's radius around 37Å. When the void volume peak was postlabelled, it was apparent that SOB could also co-elute with the oestrogen receptor possibly forming aggregates on its own or with other proteins but that [^{3}H] -oestradiol had dissociated during chromatography (Fig. 3.35). The formation of an SOB aggregate with molecular weight greater than 250,000 was confirmed using ammonium sulphate precipitation (Fig. 3.34): a decrease in the void volume peak on Sephacryl S-200 corresponded with loss of SOB in the 30% ammonium sulphate precipitate of the granulosa cell cytosol (Table 5). With Sephacryl S-300 chromatography the SOB again eluted in the void volume suggesting that it could form very large macromolecules, with molecular weight greater than or equal to 1.5×10^6 daltons.

In cytosol containing 0.4M KCl, the SOB appeared to sediment on SDG as a 5.8S species with molecular weight around 110,000. It appears that the high salt conditions

may have stablised the SOB, probably allowing any sub-units, if present, to interact in a stable manner. It is also possible that the high salt conditions could have first caused inhibitors to dissociate from the macromolecule before the SOB-units interacted. This mechanism could then be similar to that suggested for salt-induced transformation of the oestrogen receptor (Notides, 1978). On Sephacryl S-200, high salt conditions caused the SOB to elute as a 69,000 species and on Sephacryl S-300, a broad peak of radioactivity was obtained from 12 to 35Å (30-70,000). It is apparent that molecular parameters determined by sucrose density gradient centrifugations do not always agree with those made using gel filtration. The sex-steroid binding protein of the male liver cytosol have been shown to sediment as a 3-4S (60,000 mol. wt.) species but with a molecular weight of about 25,000 (gel chromatography) (Eagon et al., 1980; Singletary et al., 1982). The primary cause may be the different extents of radioligand dissociation because of (1) SOB's low affinity for oestradiol or (2) to the possibility of cold lability of endogenous enzymes over the 16.5 hr period at 2°C on SDG or 3 hr at 4°C for gel filtration (Figs. 3.21 and 3.29). In fact, even for the same method, e.g. gel filtration, the use of different lengths of column (cf 30cm and 60cm for Sephacryl S-200 and S-300 respectively) could have affected the extent of [^{3}H] - oestradiol dissociation. As the methods of determination are optimal only for the oestrogen receptor it is

possible that these conditions are not necessarily optimal for SOB fractionation. There is also extensive loss of counts from SOB on Sephacryl gels (74%) compared to 45% loss of [^{3}H] -oestradiol from oestrogen receptor sites and this might be due to the relatively low cytosol protein concentration ($\simeq$ 200µg/ml)).

The presence of plasminogen activator in the granulosa cell cytosol might have an effect on the SOB. Sherman et al. (1980) have shown that this enzyme causes cleavage of receptors and it seems likely that the plasminogen activator which is synthesised in the granulosa cell could cause similar effects on SOB.

Inspite of these drawbacks, it is possible to conclude from these studies that the granulosa cell possesses at least two oestrogen binding species which may be distinguished on the basis of their molecular forms under different ionic conditions:(see also Table 12)
1) the classical oestrogen receptor present in both the nucleus and the cytosol. In high salt conditions, the receptor isolated from nuclei and cytosol had the same molecular form, therefore there was no evidence of receptor transformation as observed in the uterus, hypothalamus, pituitary (Jensen & DeSombre, 1972; Siiteri et al., 1973; Linkie, 1977).
2) the SOB which is present as a small macromolecule on SDG (30-45,000 mol. wt.) but appears to be larger on Sephacryl S-200 (82,000) in buffers containing no salt. Under high salt conditions, the macromolecule

appears to be even larger (110,000 mol.wt.) perhaps as a product of subunit interaction or of dissociation of inhibitors.

4.9 Ontogeny of the Granulosa Cell Oestrogen Receptor

The inability to demonstrate translocation in the granulosa cell *in vitro* and *in vivo* and the inability of the granulosa cell cytosol oestrogen receptor complex to bind to oligo (dT)-cellulose suggested that the receptor activation/transformation process might be impaired. The possible existence of such a situation *in vivo* made it necessary to examine the nuclear/cytoplasmic relationships of the oestrogen receptor during postnatal development. The oestrogen receptor content (nuclear and cytosolic) of the granulosa cell was determined from 8 days of age until day 40 ± 2, (the day of vaginal opening, i.e. the animal's first pro-oestrus, Ramirez & Sawyer, 1974; Döhler & Wuttke, 1975).

4.9.1 Receptor concentration per granulosa cell during postnatal development

The graulosa cells isolated from 8 day old rats possessed oestrogen receptors and since granulosa cells appear in the ovary on day 8 (Plate 1), it may be concluded that oestrogen receptors are a constitutive property of these cells. This finding is consistent with reports of studies conducted in target tissues such as the brain (MacLusky *et al*.,1979) and the uterus (Clark & Gorski, 1971) in which oestrogen receptors have been demonstrated to be present from birth.

The granulosa cells isolated from 8 day old rats

contained the highest concentration of cytosol oestrogen receptor (35,000fmol/mg DNA; 170,000 sites/cell) and as the animals grew older, the receptor concentration declined rapidly (Fig. 3.43) until day 19 when only approximately 21,000 receptor sites/cell remained. On day 20 however a transient increase in cytosol receptor concentration occurred so that approximately 41,000 sites/cell could be measured. From day 21 onwards and until the day of pro-oestrus, the cytosol receptor content/cell remained relatively constnat varying between 10,000 and 14,000 sites.

The proportion of receptor present in the nuclear fraction of the granulosa cell was low, varying between 1% of the total receptor sites (cytosolic + nuclear)/cell on day 8 to about 24% on the morning of pro-oestrus (day 40 ± 2). The highest concentration of nuclear oestrogen receptor sites per granulosa cell was observed on days 12 and 18-20 (approximately 5400 sites/cell) and these were significantly greater than the concentration of nuclear receptor sites present on day 8 (approximately 2000 sites). The nuclear oestrogen content per cell, unlike the cytosol receptor, did not change drastically with age (Fig. 3.43).

Previous studies on the ontogeny of the oestrogen receptor in many oestrogen target tissues are in conflict in many respects, even when the same tissue has been studied more than once. Thus some workers (Kato *et al.*, 1974; Kato, 1975) claimed that the concentration of

oestrogen receptor in the hypothalamus of female rats increased between days 7 and 28, whereas others (Spona et al., 1977; Bieglmayer et al., 1978) reported a fall between days 13 and 26 and Barbanel & Assenmacher (1980) identified maximal oestrogen binding on day 6 before a decline until day 28. Studies in the immature rat uterus have shown that the cytosol receptor concentration was maximal on day 10 (Kimmel & Harmon, 1980; Medlock et al., 1981) but then fell with increasing age in accompaniment with an increase in the nuclear oestrogen receptor concentration (Medlock et al., 1981). It is difficult to compare the present findings with many previous studies, as the units in most cases are not comparable (e.g. receptor content per animal, per uterus or per mg tissue protein). Because most of these tissues are also heterogeneous, expression of the receptor concentration per mg DNA may not be comparable with the single cell type granulosa cell oestrogen receptor system. The age-related changes in receptor concentration in the granulosa cell do appear to be different from those of other tissues.

As shown in Figs. 3.43 and 4.4, the cytosol receptor concentration declines with age. Clark & Gorski (1970) found about 5,000 sites per uterine cell on day 1 and a peak of approximately 20,000 sites/cell on day 10. After day 10, the number of sites decreased to approximately 16,000 sites/cell (and this remains so up to day 22) and this concentration is comparable to the level of oestrogen receptor sites/cell present in

the granulosa cell at the same age.

It has been suggested that increases in nuclear oestrogen receptor content accompany growth of the target tissue (Buller & O'Malley, 1976) and this has beem demonstrated in the uterus (Medlock et al., 1981). In the brain which is a non-proliferating tissue, the nuclear oestrogen receptor concentration hardly changes within the first three weeks of life with the most dramatic change occurring between days 20 and 26; the levels then remaining constant through to adulthood (Plapinger et al., 1975). The cytosol receptor content of the brain was however found to increase between days 6 and 21.

These changes in receptor concentration are generally thought to reflect the changes in circulating hormones and the increase in nuclear receptor being the result of oestrogen-induced translocation (Buller & O'Malley, 1976).

4.9.2 Relationship of changes in receptor concentration per cell to serum gonadotrophins and steroids.

Comparison of receptor concentrations in the granulosa cell with the reported concentrations of circulating hormones during the prepubertal period (Fig. 4.1, derived from Döhler & Wuttke, 1975) shows that the cytosol receptor concentration per cell bore no apparent relationship to the concentration of circulating hormones.

The increased nuclear oestrogen receptor content per cell observed on day 12 appears to occur in parallel with increases in concentration of circulating LH, FSH and

oestradiol and might be the result of translocation by the increased local concentration of ovarian oestrogen (the biosynthesis of which may have commenced on days 8-12). The increase in the nuclear receptor content is however far less than the loss of cytosol oestrogen receptor over the same period suggesting that other phenomena which may include receptor "processing" (Horwitz & McGuire, 1978) may be taking place.

The increased nuclear receptor concentration in granulosa cells on days 18-20 appears to coincide with high levels of LH (serum levels are high between days 11 and 17 and on day 21) a reduction of FSH (the concentration of which is high from birth to day 17) but a low concentration of oestradiol. This gonadotrophin milieu would not be expected to induce oestrogen production (hence the low oestradiol level) but instead, might induce accumulation of androgens (Dorrington, 1979). The increased nuclear oestrogen receptor level per cell suggestes that either endogenous oestrogen synthesis had increased but had not been reflected in the circulating levels of oestradiol or the receptor translocation might have been induced by high levels of follicular androgen. This would be consistent with the report of Rochefort & Garcia (1976) and Schmidt *et al.*(1976) who showed that high concentrations of androgens could induce oestrogen receptor translocation in the immature rat uterus both *in vitro* and *in vivo*. The changes in nuclear oestrogen receptor concentration per granulosa cell observed in this study are not consistent with the observations

in other target tissues that nuclear receptor increase would accompany growth (Buller & O'Malley, 1976). The inability to observe increases in nuclear oestrogen receptor concentration in the granulosa cell might be due to the fact that it contains a high level of nuclear receptors resistant to salt extraction (Fig. 2.40, $\simeq$ 2,000 sites/cell), a concentration which is identical to the level exhibiting long term nuclear receptor stay and producing true uterine growth in the rat (Clark & Peck, 1976, 1979; Anderson et al., 1975). Thus the nuclear acceptor sites might be fully occupied and this might determine how many receptor complexes can be translocated and retained in the nucleus.

To examine what the overall changes in total ovarian nuclear receptor might mean in relation to granulosa cell proliferation the total oestrogen receptor levels in the cytosolic and nuclear compartments of the granulosa cells were computed and expressed per ovary (assuming that the same proportion of granulosa cells were removed from the ovaries irrespective of the age of the rat and that the ovarian oestrogen receptor is exclusive to the granulosa cells (Table 1).

The oestrogen receptor concentration in the cytosol compartment of the ovary increased with age with maximal levels being observed on day 20. After that the receptor concentration fell until day 35 (Fig. 3.45). Because granulosa cells proliferate rapidly during follicular development and the cytosol receptor sites per cell

declined drastically with age (Figs. 3.42 and 4 3) it is possible that the cytosol receptor is being re-distributed among the proliferating granulosa cells. This redistribution process might take place during mitosis when as part of the cytoplasmic content of the cell, the receptor might be apportioned into the daughter cells. The high steroid concentration in the follicle might then promote receptor movement out of the cytosol into the nucleus.

The nuclear oestrogen receptor content per ovary showed significant increases as the animals grew older with maximal levels being observed on day 21 and on the morning of pro-oestrus (Fig. 3.45). Thus expression of the receptor concentration per ovary made it possible to demonstrate an increasing level of nuclear receptor with ovarian growth during postnatal development. Because the nuclear oestrogen receptor content per granulosa cell did not change significantly, the increasing concentration of receptor in the nuclear compartment of the ovary with age simply reflects the increase in the number of the cells with age (Fig. 3.42). This finding also suggests that during cell division when cytosol receptor is re-distributed, the granulosa cell nuclear oestrogen receptor concentration is rigidly maintained by prompt translocation. This of course, is based on the assumption that all the granulosa cells possess an equivalent amount of nuclear receptor. Because the phenomenon of atresia occurs throughout postnatal development, it is conceivable that some cells

would possess high levels of the receptor and the atretic ones might not possess binding sites at all.

Richards et al.(1976) have suggested that increased nuclear receptor content in granulosa cells denote follicular growth while reduced levels denote either luteinisation or atresia. In the absence of a reliable method of estimating the proportion of degenerating and already degenerated follicles at the time of isolation of the granulosa cells, it will be difficult to say with certainty what fluctuations in nuclear receptor concentrations really mean. They could reflect increased atresia where increasing number of cells lose their oestrogen binding sites or it could be due to a receptor processing step in which there is an apparent loss of binding sites, subsequent to translocation.

Examination of receptor concentrations in the nuclear and cytosolic compartments of the ovary reveals changes consistent with events associated with translocation of hormone-receptor complexes (Jensen & DeSombre, 1972; King & Mainwaring, 1974; Horwitz & McGuire, 1978). These changes are not apparent when the receptor concentrations are expressed per cell: the total oestrogen receptor concentration per ovary showed very little change as the animals grew older (Fig. 3.46), varying only between 2-6 x 10^{10} receptor sites from day 8 to the morning of pro-oestrus.

From these studies on the ontogeny of the oestrogen

receptor, it can be concluded that:

1) the oestrogen receptor is a constitutive part of the granulosa cell being present when granulosa cells appear in the ovary (i.e. on day 8)

2) the oestrogen receptor might mediate oestrogen-induced growth of the ovary by translocating from the cytosol into the nucleus. The mechanism involved may encompass the following observations;

a. there is no alteration of nuclear oestrogen receptor content per granulosa cell with age

b. the total receptor contained in the nuclear compartment of the ovary increases with age presumably because of increasing number of cells (changing from approximately 160,000 on day 8 to 7 x 10^6 cells/ovary on day of pro-oestrus).

c. As the cytosol receptor content per granulosa cell declines throughout postnatal development (when granulosa cell number increases) and the overall receptor concentration shows only a 3-fold increase from day 8 to day 42, it would appear that the predominant mechanism whereby new cells acquire the oestrogen receptor involves receptor redistribution among dividing cells. During the process, the cytosol receptor content is reduced presumably because a quantity of receptor migrate into the nucleus. This proposed mechanism might explain why a very high concentration of receptor sites are present on day 8 (approximately 170,000 sites) which is about 8 times higher than the

receptor content per uterine cell at the same age (Clark & Gorski, 1970).

4.10 Control of Oestrogen Action in the Granulosa Cell

The inability to demonstrate oestrogen receptor translocation *in vitro* or *in vivo* with exogenous steroids in the granulosa cell may involve an intracellular control mechanism hitherto obscur, which perhaps protects the ovary from undue stimulation. The AFP present in the peripheral circulation does not appear to be such a controlling factor because translocation could be effected *in vivo* in the immature rat uterus using 10µg oestradiol without any apparent effect on the ovary (Saiduddin & Zassenhaus, 1977). Moreover the steroid concentration in the granulosa cell itself may be considerably higher (up to 10^{-6}M, S.G. Hillier, personal communication) than the circulating peripheral blood level. Exposure of the granulosa cell to such a concentration could lead to hyperstimulation unless the effective concentration is reduced to the normal circulating levels ($\simeq 10^{-9}$M). Such a protective mechanism may involve either controlling the level of available (free) steroid for binding to the oestrogen receptor or preventing the activation of the oestrogen receptor complex.

It has been established that binding of sex steroids to sex hormone binding globulin (SHBG) renders them inactive by reducing the circulating levels of free steroids (for review see Anderson, 1974). A similar role was ascribed to a neonatal oestrogen binding

protein in the rat brain, which had an oestrogen binding capacity of approximately 200-fold greater than the putative oestrogen receptor's binding capacity (Plapinger et al.,1973). This neonatal macromolecule appears to be identical to AFP as it also disappears with age.

The second oestrogen binding species (SOB) might be able to perform a similar protective function in the granulosa cell because of its biochemical characteristics. The presence of SOB in the cytosol but not in the nucleus suggests that it cannot stimulate genomic responses but may be able to regulate interaction of oestrogen with its receptor. SOB binds a broad range of hormones, progesterone, androgens, anti-oestrogens and oestrogen, and may therefore be able to reduce the effect of these hormones on the oestrogen receptor system of the granulosa cell.

Progesterone is known to antagonise oestrogen action by inhibiting oestrogen receptor replenishment and reducing the level of cytosol oestrogen receptor in target tissues (Hsueh et al.,1976; Coulson & Pavlik, 1977; Clark et al.,1977). Progesterone may also induce the formation of the oestrogen receptor-regulatory factor (ReRF) which inactivates the rat uterine nuclear receptor (Evans & Leavitt, 1980) possibly through acid phosphatase activity (MacDonald et al., 1982). Since atretic granulosa cells have enhanced acid phosphatase activity (Peluso & Stegger, 1978), progest-

erone might be involved in follicular degeneration in addition to androgens. Thus by binding to progesterone, SOB might be able to control oestrogen receptor replenishment and granulosa cell atresia. The fact that SOB also binds androgens might ensure no accumulation of 'free' androgens which could also cause a reduction of oestrogen biosynthesis (Leung et al., 1979a,b) or the oestrogen receptor content of both cytosol and nuclear fraction of the ovary (Saiduddin & Zassenhaus, 1978). Since androgens are also required for oestrogen biosynthesis, SOB activity might be crucial in determining whether follicular growth progresses or regresses as far as androgen concentration is concerned.

In the two-cell, two steroid theory of ovarian oestrogen biosynthesis (Baird, 1977) the androgen substrate is transported into the granulosa cell from the theca but no transport mechanism has yet been described for this process. As the local concentration of steroids in the granulosa cell is extremely high, it seems unlikely that the androgen would gain entry into the granulosa cell by a simple passive diffusion. Androgen levels are higher than oestrogen levels in the peripheral circulation (Döhler & Wuttke, 1975) probably because the thecal tissue is vascularised. It is possible that SOB may be involved in the transport of the androgens and raising the question whether SOB is truly found in the soluble fraction of the cytoplasm or is membrane-associated and is only released

during *in vitro* cell fractionation. The fact that SOB binds to Concanavalin-A Sepharose suggests that it might be a glycoprotein, associated with the plasma membrane. Although numerous reports are available describing the identification of membrane-associated glycoprotein binding species for polypeptide hormones (e.g. Sutherland, 1972) only limited evidence is available concerning membrane-associated steroid binding species.

Pietras & Szego (1977) demonstrated that specific binding sites for oestradiol-17β were present on the outer surfaces of endometrial cells. The present studies indicate that androgens displace oestradiol from its binding sites on SOB (Tables 2 and 3) indicating that their domains either coincide or overlap. Androgen 'receptor-like' macromolcules have been described for the rat ovary (Schreiber & Ross, 1976; Schreiber *et al.*, 1976) but it is unlikely that this would be responsible for transporting the androgens because (i) its low capacity and high affinity binding (10^{-9}M) would not facilitate easy interchange with intracellular sites where oestrogen biosynthesis takes place and (ii) androgen-receptor complex could be translocated into the nucleus to stimulate androgenic responses which are associated with prevention of oestrogen-induced ovarian weight gain due to granulosa-cell proliferation (Louvet *et al.*, 1975; Schreiber & Ross, 1976).

The SOB, however, could perform this function because it has a low Kd and a high binding capacity, and binds steroids rapidly. These steroids are equally able to dissociate rapidly from SOB, a factor which would facilitate rapid interchange with unoccupied oestrogen receptor. Leung et al (1979a,b)have demonstrated in vitro and in vivo that high oestrogen concentrations could inhibit thecal androgen production and it is conceivable that SOB could transport oestrogens out of the granulosa cells back into the theca. In this case SOB could be present inthe soluble fraction of the cytoplasm but because it has no nuclear binding sites no genomic responses could be stimulated. The high binding capacity of SOB (about 4 - 5-fold higher than the oestrogen receptor) would also ensure that a high proportion of intracellular steroids are bound and rendered inactive and prevented from binding to unoccupied cytoplasmic oestrogen receptor, produced as a result of de novo receptor synthesis or receptor replenishment.

The properties of SOB appear to be similar in some respects to those described for the type II cytosol receptor sites of the rat uterus (Clark et al., 1978; Eriksson et al., 1978; Clark & Peck, 1979). Type II cytosol receptor sites have a lower affinity for oestradiol (Kd $\simeq$ 30nM) but a high binding capacity ($\simeq$ 2.0pmol/uterus) which is about 3-fold higher than the binding capacity of the high affinity (Kd $\simeq$ 0.8nM) classical oestrogen receptor (Type I).

These type II sites were also found not to be translocatable after an oestradiol injection just like the SOB but unlike the SOB it binds DES. It has been suggested that the type II cytosol receptor sites might help to accumulate oestrogens within the uterus at levels higher than that in the peripheral circulation. SOB appears to be an intracellular protein whereas the type II sites may be extracellular proteins (Clark & Peck, 1979).

4.11 Effect of FSH/LH on Intracellular distribution of Oestrogen Receptor in the Granulosa Cell.

The ability of LH to augment FSH stimulation of ovarian weight in intact and hypophysectomized immature female rats is known to involve oestrogen production and enhancement of follicular growth (Simpson et al., 1941; Goldenberg et al., 1972). Recent studies have shown that FSH in the presence of testosterone can increase oestrogen accumulation by granulosa cells in culture suggesting an FSH-dependent increase in aromatase activity (Erickson & Hsueh, 1978).

Gonadotrophin action is mediated by their receptors; FSH receptor being exclusively located on granulosa cells (Richards et al., 1976; Nimrod et al., 1976) and LH receptors, normally present in theca, interstitial and luteal cells (Rajaniemi & Vanha-Pertulla, 1972; Midgley, Jr., 1973; Richards, 1980) but capable of being induced in granulosa cells of large pre-ovulatory follicles (Zeleznik et al., 1974; Amsterdam et al., 1976;

Richards, 1979, 1980). Circulating FSH levels decline sharply on day 19 and remain low for the rest of the prepubertal period (Döhler & Wuttke, 1975). Administration of 5 I.U. of a gonadotrophin mixture (70:30% FSH/LH) for 2 consecutive days to 20 day old rats caused a significant reduction of the cytosol oestrogen receptor concentration from approximately 12,000 to 1,600 sites/cell and an increase in the nuclear receptor concentration from approximately 1400 to 5800 sites/cell. This intracellular movement of receptor might have been induced by enhanced steroidogenesis: LH inducing androgen production and FSH stimulating its aromatisation to oestradiol which then may have caused translocation of the oestrogen receptor. Since this phenomenon could not be induced *in vitro* or *in vivo* with exogenous steroidal or non-steroidal oestrogens, there appears to be a strict compartmentalization device within the ovary, possibly within the granulosa cell, such that only steroids arising within the cell as a product of gonadotrophin and aromatase activity can induce translocation.

Such a device could serve to protect the granulosa cell from hyperstimulation due to oestrogen exposure especially during the first week of life when high levels of maternal oestrogens are present in the peripheral circulation (Döhler & Wuttke, 1975). AFP might participate in protecting the ovary and other target tissues in reducing the level of oestrogen that

reach them (Raynaud, 1973; Germain et al., 1978). It is possible that within the granulosa cell, where oestrogen synthesis takes place and where oestrogen is highly mitogenic, SOB might act as an additional protective facility. This topographic control of oestrogen action in the granulosa cell might have a physiological role in the selection of follicles during follicular development. Richards (1979) has suggested that follicles which ovulate in response to the gonadotrophin surge at one oestrous cycle may have been selected some 19 days earlier as part of a large pool of growing follicles. In the present study, the rats' first pro-oestrus was observed on day 40 ± 2 and Döhler & Wuttke (1975) observed that there was a pulsatile rise of gonadotrophin on day 21 postpartum. From Table 10 it can be seen that oestrogen receptor translocation appears to have taken place in coincidence with this gonadotrophin surge, presumably as a result of an enhancement of endogenous oestrogen biosynthesis. Since a period of 18-20 days elapses between the gonadotrophin surge and the first pro-oestrus, it could be concluded that this represents the time when the follicles destined to ovulate in the rats' first cycle are recruited and this event is mediated by a topographically controlled oestrogen surge. The fact that gonadotrophin treatment to older rats did not produce the same effect (i.e. receptor translocation) is perhaps an indication that the follicles are selected at a precise moment during the postnatal

period. The age-dependent sensitivity of the granulosa cells to the same doses of gonadotrophin may be due principally to the different proportions of FSH and LH receptors which would mediate their response. Recent evidence has suggested that during follicular development the action of one hormone might regulate the appearance of receptor for another hormone (Richards, 1975; Richards et al., 1976; Richards & Williams, 1976). Oestrogen treatment increases ovarian uptake of [^{3}H] -FSH in hypophysectomized rats (Goldenberg et al., 1972) and FSH treatment increased LH/hCG binding sites (Zeleznik et al., 1974). The present study indicates that the FSH/LH mixture may have induced synthesis of endogenous oestrogen which then might enhance FSH receptor level thereby propagating the effect of FSH on days 20-22. In older rats, it is possible that the proportion of LH to FSH receptors in the granulosa cells increases to such a level that the cells begin to luteinize. As a result progesterone, which antagonises oestrogen action (Buller & O'Malley, 1976; Okulicz et al., 1981) might be produced. Moreover, an enhanced LH activity might lead to accumulation of thecal androgens. This hormonal milieu would inevitably promote follicular degeneration and anti-proliferative activity of granulosa cells. This would explain the reduced granulosa cell yields obtained from rats injected after day 26 postpartum.

CHAPTER FIVE

5. SUMMARY AND CONCLUSION

The origin of granulosa cells and the ontogeny of oestrogen receptor in these cells during the postnatal development of the rat ovary has been studied. During the course of the studies a novel oestrogen binding species was demonstrated in the granulosa cell and was characterised together with the oestrogen receptor in order to establish their possible roles in the granulosa cell during ovarian follicular development.

5.1 Origin of Granulosa Cells and their Proliferation during Follicular Development.

Follicles were apparently organised both in the cortical and medullary regions of the ovary, through the differentiation of stromal cells, in agreement with the observation of Peters & Levy (1964). Granulosa cells appeared in the ovary on day 8 and increased in number from approximately 1.6×10^5 cells/ovary to 7×10^6 cells/ovary on day 40 ± 2, the time of the rats' first pro-oestrus.

5.2 Characterisation of Ovarian/Granulosa Cell Oestrogen Binding Species

The ovarian oestrogen receptor was apparently located exclusively in the granulosa cells and had a dissociation constant (10^{-9}-10^{-10}M) which was consistent with that found in other target tissues (King & Mainwaring, 1974; Buller & O'Malley, 1976). Oestradiol was only as effective as DES in displacing [^{3}H]-oestradiol from the nuclear receptor but displaced more binding than did DES in the cytosol. Saturation analysis and ligand

binding specificity studies revealed two oestrogen binding species in the granulosa cell cytosol: the classical receptor and a novel second oestrogen binding species, termed SOB. SOB binds oestrogens, progesterone, anti-oestrogens and androgens but not the non-steroidal oestrogen, DES. The SOB appears to have a single binding site for all these steroids. SOB could be distinguished from the oestrogen receptor by its relatively lower affinity for oestradiol (Kd $\simeq$ 40nM), higher binding capacity ($\simeq$ 5-fold), ability to associate with and dissociate more rapidly from oestradiol and the resistance of its ligand binding site to thermal denaturation. The novel binding species apparently had a different solubility to oestradiol receptor and could further be distinguished by its low molecular weight on SDG (30-45,000). However, it appeared larger when analysed by gel filtration. The macromolecule was stabilised by salt (0.4M KCl) producing a 110,000 molecular weight species. Like the oestrogen receptor, SOB binds to the glycoprotein-specific adsorbent, Concanavalin-A.

These characteristics were unlike any ovarian binding species hitherto described but were similar to those described for the sex-steroid binding species of the male rat liver cytosol (Dickson et al., 1978), the Z-binder of the chick oviduct cytosol (Taylor & Smith, 1982), the oestrogen-androgen binding protein of the dog prostate cytosol (Dubois, 1980) and the hepatic steroid binding

protein of the chick liver cytosol (Lazier, 1979). It was however unlike the uterine type II cytosol receptor (Smith et al., 1979; Yuh & Keyes, 1979; Clark et al., 1978; Markaverich & Clark, 1981) as SOB was measurable in the presence of sulphydryl stabilising agents and did not bind DES.

5.3 The Role of SOB

The physiological role of SOB may involve binding steroids, especially androgens, oestrogens and progesterone in the granulosa cell to reduce their active concentration and protect cytosolic oestrogen receptor in the high steroid environment of the granulosa cell. As the SOB does not appear to be present in the granulosa cell nucleus, in common with the other sex-steroid binding species described above, it is not likely to be directly involved in evoking genomic events in response to hormonal stimulation. Its low dissociation constant and rapid dissociation rate for oestradiol would facilitate interchange of ligand with receptor when required, suggesting it may be involved in regulation of ovarian follicular growth and degeneration.

SOB appears to possess carbohydrate residues and might well be a glycoprotein associated with the cell surface and released into the cytosol during in vitro cell fractionation. Endometrial cell surface oestradiol specific binding sites have been reported by Pietras & Szego (1977) although their function is unknown. A unidirectional transport mechanism for androgens from theca to granulosa cell has not been demonstrated. SOB

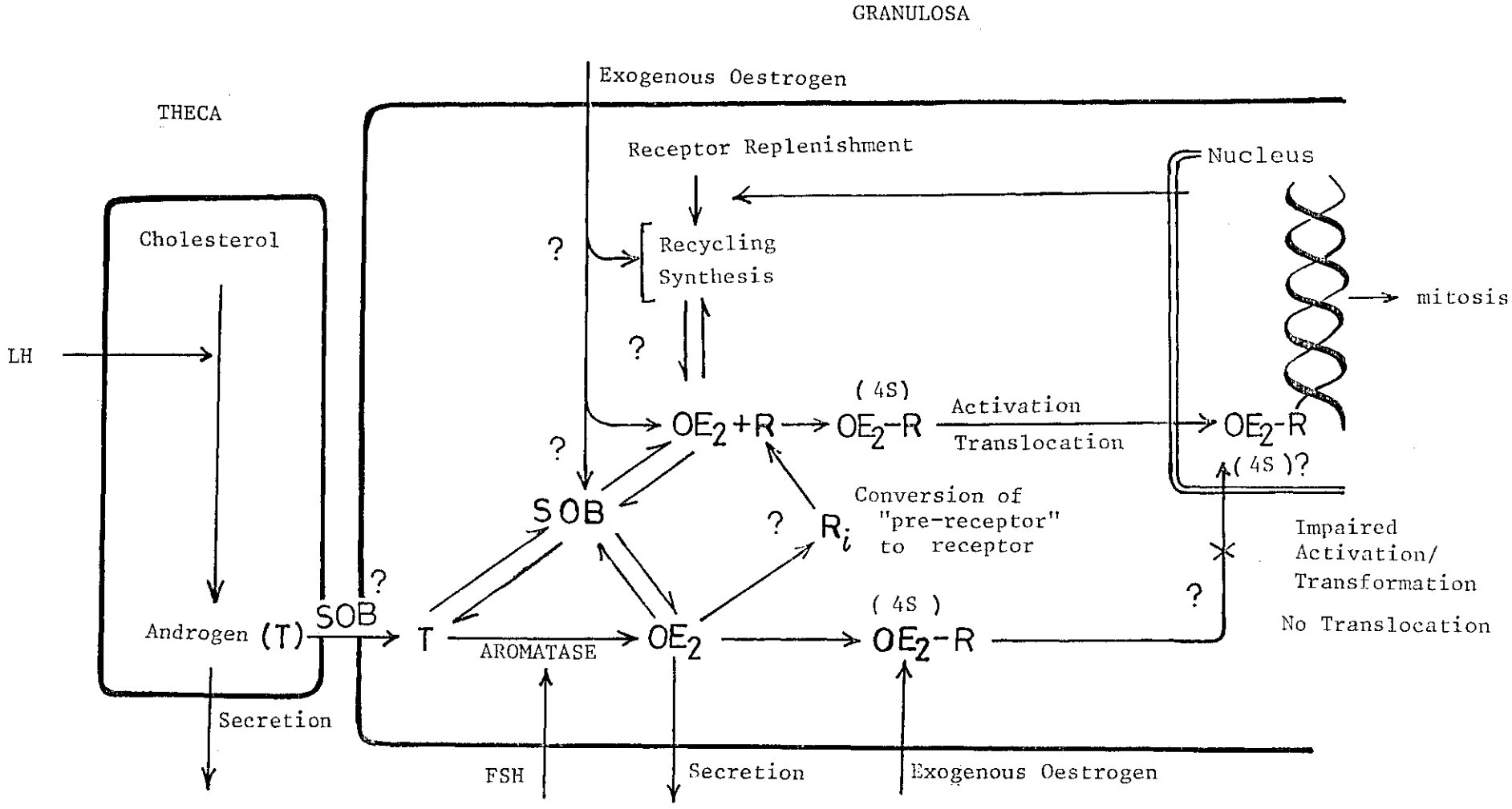


Fig.4.2. Possible roles of SOB and the oestrogen receptor in the control of oestrogen action in the granulosa cell.

? steps requiring further study.

on the granulosa cell membrane may be suited to such a role in androgen transport. However, the higher affinity of SOB for oestradiol than for testosterone argues against such a simplistic interpretation.

5.4 Ontogeny of Oestrogen Receptor

The presence of oestrogen receptor appears to be a constitutive property of the granulosa cell. The highest concentration of receptor is present on day 8 (170,000 sites /cell) but declines with age to adult levels of 10-14,000 sites/cell which are similar to those found in other target tissues. The concentration of nuclear oestrogen receptor/cell is very stable, but as a proportion of total ovarian receptor, it increases from 1% on day 8 to 24% at pro-oestrus. There is a drastic decline in cytosol receptor concentration/cell with age. As the granulosa cells proliferate, the cytosol receptor may, as part of the cytoplasm, become re-distributed amongst the daughter cells. Unlike the cytosol receptor however, the nuclear receptor concentration/cell appears to be immediately restored following cell division and as a result the receptor concentration in the nuclear compartment of the ovary increases with age. Growth of the rat uterus is also accompanied by increases in nuclear oestrogen receptor (Buller & O'Malley, 1976; Barbanel & Assenmacher, 1980). From day 8 to day 40 ± 2, the weight of the ovary changes 30- to 40-fold whilst the granulosa cell number/ ovary increases over 400-fold. Unlike the uterus where nuclear receptor content increases with growth initially

arising presumably from de novo receptor synthesis and uterine weight increases in parallel with nuclear receptor content, the granulosa cells appear to gain their receptor predominantly through a receptor-re-distribution phenomenon amongst dividing cells. Hence there is only a 3-fold increase in total ovarian receptor concentration from day 8 to day 40 ± 2. The highest concentration of receptor is observed on day 20-22 which is coincident with the selection of the first cohort of follicles for eventual ovulation at the first pro-oestrus. During this period, receptor translocation is evident in vivo apparently in parallel with changes in gonadotrophin levels in the peripheral circulation (Döhler & Wuttke, 1975). It is also during this period that translocation can be induced in vivo with the appropriate concentration of exogenous FSH/LH (70:30%), presumably due to stimulation of ovarian oestrogen biosynthesis. After day 26, the ratio of LH to FSH receptors in the ovary may rise such that luteinization of the follicle begins (i.e. progesterone production) and thecal androgen production is also enhanced leading to oestrogen antagonism. It is also possible that oestrogen biosynthesis is still enhanced but its excessive concentration possibly triggers off an intrafollicular negative feedback event and hence leads to the anti-mitotic effect of exogenous gonado-trophins over this period. The result is reduced oestrogen receptor concentration and a reduced number of granulosa cells.

5.5 Receptor Translocation and Molecular Forms

Translocation of oestrogen receptor in the granulosa cells, *in vivo* or *in vitro*, could not be induced with exogenous oestrogens (up to 50μg). Instead, oestradiol but not DES, enhanced measurable cytosol receptor binding due to either activation of conversion of pre-receptor to receptor, enhanced receptor replenishment or possibly oestrogen-induced translation of mRNA leading to receptor synthesis. The oestrogen receptor appeared not to be able to bind to oligo (dT)-cellulose and could not be heat-transformed to a 5S form *in vitro* but instead formed very large molecular aggregates (up to 1.5×10^6 daltons). The concentration of nuclear receptor that was resistant to salt extraction (75% of total sites) was a greater proportion than found in other target tissues (10-20%) but was equivalent to about 2,000 sites/cell which corresponds to the concentration (2-3,000 sites/cell) which supposedly, in the rat uterus, exhibits long term stay and promotes true uterotrophic responses. The concentration of unoccupied nuclear receptor sites found (70%) was in agreement with other target tissues and, as has been suggested for those tissues (e.g. uterus, hypothalamus, pituitary etc), supports the concept of a physiological role for unoccupied receptor. SDG and gel filtration fractionation showed that the cytosol oestrogen receptor behaved like the classical oestrogen receptor, i.e. having a '8S' form in buffers containing no salt and a '4S' form in a buffer containing 0.4M KCl which was also identical to the salt extracted nuclear

oestrogen receptor. The KCl-extractable receptor of other target tissues has usually been shown to be the '5S' form. The size of the salt-resistant species in the granulosa cell is not known. The present finding however is in agreement with the report of Linkie (1977) of the existence of 4S nuclear receptors but does not reveal if they are involved in physiological responses to oestrogen stimulation. As no nuclear receptor accumulation occurs in response to exogenous oestrogens in the granulosa cell, this suggests that either all available acceptor sites are fully occupied or the receptor activation/transformation process is impaired. However, the fact that gonadotrophins could induce translocation (albeit for only a restricted period during postnatal development) suggests that there may be a topographic arrangement of the oestrogen receptor system in the granulosa cell whereby exogenous oestrogens are excluded or bound to SOB thereby being 'inactivated' and incapable of causing translocation but endogenous oestrogen produced by aromatase action can gain access to the receptor. This facility might in particular protect the ovary from maternal oestrogens during the neonatal period of development. AFP is believed to protect other target organs and SOB may have evolved in the granulosa cell as an additional protective mechanism at the site of oestrogen biosynthesis.

Abelev, G.I., (1971) Adv. Cancer Res., 14, 295-310.

Agarwal, M.K. & Paillard, J. (1977) in Multiple Molecular forms of Steroid Hormone Receptors, Proc. XIth Acta Endocrinologica Congress Satelite Workship. (Ed. M.K. Agarwal, pp 93-112, Elsevier/North Holland, Amsterdam.

Armstrong, D.T., & Papkoff, H. (1976) Endocrinology, 99, 1144-1151.

Abul-Hajj, Y.J. (1979) Steroids, 34, 217-225.

Amsterdam, A. et al. (1975) J. Cell Biol., 67, 894-900.

Anderson, D.C, (1974) Clin. Endocrinol., 3, 69-96.

Anderson, L.D., Schaef, F.W., & Channing, C.P. (1978) In Ovarian Follicular and Corpus Luteum Function. Eds. C.P. Channing, J.M. Marsh, & W.A. Sadler, pp 241-243, Plenum Press, New York.

Anderson, J.N, Clark, J.H. & Peck, E.J. Jr. (1972) Biochem. J. 126, 561-567.

Anderson, J.N., Peck, E.J. Jr. & Clark, J.H. (1973) Endocrinology, 92, 1488-1495.

Anderson, J.N., Peck, E.J. Jr. & Clark, H.H. (1975) Endocrinology, 96, 160-167.

André, J., Pfeiffer, A. & Rochefort, H. (1976) Biochemistry, 15, 2964-2969.

Arai, H. (1920) Am. J. Anat. 27, 405-462.

Armstrong, D.T. et al. (1974) Life Sci. 14, 129-140.

Arnaud, M. et al. (1971) Biochem. Biophys. Acta. 232, 117-131.

Auricchio, F., et al. (1980) In Perspectives in Steroid Research. Ed. Bresciana F. pp 133-142, Raven Press, New York.

Auricchio, F., et al. (1981a) Biochem. Biophys. Res. Commun. 101, 1171-1178.

Auricchio, F., et al. (1981b) Biochem. J. 194, 569-574.

Auricchio, F., et al. (1982) Biochem. Biophys. Res. Commun. 106, 149-157.

Ax, R.L. and Ryan, R.J. (1979) J. Clin. Endocrinol. Metab. 49, 646-648.

Baird, D.T. (1977) J. Reprod. Fertil. 50, 359-364.

Baudendistel, L.J. & Ruh, T.S. (1976) Steroids, 28, 223-237.

Barbanel, G. & Assenmacher, I. (1980) Mol. Cell. Endocrin. 18, 227-239.

Barrack, E.R. et al. (1977) Biochem. Biophys. Res. Commun. 79, 829-836.

Bast, J.D. & Greenwald, G.S. (1977) Endocrinology, 100, 955-966.

Baulieu, E-E. et al. (1971) Rec. Prog. Horm. Res. 27, 351-419.

Beers, W.H. (1975) Cell, 6, 379-386.

Beers, W.H., Strickland, S., & Reich, E. (1975) Cell, 6, 387-394.

Bieglmayer, C., et al. (1978) Endocrinol. Exp. 12, 89-101.

Blandau, R.J. & Rumery, R.E. (1963) Fertil. Steril. 14, 330-341.

Bingel, A.S. & Schwartz, N.B. (1969) J. Reprod. Fertil. 19, 223-229.

Borthwick, N.M. & Smellie, R.M.S. (1975) Biochem. J. 147, 91-101.

Bradbury, J. (1961) Endocrinology, 68, 115-120.

British Drug House Technical Booklet on Spheroidal Hydroxylapatite.

Brown, P.S. (1968) J. Reprod. Fertil. Suppl. 4, 61-70.

Buchi, K. & Villee, C.A. (1976) J. Steroid Biochem. 7, 539-544.

Buller, R,E. & O'Malley, B.W. (1976) Biochem. Pharmacol. 25, 1-12.

Bullough, W.S. (1946) Philos. Trans. Roy. Soc. London. Ser. B., 231, 453-516.

Bullough, W.S. (1973) Natl. Cancer Inst. Monogr. 38, 5-19.

Burke, C.W. & Anderson, D.C. (1973) Nature, 240, 38-40.

Burton, K. (1956) Biochem. J. 62, 215-323.

Byskov, A.G. et al. (1977) J. Anat. 123, 77-86.

Byskov. A.F. (1979) In Ovarian Follicular Development & Function, Eds. A.R. Midgley, Jr. & W.A. Sadler, pp 41-58, Plenum Press, New York.

Carson, R.S., et al. (1979) Biol. Reprod. 21, 75-88.

Castelli, D. et al. (1982) Histochem. J. 14, 879-887.

Challis, J.R.G., Erickson, G.F. & Ruan, K.J. (1974) Prostaglandins, 7, 183-193.

Chambon, P. et al. (1975) Cold Sptring Harbor Symposium Quant. Biol. 65, 693-725.

Chamness, G.G., Jennings, A.W. & McGuire, W.L.(1974) Biochemistry, 13, 327-331.

Channing, C.P. & Letwitz-Rigby, F. (1975) in Methods in Enzymology, Eds. J.G. Hardman, & B.W. O'Malley, 39, Part D, pp. 183-230, Academic Press, New York.

Channing, C.P. & Kammerman, S. (1974) Biol. Reprod. 10, 179-198.

Channing, C.P. et al. (1978) in Receptors and Hormone Action, Vol. III, Eds. B.W. O'Malley, & L. Birnbaumer, pp 435-455, Academic Press, New York.

Chen, T.L. & Feldman, D. (1978) Endocrinology, 102, 236-244.

Chong, M.T. & Lippman, M.E. (1982) J. Receptor Res. 2, 575-600.

Cidlowski, J.A. & Muldoon, T.G. (1974) Endocrinology, 95, 1621-1629.

Cidlowski, J.A. & Muldoon, T.G.(1978) Biology of Reproduction, 18, 234-246.

Clark, E.R., MacLusky, N.J. & Naftolin, (1982) J. Endocr. 93, 327-338.

Clark, J.H., Anderson, J. & Peck. E.J. Jr. (1972) Science, 176, 528.

Clark, J.H., Anderson, J.N. & Peck, E.J. Jr. (1973a) Steroids, 22, 707-718.

Clark, J.H., Anderson, J.N. & Peck,E.J. Jr. (1973b) in Hormone Receptors and Reproduction. Eds. B.W. O'Malley, & A.R. Means. 36, pp 15-59, Adv. Explt. Med. & Biol. Plenum Press, New York.

Clark, J.H. & Gorski, J. (1970) Science, 169, 76-78.

Clark, J.H. & Peck, E.J. Jr. (1976) Nature, 260, 635-637.

Clark, J.H., Hseuh, A. & Peck, E.J. Jr. (1977) Ann. N.Y. Acad. Sci. (USA) 286, 161-179.

Clark, J.H. & Peck, E.J. Jr. (1976) Nature, 260, 635-637.

Clark, J.H. & Peck, E.J. Jr. (1977) in Receptors and Hormone Action. Eds. B.W. O'Malley, & L. Birnbaumer, pp 383-410, Plenum Press, New York.

Clark, M.R., Marsh, J.M., & LeMaire, W.J.(1978) J.Biol. Chem. 253, 7757-7761.
Das, R.M. (1972) J. Endocrinol. 55,21-30.
De Angelo, A.B. & Gorski J. (1970) Proc. Natl. Acad. Sci.(USA), 66, 693-700.
De Boer, W. et al. (1981) Endocrinology,108,206-212
De Meyts,P., Bianco, A.R. & Roth, J.(1976) J. Biol. Chem. 251, 1877-1888.
De Wit, J.C. (1953) Acta Endocrinol.(Kbh) 12,123-129.
Diaz-Infante, A.Jr., Wright K.H. & Wallach, E.E.(1974) Prostaglandins, 5, 567-581
Dickson, R.B., Aten, F.R. and Eisenfeld, A.J.(1978) Endocrinology 103, 1636-1646.
Dodson, K.S. & Watson, J. (1980) Europ. J. Obstet. Gynec. Reprod. Biol., 11, 49-56.
Dohler, K.D. & Wuttke, W. (1975) Endocrinology, 97, 898-907.
Dorrington, J.H. & Armstrong, D.T.(1979) Rec. Prog. Horm. Res. 35, 301-342.
Dorrington, J. & Gore-Langton, R.E.(1981) Nature, 290, 600-602.
Dorrington, J.H., Moon, Y.S. & Armstrong, D.T. (1975) Endocrinology, 97, 1328-1331.
Duax, W.L. et al. (1981) J. Steroid Biochem. 15,41-47.
Dube, J.Y., Lesage R. & Tremblay, R.R.(1979) J. Steroid Biochem. 10, 459-466.
Dubois, R., Dube, J.Y. & Tremblay, R.R.(1980) J. Steroid Biochem. 13, 1467-1471.
Duffy, M.J. & Duffy, G.J. (1977) Clin. Chim. Acta 74, 161-166.
Dunne, C.D. & Wood, W.A. (1975) Cur. Top. Cell. Regul. 9,65-101.
Erickson, G.F., Challis, J.R.G. & Ryan, K.J. (1977) J. Reprod. Fertil. 49, 133-134.

Erickson, G.F. & Hsueh, A.J.W. (1978) Endocrinology, 102, 1275-1282.

Erickson, G.F. & Ryan, K.J. (1976) Endocrinology, 99, 452-458.

Erickson, H. et al. (1978) Biochem. Biophys. Res. Commun. 81, 1-7.

Epsey, L.L. (1978) Biol. Reprod. 19, 540-551.

Epsey, L.L. & Lipner, H. (1963) Am. J. Physiol. 205, 1067-1072.

Evans, R.W. et al. (1980) Endocrinology, 107, 383-390.

Evans, R.W. & Leavitt, W.W. (1980) Proc. Natl. Acad. Sci. (USA) 77, 5856-5860.

Falck, B. (1953) Acta Endocr. (Kbh) 12, 115-122.

Falck, B. (1959) Acta Physiol. Scand. Suppl 163, 77-81.

Faye, J.C., Lasserre, B. & Bayard, F. (1980) Biochem. Biophys. Res. Commun. 93, 1225-1231.

Feldman, H.A. (1972) Anal. Biochem. 48, 317-338.

Flux, D.S. (1954) J. Endocrinol. 11, 223-237.

Fortune, J.E. & Armstrong, D.T. (1977) Endocrinology, 100, 1341-1347.

Fortune, J.E. & Armstrong, D.T. (1978) Endocrinology, 102, 227-235.

Fox, T.O. (1975) Nature, 258, 441-444.

Franchi, L.L., Mandl, A.M. & Zuckerman, S. (1962) in The Ovary, Vol. I., Eds. S. Zuckerman, & B.J. Weir. pp. 1-12, Academic Press.

Frost, A.A. & Pearson, R.G. (1971) in Kinetic & Mechanism, pp 163-201, New York, N.Y. Wiley.

Garcia, M. & Rochefort, H. (1977) Steroids, 29, 111-126.

Germain, B.J., Campbell, P.S. & Anderson, J.B. (1978) Endocrinology, 103, 1401-1410.

Gesell, M.S. & Roth, G.S. (1981) Endocrinology, 109, 1502-1508.

Giannopoulos, G. & Gorski, K. (1971) J. Biol. Chem. 246, 2524-2536.

Gibbons, W.E. et al. (1979) Am. J. Obstet. Gynecol. 135, 799-803.

Ginsburg, M. et al. (1974) Steroids, 23, 773-792.

Glasser, S.R., Chytil, F. & Spelsberg, T.C. (1972) Biochem. J. 130, 947-957.

Goldenberg, R.L., Vaitukaitis, J.L. & Ross, G.T. (1972) Endocrinology, 90, 1492-1498.

Gonzalez-Diddi, M., Komisanik, N. & Beyer, C. (1972) Endocrinology, 91, 1129-1132.

Gorski, J. (1964) J. Biol. Chem. 239, 889-892.

Gorski, J. et al. (1968) Rec. Prog. Horm. Res. 24, 45-80.

Gorski, J. & Raker, B. (1974) Gynecol. Oncol. 2, 249-258.

Grant, G., Vale, W. & Guillemin, R. (1972) Endocrinology, 92, 1629-1633.

Greenwald, G.S. (1974) in Handbook of Physiology, Vol. III, Eds. R.O. Greep & E.B. Astwood, pp 293-323. American Physiological Society, Washington, D.C.

Greenwald, G.S. (1980) in Animal Models for Research on Contraception and Fertility. Ed. N.J. Alexander, pp 176-186, Harper & Row, Cambridge.

Greep, R.O., van Dyke, H.B., & Chow, B.F. (1942) Endocrinology, 30, 635-649.

Gurpide, E., Gusberg, S.B., & Tseng, L. (1976) J. Steroid Biochem. 7, 891-896.

Hamilton, T.H. (1963) Proc. Natl. Acad. Sci. (USA) 49, 373-379.

Hamilton, T.C., Davies, P. and Griffiths, K. (1982) J. Endocr. 95, 377-385.

Hannah, P. (1969) Acta Endocrinol., 62, 98-116.

Hardin, J.W., et al. (1979) in Ontogeny of Receptors and Reproductive Hormone Action. Ads. T.H. Hamilton, J.H. Clark & W.A. Sadler, pp 103-117, Raven Press, New York.

Harman, S.M., Louvet, J.P. & Ross, G.T. (1975) Endocrinology, 96, 1145-1152.

Haselbacher, G.K. & Eisenfeld, A.J. (1976) Biochem. Pharmacol., 25, 2571-2581.

Hemminki, K. (1976) J. Steroid Biochem., 7, 418-418.

Hemreck, A.S. & Greenwald, G.S. (1964) Anat. Rec. 148, 171-176.

Higgins, S.J. et al. (1973) J. Biol. Chem. 248, 5873-5879.

Hillier, S.G. et al. (1980) J. Reprod. Fertil. 60, 219-229.

Hillier, S.G. Reichert, L.E. Jr., & Van Hall, V.E. (1981) J. Clin. Endocrinol. Metab., 52, 847-856.

Hillier, S.G. et al. (1982a) in Follicular Maturation and Ovulation. Eds. R. Roland, E.G. van Hall, S.G. Hiller, K.P. McNatty & J. Schoemaker, pp 51-70, Excerpta Medica, International Congress Series, No. 560.

Hillier, S.G. & de Zwart, F.A. (1982b) Mol. Cell. Endocrinol. 28, 347-361.

Hogden, G.D. (1982) Fertil. Steril. 38, (3), 281-300.

Housley, P.R., Dahmer, M.K. & Pratt, W.B. (1982) J. Biol. Chem. 257, 8615-8616.

Horwitz, K.B. & McGuire, W.L. (1978) J. Biol. Chem. 253, 2223-2228.

Hospital, M. et al. (1975) J. Steroid Chem. 6, 221-225.

Hsueh, A.J., Peck, E.J. Jr. & Clark, J.H. (1976) Endocrinology, 98, 438-444.

Ikeda, M. (1982) Biophys. Biochem. Acta, 718, 66-73.

Ireland, J.J. & Richards, J.S. (1978) Endocrinology, 102, 876-883.

Janes, G.R., Koenders, A.J. & Benraad, Th.H. (1982) J. Steroid. Biochem. 17, 387-392.

Jensen, E.V. & Desombre, E.R. (1972) Ann Rev. Biochem. 41, 203-230.

Jensen, E.V. & Jacobson, J.I. (1962) Rec. Prog. Horm. Res., 18, 387-414.

Jensen, E.V. et al. (1968) Proc. Natl. Acad. Sci. (USA) 59, 632-638.

Jensen, C.E. & Zachariae, F. (1958) Acta Endocrinol. 27, 343-353.

Juliano, J.V. & Stancel, G.M. (1976) Biochemistry, 15, 915-920.

Kallos,J. & Hollander, V.P. (1978) Nature, 272, 177-179.

Kallos, J., Fasy, T.M. & Hollander, V.P. (1981) Proc. Natl. Acad. Sci. (USA) 78, (5), 2874-2878.

Kato, J., Sugimara, N. & Kobayashi, T. (1971) in Hormones in Development, Eds. M. Hamburgh, & E.J.W. Barrington, pp 689-703, Appleton, New York.

Knowlie, J.T. & Smellier, R.M.S. (1971) Biochem. J. 125, 605-614.

Kassis, J.A. & Gorski, J. (1981) J. Biol. Chem. 256, 7378-7382.

Katzenellenbogen, B.S. (1975) Endocrinology, 96, 289-297.

Katzenellenbogen, B.S. & Ferguson, E.R. (1975) Endocrinology, 97, 1-12.

Katzenellenbogen, B.S. & Gorski, J. (1975) in Biochemical Actions of Hormones, Ed. Litwack, G. pp 187-243. New York Academic Press.

Katzenellenbogen, B.S. et al. (1978) J. Biol. Chem., 253, 697-707.

Kawakami, M. & Sawyer, C.H. (1967) Endocrinology, 80, 857-971.

Kenny, A.J. (1977) in Proteinases in Mammalian Cells & Tissues. Ed. A.J. Barret, Research Monographs in Cell and Tissue Physiology, Vol 2, pp 393-444. Elsevier/North-Holland Biomedical Press, Amsterdam.

Kimmel, G.L. & Harman, J.R. (1980) J. Steroid. Biochem. 12, 73-75.

King, R.J.B., Gordon, J. & Inman, D.R. (1965) J. Endocrinol. 32, 9-15.

King, R.J.B. & Mainwaring, W.I.P. (1974) in Steroid Cell Interactions, pp 190-262, Butterworth & Co., London.

Kohler, P.O., Grimley, P.M., & O'Malley, B.W. (1969) J. Cell Biol., 40, 8-27.

Kon, O.L. & Spelsberg, T.C. (1982) Endocrinology, 111, 1925-1935.

Korenman, S.G. (1969) Steroids, 13, 163-171.

Kornberg, R.D. (1977) Ann. Rev. Biochem. 48, 931-954.

Kuehl, F.A. et al. (1970) Science, 169, 883-884.

Kumar, S.A., Beach, T.A. & Dickerman, H.W. (1979) Proc. Natl. Acad. Sci. (USA) 76, 2199-2203.

Kumar, S.A., Beach, T.A. & Dickerman, H.W. (1980) Proc. Natl. Acad. Sci. (USA) 77, 3341-3345.

LaBarbera A.R., & Linkie, D.M. (1978) J. Steroid Biochm. 9, 1055-1060.

Lazier, C.B. (1979) in Ontogeny of Receptors and Reproductive Hormone Action. Eds. T.H. Hamilton, J.H. Clark, & W.A. Sadler, pp 353-368. Raven Press, New York.

Lee, W. & Novy, M.J. (1978) Biol. Reprod. 17, 799-807.

Legan, S.J., Coon, G.A. & Karsch, F.J. (1975) Endocrinology, 96, 50-56.

Le Maire, W.J. et al. (1973) Prostaglandins, 3, 367-376.

Le Maire, W.J., Leidner, R. & Marsh, J.M. (1975) Prostaglandins, 9, 221-229.

Lenz, R.W., Ax, R.L. & First, N.L. (1982) Biochem. Biophys. Res. Commun. 106, 1092-1098.

Lerner, L.J. et al. (1966) Endocrinology, 78, 111-124.

Leung, P.C.K., Tsang, B.K. & Armstrong, D.T. (1978) Adv. Exp. Med. Biol. 112, 241-243.

Leung, P.C.K., Goff, A.K. & Armstrong, D.T. (1979a) Endocrinology, 104, 1119-1123.

Leung, P.C., et al. (1979b) Biol. Reprod. 19, 641-647.

Lindner, H.R. et al. (1974) Recent Prog. Horm. Res. 30, 79-127.

Linkie, D.M. (1977) Endocrinology, 101, 1862-1870.

Linkie, D.M. & Siiteri, P.K. (1978) J. Steroid Biochem. 9, 1071-1078.

Lovgren, T., Pettersson, K. & Punnonen, R. (1979) J. Steroid Biochem. 10, 155-160.

Liu, W-K, Burleigh, B.D. & Ward, D.N. (1981) Mol. Cell. Endocr. 21, 63-73.

Louvet, J.P., et al. (1975) Endocrinology, 97, 366-372.

Lowry, O.H., et al. (1951) J. Biol. Chem. 193, 265-275.

Luck, D.N. (1979) in Ontogeny of Receptors and Reproductive Hormone Action. Eds. T.H. Hamilton, J.H. Clark, & W.A. Sadler. pp 133-148, Raven Press, New York.

McEwen, B.S. (1980) Biol. Reprod. 22, 43-48.

McGuire, W.L., De La Garza, M. & Chamness, G.C. (1977) Cancer Research 37, 637-639.

McNatty, K.P. & Baird, D.T. (1978) J. Endocrinol. 76, 527-531.

McNatty, K.P. et al. (1979) J. Endocrinol. Metab. 49, 851-860.

MacDonald, R.G., Okulicz, W.C. & Leavitt, W.W. (1982) Biochem. Biophys. Res. Commun. 104, 570-576.

Makris, A. & Ryan, K.J. (1975) Endocrinology, 96, 694-701.

MacLusky, N.J., Chaptal, C. & McEwen, B.S. (1979a) Brain Res. 178, 143-160.

MacLuskey, N.J., Lieberburg, I. & McEwen, B.S. (1979b) Brain Res. 178, 129-142.

Makinen, K.K. (1972) Int. J. Protein Res. 4, 21-28.

Markaverich, B.M. & Clark, J.H. (1979) Endocrinology, 105, 1458-1462.

Markaverich, B.M., Upchurch, S. & Clark, J.H. (1980) in Perspectives in Steroid Research, Ed. F. Bresciani, pp 143-166. Raven Press, New York.

Markaverich, B.M. & Clark, J.H. (1981) J. Steroid Biochem. J. Steroid Biochem. 15, 289-292.

Markaverich, B.M. et al. (1981) Biol. Reprod. 23, 171-181.

Markaverich, B.M. & Clark, J.H. (1981) J. Steroid Biochem. 15, 289-292.

Martin, B. et al. (1981) J. Clin. Endocrinol. Metab. 53, 443-447.

Martin, R.G. & Ames, B.N. (1961) J. Biol. Chem., 236, 1372-1379.

Masseyeff, R. et al. (1974) in Alpha-fetoprotein, ed. R. Masseyeff, pp 313-322, Paris: INSERM

Mauck, L.A., Day, R.N. & Notides, A.C. (1982) Biochemistry, 21, 1788-1793.

Maurer, H.R. & Chalkley, G.R. (1967) J. Mol. Biol. 27, 431-441.

Means, A.R. & Hamilton, T.H. (1966a)Proc. Natl. Acad. Sci. (USA) 56, 686-693.

Means, A.R. & Hamilton, T.H. (1966b) Biochem. Biophys. Acta. 129, 432-435.

Medlock, K.L., Sheenan, D.M. & Branham, W.S. (1981) J. Steroid Biochem. 15, 285-288.

Mester, J., et al. (1970) Biochem. J. 120, 831-836.

Mester, J. & Baulieu, E-E. (1975) Biochem. J., 146, 617-623.

Michel, G., et al. (1974) Steroids, 24, 437-449.

Midgley, A.R,J. (1973) Adv. Exp. Med. Biol. 36, 365-378.

Migliaccio, A., et al. (1982) Biochem. Biophys. Res. Commun., 109, 1002-1010.

Miroshnichenko, M.L. et al. (1983) J. Steroid Biochem. 18, 403-409.

Mohla, S., DeSombre, E.R. & Jensen, E.V. (1972) Biochem. Biophys. Res. Commun., 46, 661-667.

Moon, Y.S., Dorrington, J.H. & Armstrong, D.T. (1975) Endocrinology, 97, 244-247.

Moon, Y.S., et al. (1978) J. Clin. Endocrinol. Metab. 47, 263-267.

Moor, R.M. (1977) J. Endocrin. 73, 143-150.

Mon charmont, B. et al. (1982) J. Steroid Biochem., 16, 361-368.

Mouridsen, H. et al. (1978) Cancer Treat. Rev. 5, 131-141.

Mueller, P.L. (1978) Endocrinology, 102, 824-831.

Muldoon, T.G. (1971) Biochemistry, 10, 3780-3784.

Muldoon, T.G. (1979) in Ontogeny of Receptors & Reproductive Hormone Action, Eds. T.H. Hamilton, J.H. Clark & W.A. Sadler. pp 225-247, Raven Press, New York.

Musliner, T.A., Chader, G.J., & Vilec, C.A. (1970) Biochemistry, 9, 4448-4453.

Myatt, L. (1979) PhD Thesis, University of London.

Myatt, L., Elder, M.G. & Lim, L. (1979) Biochem. Soc. Trans. 7, 909-910.

Myatt, L., et al. (1982) Biochem. J. 202, 203-209.

Nielsen, C.J. et al. (1977) J. Biol. Chem. 252, 7568-7578.

Nimrod, A., Erickson, E.F. & Ryan, K.J. (1976) Endocrinology, 98, 56-64.

Nishizawa, Y. et al. (1981) Endocrinology, 109, 1463-1472.

Nisula, B.C., Loriaux, D.L. & Wilson, Y.A. (1978) Steroids, 31, 681-690.

Noma, K., et al. (1980) Endocrinology, 107, 1205-1211.

Noteboom, W.D. & Gorski, J. (1963) Proc. Natl. Acad. Sci. (USA) 51, 83-89.

Notides, A.C. (1978) in Receptors and Hormone Action, Vol. II, Eds. B.W. O'Malley & L. Birnbaumer, pp 33-61, Academic Press

Notides, A. and Gorski, J. (1966) Proc. Natl. Acad. Sci. (USA) 56, 203-235.

Notides, A., Hamilton, D.E. & Auer, H.E. (1975) J. Biol. Chem. 250, 3945-3950.

Notides, A.C. & Nielsen, S. (1974) J. Biol. Chem. 249, 1866-1873.

Notides, A.C., Lerner, N. & Hamilton, D.E. (1981) Proc. Natl. Acad. Sci. (USA) 78, 4926-4930.

Odor, D.L. & Blandau, R.J. (1969) Am. J. Anat. 124, 163-186.

Okulicz, W.C., Evans, R.W. & Leavitt, W.W. (1981) Steroids, 37, 463-470.

Okulicz, W.C., MacDonald, R.C. & Leavitt, W.W. (1982) Endocrinology, 109, 2273-2275.

O'Malley, B.W. & McGuire, W.L. (1968) J. Clin. Invest. 47, 654-664.

O'Malley, B.W. & Means, A.R. (1976) Science, 183, 610-620.

O'Malley, B.W., et al. (1972) Nature 235, 141-144.

Orczyk, G.P. & Behrman, H.R. (1972) Prostaglandins 1, 3-20.

Oster, G. (1978) Res. Reprod., 10, No. 1.

Palmiter, R.D., et al. (1981) J. Biol. Chem. 256, 7910-7916.

Panko, W.B. & McLeod, R.M. (1978) Cancer Res. 38, 1948-1951.

Payne, R.W. & Runser, R.H. (1958) Endocrinology, 62, 313-321.

Peck, E.J. Jr., Burgner, J. & Clark, H.J. Biochemistry, 12, 4596-4603.

Pederson, T. (1969) Acta Endocrinol. (Kbh) 62, 117-132.

Peleg, S., De Boever, J. & Kaye, A.M. (1979) Biochem. Biophys. Acta, 587, 67-74.

Peluso, J.J. & Steger, R.W. (1978) J. Reprod. Fertil. 54, 275-278.

Pencharz, R.I. (1940) Science, 91, 554-555.

Peppler, R.D. & Greenwald, G.S. (1970) Am. J. Anat. 127, 1-7.

Peters, H. & Levy, E. (1964) J. Reprod. Fertil. 7, 37-45.

Peters, H. et al. (1975) J. Reprod. Fertil., 45, 559-566.

Peters, H. & Pederson, T. (1967) Fertil. Steril. 18, 309-313.

Petterson, K. et al. (1982) J. Steroid Biochem. 16, 369-372.

Pietras, R.J. & Szego, C.M. (1977) Nature, 265, 69-72.

Powell, B.L. et al. (1981) Breast Cancer Res. Treatment. 1, 33-35.

Plapinger, L. & McEwen, B.S. (1973) Endocrinology, 93, 1119-1128.

Presl, J. et al. (1965) J. Endocrinol. 31, 293-294.

Price, D. (1947) Physiol. Zool. 20, 213-247.

Puca, G.A. & Bresciani, F. (1968) Nature, 218, 967-969.

Puca, G.A. & Bresciani, F. (1969) Nature, 223, 745-747.

Puca, G.A. et al. (1971) Biochemistry, 10, 3769-3779.

Radanyi, C., et al. (1977) Proc. Natl. Acad. Sci. (USA) 74, 2269-2272.

Rajaniemi, H. & Vanha-Pertulla, T. (1972) Endocrinology, 90, 1-9.

Ramirez, V.D. & Sawyer, C.H. (1965) Endocrinology, 76, 1158-1168.

Rao, M.C., Midgley, A.R. Jr. & Richards, J.S. (1978) Cell, 14, 71-78.

Raynaud, J.P. (1973) Steroids, 21, 249-256.

Richards, J.S. (1975) Endocrinology, 97, 1174-1184.

Richards, J.S. (1979) Rec. Prog. Horm. Res., 35,343-370.

Richards, J.S. (1980) Physiol. Rev., 60, 51-89.

Richards, J.S. & Williams, J.J. (1976) Endocrinology, 99, 1571-1581.

Richards, J.S. et al. (1976) Endocrinology, 99, 1562-1570.

Rochefort, H. & Garcia, M. (1976) Steroids, 28, 549-560.

Roeder, R.G. & Rutter, W.J. (1979) Biochemistry, 9, 2543-2553.

Rondell, R. (1970) Biol. & Reprod. 10, 199-215.

Rosenthal, H.E. (1967) Anal. Biochem., 20, 525-532.

Rosner, A.L. et al. (1979) Arch. Biochem. Biophys. 198, 153-163.

Ruh, T.S. & Ruh, M.F. (1974) Steroids, 24, 209-224.

Ruh, T.S. & Baudendistel, L.J. (1977) Endocrinology, 100, 420-426.

Ruh, T.S. & Baudendistel, L.J. (1978) Endocrinology, 102, 1838-1846.

Ruh, T.S., Baudendistel, L.J. & Ruh, M.F. (1979) in Anti-hormones, Ed. M.K. Agarwal, pp 199-217, Elsevier/ North Holland Biomedical Press.

Ruh, T.S., Wassilak, S.G. & Ruh, M.F. (1975) Steroids, 25, 257-273.

Ryle, M., Court, J. & Morris, R.J. (1974) J. Endocrinol. 61, xxiii.

Saiduddin, S. & Zassenhaus, H.P. (1977) Steroids, 29, 197-213.

Saiduddin, S. & Zassenhaus, H.P. (1978) Endocrinology, 102, 1069-1076.

Sala-Trépat, J.M. & Vallée-Strouve, C. (1974) Biochem. Biophys. Acta. 371, 186-202.

Sanborn, B.M., Rao, B.R. & Korenman, S.G. (1971) Biochemistry, 10, 4955-4961.

Sando, J.J., La Forest, A.C. & Pratt, W.B. (1979) J. Biol. Chem. 11, 4772-4778.

Sato, B., Huseby, R.A. & Samuels, L.T. (1978) Endocrinology, 102, 545-555.

Sato, B. et al. (1979) Endocrinology, 104, 1474-1479.
Sato, B. et al. (1980) Endocrinology, 106, 1142-1148.
Sato, B. et al. (1981) Endocrinology, 108, 612-619.
Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-672.
Schmidt, W.N., Sadler, M.A. & Katzenellenbogen, B.S. (1976) Endocrinology, 98, 702-716.
Schreiber, J.R., Reid, R. & Ross, G.T. (1976) Endocrinology, 98, 1206-1213.
Schreiber, J.R. & Ross, G.T.(1976) 99, 590-596.
Schwartz, N.B. (1973) in Handbook of Physiology, Vol. 10, part 1., Eds. R.O. Greep, & E.B. Astwood, pp 125-141. American Physiological Society, Washington, D.C.
Sell, S. et al. (1976) Cancer Res. 36, 4239-4249.
Sherman, M.R., Tuazon, F.B. & Miller, L.K. (1980) Endocrinology, 106, 1715-1727.
Shyamala, G. & Gorski, J. (1967) J. Biol. Chem. 244, 1097-1103.
Shyamala, G. & Leonard, L. (1980) J. Biol. Chem. 255, 6028-6031.
Siegel, L.M. & Monty, K.J. (1966) Biochem. Biophys. Acta. 112, 346-362.
Siiteri, P.K. et al. (1973) in Hormone Receptors and Reproduction. Eds. B.W. O'Malley & A.R, Means. pp 97-112, Plenum Press, New York.

Simpson, M.E., et al. (1941) Endocrinology, 28, 37-41.
Singletary, B.K. et al. (1982) Hormone Res., 16, 377-384.
Smith, R.G. et al. (1979) J. Steroid Biochem. 10, 31-35.
Sobell, H.M. & Jain, S.C. (1972) J. Molec. Biol. 68, 21-34.
Somjen, D. et al. (1973) Biochem. J. 136, 25-33.
Sonnenschein, C. & Soto, A.M. (1980) J. Natl. Cancer. Inst. 63, 835-841.
Soto, A.M. & Sonnenschein, C. (1980) Proc. Natl. Acad. Sci (USA) 77, 2084-2087.
Spelsberg, T.C. et al. (1972) J. Biol. Chem. 247, 1368-1374.
Spelsberg, T.C. et al. (1976a) J. Steroid Biochem. 7, 1091-1101.

Spelsberg, T.C., Webster, R.A. & Pikler, G.M. (1976b) Nature, 262, 65-67.

Spona, J., et al. (1977) FEBS lett. 76, 306-310.

Stancel, G.M. et al. (1973) Biochemistry, 12, 2130-2136.

Stancel, G.M. et al. (1979) Biol. Reprod. 21, 269-272.

Steggles, A.W. & King, R.J.B. (1970) Biochem. J. 118, 695-701.

Strickland, S. & Beers, W.H. (1976) J. Biol. Chem. 251, 5694-5702.

Stone, G.M. (1963) J. Endocrinol. 27, 281-288.

Stormshak, F. et al. (1976) Endocrinology, 99, 1501-1511.

Stormshak, F., Harris, J.N. & Gorski, J. (1978) in Receptors and Hormone Action, Vol. II. Eds. B.W. O'Malley & L. Birnbaumer, L. pp 63-81, Academic Press.

Stumpf, W.E. (1969) Endocrinology, 85, 31-37.

Sudo, K., Monsma, F.J. & Katzenellenbogen, B.S. (1983) Endocrinology, 112, 425-434.

Sutherland, R.L. et al. (1980) Nature, 288, 273-275.

Sutherland, E.W. (1972) Sciences, 177, 401-408.

Sutton, G.P., Lyles, K.W. & Wiebe, R.H. (1981) Am. J. Obstet. Gynecol. 141, 535-538.

Tate, R.L. et al. (1975) J. Biol. Chem. 250, 6527-6533.

Taylor, R.N. & Smith, R.G. (1982a) Proc. Natl. Acad. Sci. (USA) 79, 1742-1746.

Taylor, R,N. & Smith, R.G. (1982b) Biochemistry, 26 (8), 1781-1787.

Thanki, K.H., Beach, T.A. & Dickerman, H.W. (1978) J. Biol. Chem. 253, 7744-7750.

Thrower, S., et al. (1976) Biochem. J. 160, 271-280.

Thrower, S. & Lim, L. (1980) Biochem. J. 186, 295-300.

Thrower, S., et al. (1981) Biochem. J. 194, 667-671.

Toft, D. & Gorski, J. (1966) Proc. Natl. Acad. Sci. (USA) 55, 1574-1581.

Toft, D.O. & Tomasi, T.B. Jr. (1978) Proc. Soc. Exp. Biol. Med. 157, 594-598.

Triebwasser, W.F. et al. (1978) Prostaglandins, 16, 621-632.

Tsafriri, A., et al. (1972) Prostaglandins, 2, 1-10.

Tsai, S.Y. et al. (1975) Proc. Natl. Acad. Sci. (USA) 72, 4228-4232.

Tseng, L. & Gurpide, F. (1976) J. Steroid Biochem. 7, 817-822.

Ui, H. & Mueller, G.C. (1974) Proc. Natl. Acad. Sci. (USA) 50, 256-260.

Uilenbrock, J.Th.J. (1982) J. Endocrinol. Suppl 94, Abstract 31P.

Uriel, J. (1969) Path. Biol. 17, 877-882.

Uriel, J. et al. (1976) Proc. Natl. Acad. Sci. (USA) 73, 1452-1456.

Van Doorn, L.G. et al. (1982) J. Steroid Biochem. 16, 661-671.

Versee, V. & Barel, (1978) FEBS Lett. 96, 155-158.

Vigersky, R.A. & Loriaux, D.L. (1976) J. Clin. Endocrinol. Metal. 43, 817-823.

Vito, C.C., Bates, S.E. & Fox, T.O. (1979) Soc. Neuro-Science Atlanta, Abstract 1575.

Wang, C. & Leung, A. (1982) Endocrinology, 112, 1201-1207.

Webster, R.A., Pikler, G.M. & Spelsberg, T.C. (1976) Biochem. J. 156, 409-418.

Weichman, B.M. & Notides, A.C. (1977) J. Biol. Chem. 252, 8856-8861.

Weichman, B.M. & Notides, A.C. (1979) Biochemistry, 18, 221-225.

Weichman, B.M. & Notides, A.C. (1980) Biochemistry, 106, 434-439.

Weil, P.A., et al. (1977) J. Biol. Chem. 252, 1092-1098.

Weir, B.J. & Rowlands, I.W. (1977) in The Ovary, Eds. S. Zuckerman, & B.J. Weir, Vol. 1. pp 265-302. Academic Press, New York.

Weiss, S.B. (1960) Proc. Natl. Acad. Sci. (USA) 46, 1020-1026.

Whelly, S.M. & Barker, K.L. (1974) Biochemistry, 13, 341-346.

Whelly, S.M. & Barker, K.L. (1982) J. Steroid Biochem. 16, 495-501.

White, J.O., Thrower, S., & Lim, L. (1978) Biochem. J. 172, 37-47.

White, J.O. & Lim, L. (1978) Biochem. Soc. Trans. 6, 1310-1312.

White, J.O. & Lim, L. (1980) Biochem. J. 190, 833-837.

Williams, D. & Gorski, J. (1972) Proc. Natl. Acad. Sci. (USA) 69, 3453-3468.

Williams, P.C. (1940) Nature, 145, 388-389.

Wira, C.R. & Baulieu, E.E. (1971) C.R. Acad. Sci. (Paris) 273, 218-223.

Yamamoto, K.R. (1974) J. Biol. Chem. 249, 7068-7075.

Yamamoto, K.R. & Alberts, B. (1974) J. Biol. Chem. 249, 7076-7086.

Yanagishita, M., Hascall, V.C. & Rodbard, D. (1981) Endocrinology, 109, 1461-1649.

Yuh, K-C. & Keyes, P.L. (1979) Endocrinology, 105, 690-696.

Zachariae, F. (1959) Acta Endocrinol. Suppl. 47

Zava, D.T. & McGuire, W.F. (1977) J. Biol. Chem. 252, 3703-3708.

Zeleznik,A.J., Midgley, A.R. Jr. & Reichert, L.D. Jr. (1974) Endocrinology, 95, 818-825.

Zuckerman, S. & Baker, T.G. (1977) in The Ovary, Eds. S. Zuckerman, & B.J. Weir, 2nd Edition, Vol. 1. ppl02-137, Academic Press, New York.