MOLECULAR PROPERTIES OF UTERINE CYTOSOLIC AND NUCLEAR OESTROGEN AND PROGESTERONE RECEPTORS OF THE PRIMATE *CERCOPITHECUS AETHIOPS PYGERYTHRUS*

by

TIRZA KLEIN

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To my husband, Baruch for being encouraging, supporting and being my best friend and to my children : Lehavit, Amit, Asi and Jonathan - with all my love.

לברוך, חברי הטוב ביותר על עדודו ותמיכתו
ולילדי: להבית, עמית, אסי ויונה - באהבה.

DECLARATION

I declare that this dissertation is my own, unaided work.

It is being submitted for the degree of Ph.D. in the University of Witwatersrand. It has not been submitted before for any degree or examination in any other university.

T. Klein

28 day of February, 1985.

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ABSTRACT

Molecular properties of uterine cytosolic and nuclear oestrogen and progesterone receptors of the primate Cercopithecus aethiops pygerythrus.

T. Klein

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The uterine cytosolic oestrogen receptor(ER) from the Vervet monkey (Cercopithecus aethiops pygerethrus) has been partially characterised by means of competitive protein binding studies, sucrose density gradients, gel permeation chromatography iso-electric focussing, chromatofocussing and anion exchange chromatography. Mean concentration of ER (B_{max}) for a batch of 21 uteri was found to be 327±165 fmol/mg protein with a dissociation constant (K_d) of $3.15\pm1.4\times10^{-10}$M for oestradiol using the same batch of uteri. Mean B_{max} and K_d values for uterine tissue powders, representing binding data from 95 uteri in 77 assays were the following : ER : B_{max}=224±61 fmol/mg protein and K_d=3.8±0.9x 10^{-10}M; progesterone receptor (PR) : B_{max}=742±283 fmol/mg protein and $K_d=2.9\pm0.71\times10^{-9}$M. Mean sedimentation coefficients (Beckman VTi-80 rotor; 520000xg'x120min at 2°C), calculated from sucrose density gradient analyses on 10-35% hypotonic sucrose gradients were 8.4±0.4S for the ER and 7.6±0.5S for the PR.The mean sedimentation coefficient of the nuclear ER, determined in hypertonic 10-35% sucrose gradients was calculated to be 4.6±1.2S. The mean sedimentation coefficient (Beckman VTi rotor) in low ionic strength media in the absence or presence of 10mM sodium molybdate was 8.4±0.4S. A marked protective effect by sodium molybdate on the ∿ 8S component could be illustrated. Exposure of the oestradiol charged cytosolic ER complexes to 0.4 M KCl produced a time dependent loss of the ∿ 8S moiety with a resultant formation of more than one entity in the 3-5S range. Chromatography on Ultrogel AcA-22 in low ionic strength buffers yielded high molecular mass

components of 4.68×10^5 daltons with a Stokes radius of 63.2Å in the absence of sodium molybdate and 3.87×10^5 daltons with a Stokes radius of 60.0Å in the presence of sodium molybdate. In the same system using higher ionic strength buffers a much smaller component was observed : 8.61×10^4 daltons with a Stokes radius of 41.8Å in 0.154 M KCl and 9.22×10^4 daltons with a Stokes radius of 42.3Å in buffers containing 0.4 M KCl. HPLC using a Waters I-250 gel column showed a high molecular mass entities (4.85×10^5 and 2.01×10^5 daltons) in low ionic strength buffers not containing sodium molybdate, while only the 2.01×10^5 dalton component appeared in buffers containing 100mM KCl. High ionic strength conditions (0.4 M KCl) produced high molecular mass aggregates (7.2×10^5 daltons) in the absence of sodium molybdate and only one macromolecular entity of 1.52×10^5 daltons in the presence of sodium molybdate. Disc gel electrophoresis on polyacrylamide gels yielded only one symmetrical peak displacable with 100 times excess levels of diethylstilbestrol. Upon iso-electric focussing on agarose gels two peaks were obtained : a major peak with a pI of 6.6±0.2 and a minor peak at pI=5.5±0.3.

Considerable receptor heterogeneity was revealed upon chromatofocussing (Ax-500 polyamine column) of cytosols, prepared in molybdate-free buffer (CYT(+)). At least 7 components could be distinguished (pI=8.2, 8.0, 7.4, 7.1, 6.5, 5.8 and 4.5-5.4). Cytosols prepared in buffers containing molybdate (20mM) (CYT(+)) revealed much simpler chromatograms with peaks shifted to more acidic pI values (pI=6.1 and 5.5). These data were corroborated by iso-electric focussing on agarose gels. CYT(-) cytosols displayed 3 major peaks (pI 6.8,6.2 and 5.9) while CYT(+) cytosols exhibited a single major high affinity binding component (pI=5.9). The value of the findings was underlined when anion exchange chromatography yielded two distinct forms; one eluted at 90 mM and the other at 230 mM HPO_4^- for CYT(-) cytosols in contrast to only one single component eluting at 189 mM HPO_4^- for the CYT(+) cytosols.

Sodium molybdate affected the stability of vervet monkey uterine oestrogen (ER) and progesterone (PR) receptors. Yields of recep-

tors were invariably higher (20 - 40 %) when cytosols were prepared in the presence of 10mM sodium molybdate. No changes were observed in the binding affinities for the natural ligands as reflected in dissociation constants. Receptor ligand association at 0°C and 20°C was not affected in the presence or absence of molybdate. Stability studies at 37°C indicated that both receptors were more resistant to inactivation in the presence of molybdate. Dissociation of ER and PR was biphasic indicating the existence of slow (SDC) as well as fast dissociating (FDC) complexes. Rate constants of dissociation were significantly affected by the presence of sodium molybdate. Although no significant changes in the sedimentation coefficients were observed, marked differences in the actual gradient profiles could be illustrated in the presence or absence of sodium molybdate. The effects observed could be partially reversed in sedimentation dialysis experiments. Proteolytic inhibitors phenylmethylsulfonylfluoride (PMSF) and leupeptin had no inhibitive effect on the molybdate stabilization of ER and PR.

It could be illustrated by in vitro studies that temperature-induced activation of both ER and PR lead to the formation of a 4.5S (ER) or a 4.6S (PR) complex maximally at 15 min of temperature activation (SDG analysis). Simultaneously, binding affinity of the activated complexes for either uterine nuclei or DNA-cellulose reached a maximum at 15 min of temperature induced activation. Both the sedimentation shift and binding of the activated complexes to nuclei and DNA-cellulose were severely inhibited in the presence of 10mM sodium molybdate. Furthermore it could be demonstrated that activation of the human uterine PR-ligand complexes was also accompanied by a sedimentation shift (3.7-4.6) and to maximum binding of the activated complexes to human uterine nuclei and to DNA-cellulose. A limited number of pathological human uterine samples was investigated in a similar manner and it was found that in all the cases studied (leiomyomas and cervix carcinomas) the sedimentation shift of the activated complexes

appeared to be absent, while binding of these complexes to nuclei and DNA-cellulose were sub-normal.

PREFACE

In the past 25 years, a large volume of data was collected on steroid hormone receptors and their role in the action mechanisms of steroid hormones. Efforts were made to use the principles, derived from fundamental investigations into the actual function of steroid hormone receptors in target tissues, (e.g. breast and uterine tissues) to provide a comprehensive understanding of the nature of hormone responsive tumours (e.g. breast tumours) and to formulate successful endocrine treatment regimes for e.g. breast cancer patients. Although some measure of success was achieved with antihormonal drugs like tamoxifen, clomiphene, medroxyprogesterone acetate, etc. a statistical analysis of patient response data has revealed disappointingly low median response times for breast cancer patients. A number of reasons can be offered in explanation of the discrepancies between theory and practice, however, it was felt that the two most obvious ones are the lack of a proper animal model, closer to man on the species scale, and insufficient understanding of the actual *in vivo* mechanisms of steroid hormone action.

For this very reason it was decided to launch an in depth investigation into the properties and functions of steroid hormone receptors in the biological action of steroid hormones, especially the sex steroid hormone receptors, like the oestrogen and progesterone receptors. Furthermore, it was decided to employ a non-human primate, *Cercopithecus aethiops pygerythrus*, commonly called the Vervet monkey, as the animal model, instead of the more common Sprague-Dawley rat. Since the field of investigation defined is both broad and deep, it is obvious that this thesis could not contain all the answers.

So, in view of the obvious limitation in time and man-power, the prime object for this thesis was defined as the laying of sound foundations for future research on the topic specified above. Thus, it was decided to concentrate on the molecular properties of the uterine oestrogen and progesterone receptors of the Vervet

monkey. The basic arsenal of biochemical technology used in protein chemistry (competitive protein binding assays, sucrose density gradient analysis, size exclusion chromatography, ion exchange chromatography, chromatofocussing, isoelectric focussing) and available in the Institute of Life Sciences, Faculty of Medicine, University of Pretoria at the time of this investigation, was employed. In view of the information generated in the course of the study, attention was focussed upon a small, but important aspect of the mechanism of steroid hormone action, namely the process of activation or tranformation of receptor-ligand complexes and their interaction with the nuclear compartment. Since disturbance(s) in these processes in abnormal uterine tissues may exist, a limited study of the oestrogen and progesterone receptors in human uterine tissues was undertaken, because no abnormal Vervet monkey uterine tissues were available. Unfortunately it was also extremely difficult to obtain an abundance of pathological human uterine tissues necessary for an adequate study. The investigator consequently had to be content to study only a small number of pathological uterine specimens. Thus the latter part of this thesis only skims the surface of what could have been a major fait accompli with possible clinical significance.

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LIST OF ABBREVIATIONS

Å	angstrom unit (1 A=10^{-8}cm)
B_{max}	maximum binding capacity
B/F	bound to free ratio
BSA	bovine serum albumin
CYT(+)	cytosols prepared in the presence of sodium molybdate
CYT(-)	cytosol prepared in the absence of sodium molybdate
DCC	dextran coated charcoal
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHT	dehydrotestosterone
DTNB	dithionitrobenzene
DTT	dithiothreitol
EE_2	ethinyloestradiol, ethynyloestradiol
E_1	estrone
E_2	17β-oestradiol
E_3	estriol
EDTA	ethylenediaminetetraacetic acid disodium salt
ER	oestrogen receptors
FDC	fast dissociating component
fmole	femtomoles (1 fmole=10^{-15}mole)
g	gram(s)
g'	gravitational constant
GPC	gel permeation chromatography
GRAD(+)	sucrose density gradient prepared with buffer containing sodium molybdate
GRAD(-)	sucrose density gradient prepared with buffer, free of sodium molybdate
h	hour(s)
HAP	hydroxylapatite assay
^{3}H-E_2	[2,4,6,7^{3}H(N)]-17β-oestradiol
^{3}H-R5020	[17-methyl-^{3}H]-promogestone
^{3}H-P	[1,2,6,7,2 ^{3}H(N)]-progesterone
HC	hydrocortisone,cortisol
HPLC	high performance liquid chromatography
IAA	iodoacetic acid

%I	percent inactivation of ligand binding capacity
$^{125}I\text{-}E_2$	16α-[^{125}I]-Iodooestradiol
IEF	isoelectric focussing
K_d	dissociation constant
K_D	distribution coefficient
Leu	leupeptin
M	mole per liter
mℓ	millilitre
min	minute(s)
mole	molecular mass of compound in grams
M W	molecular mass
NE	norethindrone
NEM	N-ethylmaleimide
O-phen	O-phenanthroline, ortho-phenanthroline
P	progesterone
PEG	phosphate buffer (25mM) containing 1.5mM EDTA and 10% glycerol
$PEGK_{100}$	PEG buffer containing 0.1 M KCl
$PEGK_{400}$	PEG buffer containing 0.4 M KCl
$PEGK^{\circ}_{400}$	cytosols incubated zero minutes with 0.4 M KCl and run on columns eluted with $PEGK_{400}$ buffer
$PEGK^{\circ}_{400}$	cytosols incubated 30 minutes with 0.4 M KCl and run on columns eluted with $PEGK_{400}$ buffer
pI	isoelectric point
PMSF	phenylmethylsulfonyl fluoride
pM	picomole per litre ($1pM=10^{-12}M$)
PR	progesterone receptors
R5020	promogestone
r	correlation coefficient
RBA	relative binding affinity
R or r*	Stokes radius
SDC	slow dissociating component
S	svedberg ($1S=10^{-13}$seconds)
SDG	sucrose density gradient
TLCK	N-α-p-tosyl-L-chloromethylketone
$TEDAG_{10}$	Tris-HCl buffer containing 10mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol, 1mM sodium azide and 10% (w/v) glycerol

$TEDAG_{10}$	Tris-HCl buffer (10mM) containing 1.5 mM EDTA, 1mM DTT, 1mM sodium azide and 60 % (w/v) glycerol
TEDAG-MO	$TEDAG_{10}$ buffer containing 20mM sodium molybdate
T	testosterone
V_e	elution volume
V_o	column void volume
V_t	column total volume
VMU	Vervet monkey uterus
w/v	weight per volume

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CHAPTER 1

INTRODUCTION.

PHYSIOLOGY AND ANATOMY OF THE FEMALE SEXUAL ORGANS

The sexual organs of the human female includes : the ovaries, the fallopian tubes, the uterus, the vagina and the breasts. Reproduction begins with the development of ova in the ovaries. A single ovum is expelled from an ovarian follicle into the abdominal cavity in the middle of each monthly sexual cycle. This ovum then passes through one of the fallopian tubes into the uterus, and if it has been fertilized by a sperm, it implants in the uterus where it develops into a foetus, a placenta and fetal membranes.

The breasts begin to develop at puberty, a development which is stimulated by the sex hormones produced by the ovaries.

These hormones stimulate the growth of the mammary stroma and the ductile system. During pregnancy further development of the ductile system occurs in preparation for lactation. Throughout pregnancy, large quantities of oestrogens are secreted by the placenta causing the ductile system of the breasts to grow and to branch. Moderate quantities of growth hormone work in conjunction with the oestrogens to produce their effect on the breasts. Simultaneously progesterone affects the growth and development of the lobules and the development of secretory characteristics within the cells of the mammary alveoli. All these changes are analogous to the secretory effects of progesterone on the endometrium of the uterus. Other hormones exerting a controllig effect on the function and maintenance of the breasts are prolactin and oxytocin. The exact interaction of these hormones with the steroid hormones appear to be very complex. For the purpose of this introduction it suffices to point out that all the female sex organs are under strict control of several

hormones, including the most important steroid sex hormones oestrogen and progesterone.

THE FEMALE HORMONAL SYSTEM

The female hormonal system, consists of three different hierarchies of hormones:

1) The hypothalamic releasing factors : follicle-stimulating hormone releasing factor (FRF) and luteinizing hormone releasing factor (LRF) or one factor responsible for the secretion of both FSH and LH by the pituitary gland the gonadotrophin releasing hormone (GnRH).
2) The anterior pituitary hormones : follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which are secreted in response to the releasing factors from the hypothalamus.
3) The ovarian hormones : oestrogen and progesterone, which are secreted by the ovaries in response to the two hormones from the anterior pituitary gland.

The various hormones are not secreted in constant, steady amounts but instead at drastically differing rates during different parts of the female cycle.

THE MONTHLY OVARIAN CYCLE AND FUNCTION OF THE GONADOTROPIC HORMONES.

The normal reproductive years of the female are characterized by monthly rhythmic changes in the rates of secretion of the female hormones and corresponding changes in the sexual organs themselves. This rhythmic pattern is called : the menstrual cycle. The duration of the cycle averages 28 days. The two significant results of the female sexual cycle are : first, only a single mature ovum is normally released from the ovaries each month so that only a single fetus can begin to grow at a time. Second, the uterine endometrium is pre-

pared for implantation of the fertilized ovum at the required time of the month.

1.3.1 The gonadotropic hormones:

The sexual cycle is completely dependent on gonadotropic hormones secreted by the anterior pituitary gland. Ovaries that are not stimulated by gonadotropic hormones remain completely inactive.

The anterior pituitary secretes two different hormones that are known to be essential for full function of the ovaries:

1) follicle-stimulating hormone (FSH), and
2) luteinizing hormone (LH). Both of these are small glycoproteins having molecular weights about 30,000. During each month of the female sexual cycle, there is a cyclic increase and decrease of FSH and LH as illustrated below. These cyclic variations in turn cause cyclic ovarian changes.

Throughout childhood the primordial follicles (primary follicle) do not grow, but at puberty, when FSH from the anterior pituitary gland begins to be secreted in large quantity, the entire ovaries and especially the follicles within them begin to grow. The first stage of follicular growth is enlargement of the ovum itself. This is followed by development of additional layers of granulosa cells around each ovum and development of several layers of theca cells originating from the stroma of the ovary and which soon take on epitheloid characteristics. It is mainly these cells that are destinated to secrete most of the female hormones, the oestrogens and progesterone. Luteinizing hormone (LH) is necessary for final follicular growth and ovulation. Without this hormone, even though large quantities of FSH are available, the follicle will not progress to the strength of ovulation. The change of follicular cells into lutein cells

is completely dependent on the LH secreted by the anterior pituitary gland. The corpus luteum is a highly secretory organ, secreting large amounts of both progesterone and oestrogen. In the presence of LH the degree of growth of the corpus luteum is enhanced, its secretion is greater and its life is extended. During the luteal phase of the ovarian cycle, the large amount of oestrogen (and perhaps to a very slight extent, the progesterone as well) secreted by the corpus luteum causes a feedback decrease in secretion of both FSH and LH. Therefore, during this period no new follicles begin to grow in the ovary. However, when the corpus luteum degenerates completely at the end of 12 days of its life, the loss of feedback suppression now allows the anterior pituitary gland to secrete several times as much as FSH as well as moderately increased quantitities of LH. The FSH and LH initiate growth of new follicles to begin a new ovarian cycle. At the same time, the paucity of secretion of progesterone and oestrogen leads to menstruation by the uterus. In summary: Approximately each 28 days, gonadotropic hormones from the anterior pituitary gland cause new follicles to begin to grow in the ovaries, one of which finally ovulates at the 14th day of the cycle. During growth of the follicles, oestrogen is secreted. Following ovulation, the secretory cells of the follicle develop into a corpus luteum that secretes large quantitites of the female hormones progesterone and oestrogen. After another two weeks the corpus luteum degenerates, whereupon the ovarian hormones, oestrogen and progesterone, decrease greatly and menstruation begins. A new ovarian cycle then follows.

1.3.2. The ovarian hormones - oestrogen and progesterone.

The two types of ovarian hormones are the oestrogens and progesterone. The oestrogens mainly promote proliferation and growth of specific cells in the body and are responsible for development of most secondary sexual characteristics of

the female. On the other hand, progesterone is concerned almost entirely with final preparation of the uterus for pregnancy and of the breasts for lactation.

1.4 CHEMISTRY OF THE SEX STEROID HORMONES:

1.4.1 The oestrogens: In the normal, non-pregnant female, oestrogens are secreted in major quantitites only by the ovaries, though minute amounts are also secreted by the adrenal cortices. In pregnancy tremendous quantities are also secreted by the placenta. At least six different natural oestrogens have been isolated from the plasma of the human female, but only three are present in significant quantitites : β-oestradiol, oestrone and oestriol. However, the oestrogenic potency of β-oestradiol is 12-times that of oestrone and 80 times that of oestriol, so that the total oestrogenic effect of β-oestradiol is usually many times that of the other two together.

1.4.2 Progesterone:Almost all the progesterone in the non-pregnant female is secreted by the corpus luteum during the latter half of each ovarian cycle. However, during pregnancy progesterone is formed in extreme quantities by the placenta, about 10 times the normal monthly amount, especially after the fourth month of gestation.

1.5 FUNCTIONS OF OESTROGEN AND PROGESTERONE

1.5.1 Functions of oestrogens:

The principal function of the oestrogens is to cause cellular proliferation and growth to the tissues of the sexual organs and of other tissues related to reproduction. During childhood oestrogens are secreted only in small quantities, but following puberty the quantity of oestrogens secreted under the influence of the pituitary a gonadotropic hormones increases some 20-fold or more. At this time the female

sexual organs change from those of a child to those of an adult. In addition, oestrogens change the vaginal epithelium from a cuboidal into stratified type. More important however, are the changes that take place in the endometrium under the influence of oestrogens, for oestrogens cause marked proliferation of the endometrium and development of glands that will later be used to aid in nutrition of the implanting ovum. Oestrogens cause fat deposition in the breasts, development of stromal tissues of the breasts, and growth of an extensive ductile system. The lobules and alveoli of the breasts develop to a slight extent, but it is progesterone and prolactin that cause the determinative growth and function of these structures.Oestrogens cause increased osteoblastic activity. However, oestrogens have an other potent effect on skeletal growth: they cause early uniting of the epiphyses with the shafts of the long bones. This effect is much stronger in the female than is the similar effect of testosterone in the male. Oestrogens cause deposition of increased quantities of fat in the subcutaneous tissues. Oestrogens cause the skin to become more vascular than normal; this effect is often associated with increased warmth of the skin.

1.5.2 Functions of the progesterone :

The most important function of progesterone is to promote secretory changes in the endometrium, thus preparing the uterus for implantation of the fertilized ovum. Progesterone also promotes secretory changes in the mucosal lining of the fallopian tubes. Progesterone promotes development of the lobules and alveoli of the breasts, causing the alveolar cells to proliferate, to enlarge and to become secretory in nature.

1.6 THE ENDOMETRIAL CYCLE AND MENSTRUATION:

Associated with the cyclic production of oestrogens and

progesterone by the ovaries is an endometrial cycle operating through the following stages: First, proliferation of the uterine endometrium; second, secretory changes in the endometrium; and third, desquamation of the endometrium,a which is known as menstruation.The different stages will now be briefly discussed.

1.6.1 Proliferative phase (oestrogen phase) of the endometrial cycle:

At the beginning of each menstrual cycle, most of the endometrium is desquamated by the process of menstruation. After menstruation, only a thin layer of endometrial stroma remains at the base of the original endometrium and the only epithelial cells left are those located in the remaining deep portions of the glands and crypts of the endometrium. Under the influence of oestrogens, secreted in increasing quantities by the ovary during the first part of the ovarian cycle, the stromal cells and the epithelial cells proliferate rapidly. The endometrial surface is re-epithelialized within three to seven days after the beginnning of menstruation.,

1.6.2 Secretory phase (progestational phase) of the endometrial cycle.

During the latter half of the sexual cycle, progesterone as well as oestrogen is secreted in large quantity by the corpus luteum. The oestrogens cause additional cellular proliferation, and progesterone causes considerable swelling and secretory development of the endometrium. The thickness of the endometrium approximately doubles during the secretory phase so that toward the end of the monthly cycle the endometrium has a thickness of 4-6 mm.

1.6.3 Menstruation.

Approximately 2 days before the end of the monthly cycle, the ovarian hormones, oestrogens and progesterone, decrease sharply to low levels of secretion. The first effect has decreased stimulation of the endometrial cells by these two hormones, followed rapidly by involution of the endometrium itself to about 65 % of its previous thickness. Vasospasm and loss of hormonal stimulation cause beginning necrosis in the endometrium, as a result blood seeps into the vascular layer of the endometrium. Within 3-7 days after menstruation starts, the loss of blood ceases, for by this time the endometrium has become completely reepithelialized.

Secretion of most of the anterior pituitary hormones is controlled by releasing factors formed in the hypothalamus and transmitted to the anterior pituitary gland by way of the hypothalamic-hypophyses portal system.

1.7 HISTORY OF STEROID HORMONE RECEPTORS

As early as 1835 (1), Sir Astley Cooper in his book "Lectures on the Principles and Practice of Surgery" noted a correlation between the size of breast tumours and the phase of the menstrual cycle. He also observed a relationship between multiparity and an increased risk of breast cancer an observation which stood the test of time.

More than 50 years later, Schinzinger (1889) (2) noted that the mortality among premenopausal breast cancer patients was higher than that in postmenopausal patients. He suggested that the life expectancy of the younger patients could be increased by prophylactic oophorectomy.

A great advance in the pioneering work of establishing the

hormonal dependence of some breast cancers came in 1896 when Sir George Beatson (3) reported the beneficial effect of oophorectomy in two premenopausal women with advanced breast cancer. This finding preceded the isolation of oestrogen from ovarian tissue and the explanation for Beatson's observation was therefore not realized at the time. During his studies of lactating cows, Beatson noted that cells from cancerous breasts multiplied rapidly and often penetrated the breasts lymph system instead of undergoing fatty degeneration and being cast off in the milk. He reasoned that since farmers spayed lactating cows in order to maintain a long period of fatty degeneration and milk production the same procedure might induce fatty generation of the cancerous breast. In 1895, Beatson performed an oophorectomy on a premenopausal women with advanced breast cancer and found to his amazement that her metastatic chest wall lesion also disappeared. After Beatson's now classical paper, "On the treatment of inoperable cases of carcinoma of the mamma : suggestions for a new method of treatment with illustrative cases" was published in the Lancet several workers tried to repeat the same treatment, but noted that it was difficult to predict which patients would respond to oophorectomy (4-5).

Decourmelles (1926) and others (6) subsequently popularized irradiation castration rather than oophorectomy, but because some patients continued to manifest reproductive function, surgical oophorectomy again came into vogue in the 1950s (7).

A significant advance in the understanding of the chemical basis of endocrine response occurred with the isolation and identification of oestrogen from sow ovaries by McCorquodale, Thayer & Doise (1926)), in which they reported the isolation of crystalline oestrogenic hormone from sow ovaries, (8)

The modern era of endocrine therapy; based on the concept that hormones may influence the growth of certain forms of cancer, commenced when Charles Huggins showed that orchidectomy or the administration of estrogens caused regression of canine prostatic cancer (9). After several important contributions in the field of prostatic cancer (10-11) Huggins turned his attention to mammary cancer, and illustrated that adrenalectomy was often effective after a relapse following oophorectomy (12-13).

Huggins' concepts can best be summarized from his Nobel lecture given in 1967, entitled "Cancer is not necessary autonomous and intrincisally selfperpetuating. Its growth can be sustained and propagated by hormonal function in the host" (14).

Until 1953, only sporadic reports of successful hypophysectomy in humans were published. Luyt and Olivecrona (1953) were the first to report that hyphophysectomy induced remissions similar to that obtained by oophorectomy and adrenalectomy in metastatic breast cancer (15). These results were later confirmed by Pearson and Ray who reported objective remission following hypophysectomy in 21 out of 41 cases of advanced mammary cancer (16-17).

Glascock and Hoekstra (1959) (18) succeeded in synthesizing tritium-labelled hexoestrol (anti-estrogen) and using this labelled hormone analogue in goats, noted the hormone was concentrated and retained by 'target' organs such as the uterus and vagina. During the past two decades, there has been an overwhelming proliferation of research contributions made towards the understanding of the mechanism of steroid hormone action. Jensen and Jacobson made an invaluable contribution when they synthesized [^{3}H]-oestradiol with a high specific activity, which made it possible to observe differential accumulation and retention of [^{3}H]oestra-

diol by the uterus and other target tissues (19). Mobbs (20) found that endocrine-dependent dimethylbenzanthracene-induced (DMBA-induced) rat mammary tumours retained labelled oestradiol, in contrast to autonomous tumours which did not.

Jensen, De Sombre and Jungblut (21) and Terenius (22), independently demonstrated, the enhanced specific binding of radioactively labelled estrogens to breast cancer specimens *in vitro* which exhibited objective clinical responsiveness. These experiments indicated that there were at least two general classes of breast cancer, those that exhibited a significant uptake of labelled estradiol *in vitro* and those that showed no or very low hormonal uptake.

Toft and Gorski (23) established the usefulness of sucrose gradient sedimentation analysis in the characterization of the oestrogen receptors present in the cytosol of rat uterus. A year later Jensen, De Sombre and Jungblut (24) using sucrose gradient analysis could demonstrate that tumours from patients who responded to hormonal therapy contained relatively high levels of oestrogen receptor (ER) in the cytoplasm, while unresponsive tumours lacked oestrogen receptors. These experiments provided a method for distinguishing hormonedependent from autonomous tumours prior to actual therapy. The careful observations of Jensen *et al* (21) and Gorski *et al* (25) led to the proposal of the classical 'two-step mechanism' for the hormone-induced transfer' of the receptor from the cytoplasm to the nucleus. Stumpf (26) employed autoradiography to confirm the rapid movement of steroid hormone to the nucleus.

Initial binding of steroid to the cytosolic receptor seems to initiate a cascade reaction currently incompletely underunderstood. Toft and Gorski (23), as well as Erdos (27), observed that the ∿ 8 S oestrogen receptor species, usually obtained in low ionic strength SDG could be dissociated into ∿ 4 S complexes at higher ionic strength condi-

tions. Jensen et al (28) found that cytosolic receptors could be 'transformed' into a 5 S form, by a hormone-dependent, temperatureinduced process. Higgins et al (29) and Milgrom, Atger and Baulieu (30) furthermore proved that the 'transformed' receptor showed an increased affinity for polyanions especially DNA, following this process, which they termed receptor 'activation' .

Up to this time very little fruitful information had been obtained on the progesterone receptor mainly due to technical problems experienced with the receptor assays.Milgrom et al (31) however first reported the presence of PR in guinea pig uterus. Subsequently, a synthetic progestin (promogestone, R 5020) was developed by Philibert and Raynaud (32) and employed in progesterone receptor studies. This progestin appeared to form significantly more stable complexes with the PR than the natural ligand progesterone. Horwitz and Mc Guire (33) subsequently demonstrated the presence of PR in breast cancer tissues, and demonstrated that the synthesis of this receptor can serve as a means of monitoring oestrogen responsiveness.

In 1974, a workshop was held under the auspices of the Breast Cancer Task Force. The results of 14 different institutions were analysed and were found to be in general agreement with each other, namely that patients with breast cancers lacking in significant amounts of ER had very little chance of responding to hormonal manipulations. At the same time they concluded that the majority of patients with receptor-positive cancers would benefit from such treatments.

Numerous subsequent investigations have confirmed, amplified, elucidated and embelished these pioneering investigations.

One of the functions of the serum steroid-binding proteins is the transport of the specific hormone in a bound state, acting as a resrvoir from which the active hormone can be mobilized rapidly by dissociation in the proximity of the target cell. The steroid-binding proteins also conserve and protect the steroid against enzymatic attack, against adsorption to the walls of blood vessels, and against excretion by the kidney and metabolism by the liver.

In some cases, metabolism of hormones is important after entrance into target cells, as has been shown for the conversion of testosterone to dihydrotestosterone (by the enzyme 5α-reductase) and 4-androstenedione (38).

Oestrogen and progesterone do not however require metabolic conversion for biological activity (19), but metabolism may be involved in the interconversion of various forms of oestrogen and in the elimination of hormone from target tissues (39).

1.8 THE MECHANISM OF ACTION OF STEROID HORMONE RECEPTOR

A multitude of different techniques are presently employed in the investigation of the action mechanism of steroid hormones, e.g. oestradiol, in normal and neoplastic tissues. The analytical techniques that were established in the 1960's and early 1970's to study the ER through the binding of [^{3}H]-oestradiol have provided much valuable information. However, the studies over the past 20 years have generally served only to consolidate the established ER model because each technique used to study the receptor depends upon cell disruption and extraction of receptors followed by detection methods that require binding of [^{3}H]-oestradiol to the ER. Clearly further progress and a critical study on the subcellular effects and mechanisms of oestradiol action via the ER would require new approaches. Receptor dynamics must be studied in intact cells in order to investigate subcellular

compartmentalization, receptor recycling or resynthesis. The development of monoclonal and polyclonal antibodies which can be labelled to identify the position of unoccupied and occupied receptors in the cell is one possible approach. Similarly, the recent synthesis of a radioactive covalent antioestrogen affinity label, [^{3}H]-tamoxifen aziridine (40), should prove to be an extremely useful tool to study conformation, physicochemical properties, and subcellular localization of ER. The availability of a broad spectrum of high-affinity, radiolabelled anti-oestrogens will facilitate studies on the physicochemical properties of oestrogen- and antioestrogen-receptor complexes. Although it is well-established that the ER is present in oestrogen target tissues, the exact role of the receptor and its interactions with other subcellular components to modulate oestrogen-induced events is unclear.

Examination of steroid-induced responses in mutant cell lines that contain reduced levels or abnormal receptor (41) is a useful approach to determine which steroid-stimulated events are receptor-dependent. Furthermore, the study of oestrogen-induced events in antioestrogen-sensitive and resistant cell lines may provide additional information about the molecular biology of the oestrogen receptor mechanism (42-43). The ultimate site of action of steroid receptor in the nucleus is not known (44). No doubt the rapid advances in molecular biological techniques will elucidate the structure and control of the ER gene as well as ER control of oestrogen-stimulated gene products. A critical application of new methodologies will confirm or evolve the models for ER mechanism.

The rapid application of these investigations to the clinic may offer more precise and accurate detection of hormone-dependent cancer and the possibility of improved or novel treatment regimes that might exploit the ER system.

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1.8.1 Possible models for steroid hormone receptors.

One aspect of steroid hormone receptor action has been elevated to the status of dogma since its elucidation fifteen years ago, following the first detection of oestradiol receptor in rat uterine cytosol (23). E. Jensen and his collaborators demonstrated by cell fractionation and autoradiography that within an hour of hormone administration, the receptor accumulates in the nucleus of target cells (24). Their finding led to the 'two step' hypothesis. According to this model, represented schematically in Fig. 1.1, oestradiol enters the uterine cell, binds to a specific cytoplasmic receptor and then is translocated to the nucleus by a temperature-dependent 'activation' process. This 'activation' of the steroid receptor complex is reflected both by a change in its sedimentation coefficient from 4 S to 5 S (in the presence of high ionic strength buffer) and by its ability to bind to isolated uterine receptors which can exist either as steroid filled or unfilled receptors, but nuclear receptors can exist only when steroid filled. The investigations were repeated and extended to numerous other steroid hormone receptor systems with similar conclusions. Now a number of workers (45-47) challenge the dogma : it would seem that steroid receptors may not be cytoplasmic proteins after all, and a number of reports have appeared which are difficult to reconcile with the two-step model of steroid hormone intracellular interaction. Important amongst these is the observation that unfilled receptors do in fact exist in the nucleus of both normal (49-51) and tumourous oestrogen target tissues (52-53). In these studies careful attention was paid to the purity of the nuclei, thereby ensuring no contamination of the preparation with cytoplasmic receptors. Physicochemical analyses indicated that the unfilled sites possess properties characteristic of 'classical' type 1 (54) nuclear oestrogen receptors in terms of their binding affinity, association (dissociation) kinetics, specificity and sedimentation behaviour in high ionic

strength sucrose density gradients; However they differ in one important respect : they are not steroid filled (unoccupied receptors).

The origin of these unfilled sites is unknown, although several authors have reported that oestrogen receptor binding sites are present in purified preparations of nuclei (55-56). In the rat uterus, autoradiography shows tritiated oestradiol, even after short periods of in vitro incubations at low temperatures, conditions which normally minimize the translocation of receptor complexes from the cytoplasm (57) to the nucleus. These observations have led to the hypothesis that in intact cells, unfilled receptors may exist in a state of equilibrium between the cytoplasm and nucleus. This equilibrium can be shifted in favour of the cytoplasm during tissue homogenization (57). Based on this model, (Fig. 1.1 b) unfilled nuclear receptors assayed using conventional techniques may only represent a small fraction of the total cellular content of unfilled sites actually present, with the bulk of unfilled sites having been solubilized during the course of nuclear isolation.

In order to overcome the inherent problem of receptor solubilization during preparation of cell nuclei,a technique was recently described by Welshons et al (48): Cytochalasin B-induced enucleation was employed in obtaining cytoplasts and nucleoplasts from receptor - rich CH_3 cells derived from rat pituitary tumours.In contrast to the predictions of the two-step model, the cytoplasts were found to contain little oestrogen-binding activity, with most of the unfilled receptor residing within the nucleoplasts. This finding supports the notion that unfilled oestrogen receptors do reside within the cell nucleus of steroid-sensitive cells. These observations led Welshons et al (48) to propose that no nuclear translocation of receptor can be found in the intact cell as part of the steroid response, but rather an increase in receptor affinity for nuclear elements.

One of the most convincing studies indicating that'oestrogen receptors may reside exclusively in target cell nuclei of oestrogen sensitive tissues' is the study of King and Greene(47). Here, rather than using radiolabelled ligands to detect the oestrogen receptor, they used immunochemical staining with a monoclonal antibody generated against the oestrogen receptor protein. Specific staining was found to be confined to the nucleus of all stained cells, which included human breast tumours and uterus, rabbit uterus, oviduct, corpus luteum. mammary gland, pituitary and liver, as well as MCF-7 cell cultures. The distribution of oestrogen receptors as would be predicted from the study described above can be schematically represented by Fig 1.1. The application of the new model (1.1.c) on the process of activation will be discussed in more detail later in Chapter 5.

It is an experimental fact that hormone agonists alter gene expression, and hence some aspect of the receptor structure must change by interaction with its ligand. Receptors are probably permanently associated with the gene they regulate, and thus have only to rotate or change conformation to achieve their effects. There still are two identifiable states of a steroid receptor : an active (ligand associated) state and an inactive (unliganded) one. The real significance of the original two-step idea is retained: Steroid-receptor complexes are nuclear regulatory elements wherever they originate in the cell.

It is obvious that much work is still to be done in unravelling the nature of the interaction between steroid hormones and their receptors.

1.8.2 Common events in steroid hormone-receptors mechanism of binding.

Most workers agree that the entry of steroids into cells

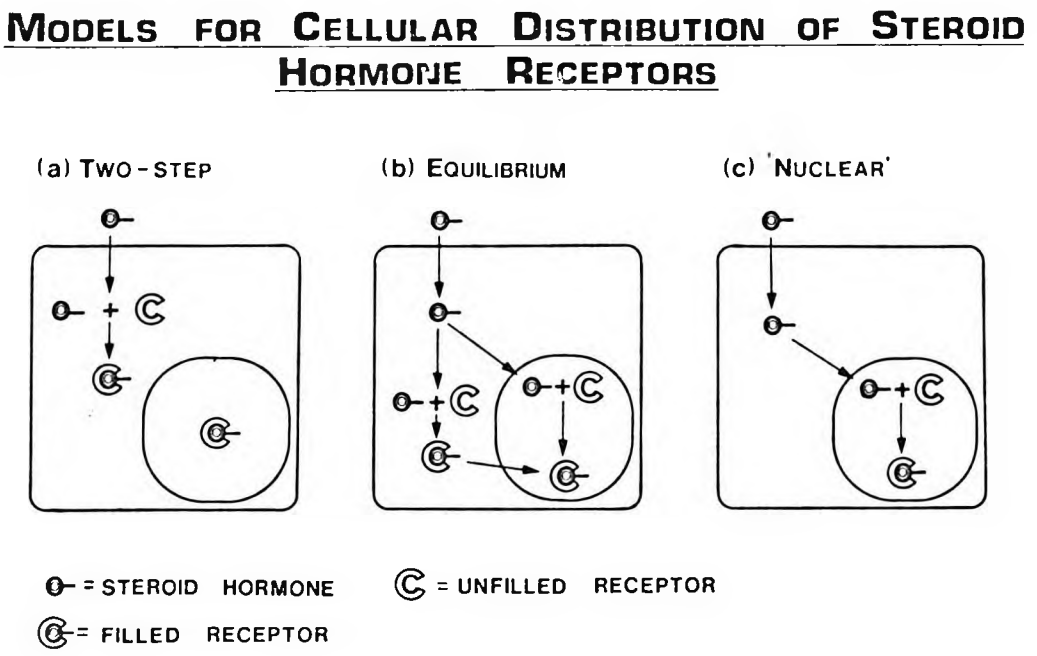


Figure 1.1: Three models depicting the possible cellular distribution of steroid hormone receptors within a steroid target cell (from Clark (46)).

from the extra cellular space appears to occur by diffusion. Gurpide and Welch (58) studied uptake dynamics using a double-isotope steady-state technique, and found that target and non-target tissues accumulated steroid with equal facility, at a rate proportional to steroid concentration, suggesting a mechanism of simple diffusion. However, the possibility of specific recognition sites on the plasma membrane of target cells has not been finally excluded. Zanker, Prokscha and Blumel (59) proposed that plasma membranes from breast cancer cells contain receptors of relatively high affinity (K_d = $6.4x10^{-10}$M) and high capacity,working apparently in tandem with the high-affinity and low capacity receptors described for the cytosol. They postulated that these membrane receptors may serve a modulator function.

Following entry into the target cell, the hormone may either bind to its specific cytoplasmic receptor or bind to the more abundant, lower affinity binding sites. Sometimes a given hormone may interact with more than one high-affinity receptor.Baulieu (60) demonstrated that oestradiol interacts with androgen receptor with a K_d of approximately 3nM, while oestradiol binds to oestrogen receptor with a K_d of 0.1 nM. Several hormones may bind to one class of receptor, and receptor concentrations may vary under different conditions such as changes in the menstrual cycle.

The K_d is a measure of the affinity of the receptor for the steroid and is approximately the concentration of free steroid at which one-half of the receptors are saturated. The K_d which is the reciprocal of the association constant (K_a) can also be obtained from a Scatchard plot (61).

After oestrogen enters a target cell, the physiological form of the receptor is at present uncertain. Such factors as ionic strength, temperature, presence of molybdate, or enzyme inhibitors such a leupeptin, can cause the receptor to exist in a certain form or to interconvert between several

molecular forms, any or none of which may be the true endogeneous receptor(62). After reaction with oestrogen, the ER complex undergoes a temperature-dependent conformational change or 'transformation' that is associated with receptor activation and an increase avidity for nuclear binding.

The dissociation kinetics of a hormone from the receptor provides a sensitive criterion for measuring receptor activation. The rate of dissociation of a hormone from its activated receptor complex is an indication of the effectiveness of that particular hormone in maintaining the receptor in the activated state. The dissociation of oestradiol from the receptor occurs as a biphasic process (63). The low affinity for oestradiol binding by the non-activated receptor causes the first,rapid exponential phase, while the higher affinity of the activated state of the receptor produces the second, slower phase (64).Oestradiol binding, then, shifts the equilibrium from a lower-affinity state to a high-affinity, activated, slower-dissociating phase (63).

Several investigators have suggested that the multiple forms of ER detected in human breast cancer may be due to degradation of native receptors. Sherman et al (65) have shown that the steroid receptors degraded by endogeneous proteases to lower-molecular weight 'meroreceptors' contained hormone-binding sites. Puca et al (66) described a partially purified 'receptor-transforming factor' which was responsible for degradation of ER in calf uterus to a smaller size.

After entering the cell nucleus, the 5 S oestrogen-receptor complex binds to 'putative acceptor' sites on chromatin (30), where the expression of specific genes occurs at a different locus on the chromosomes called the 'effector' site.

Recently two groups have suggested preferential binding of steroid hormone receptors to specific DNA sequences (68). Mulvihill, Le Pennec and Chambon (69) found that specific

double-stranded DNA sequences from hormonally responsive genes coding for egg-white proteins were recognized by oviduct progesterone receptor in vivo. These works are significant, because they suggest that steroid receptors may modulate transcription by recognizing specific DNA sequences within or near hormone-regulated genes. Acceptor sites on chromosomes have been suggested to be a specific complex of DNA and non-histone proteins, which the oestrogen receptor complex recognizes and binds with a very high affinity (70). There are also believed to be secondary sites present in large numbers on chromatin, where the ER-complex can bind with lower affinity (71). Besides primary binding sites associated with activated ER-complexes which are translocated to the nucleus, called type I sites, secondary sites also appear, called type II sites (72). These type II sites are found in increased number after oestradiol stimulation and appear to be a specific hormonally-induced nuclear response. The type II sites, which are not translocated from the cytoplasm, are present in larger quantities than type I sites and remain elevated for a longer period.

The binding of ER to acceptor sites is thought to increase the rate of transcription of specific genes, possibly by altering their affinity for interaction with RNA polymerase. In some systems, rapid increases in transcription are apparent after oestrogen stimulation (73) and mitosis is accelarated (74).

One of the important actions of oestrogen seen in some hormone-sensitive target cells, including oestrogen-sensitive human breast cancer cells is the induction of progesterone activity (75), which may be involved in the further hormonal regulation of the cells. Other workers (76) have demonstrated that peroxidase also appeared to be an oestrogen-inducible enzyme in both normal malignant tissues. It seems that most steroid effects are regulated at the level of primary transcription, however, some of the short term effects like water inhibition and eosinophilia may not necessitate regulation at the genetic level.

When oestradiol enters the responsive breast cancer cell or uterine tissue in vitro, the newly formed hormone-receptor complex is rapidly translocated to nuclear acceptor sites, from which it can only be extracted using high ionic strength buffers. Horwitz and McGuire (77) have proposed that bound nuclear receptors then undergo rapid 'processing' in which approximately 70 % of the nuclear receptor sites are lost from the cells over a few hours' duration without reappearance of unfilled sites. They found that this 'processing' was essentially complete by five hours, after which time the nuclear receptor complex stabilizes at a new steady state level (78). Lippman et al (79) found that processing is not a disappearance of the nuclear receptor, as had been previously proposed, but a receptor modification to a less KCℓ-soluble and less exchangeable form. Oestrogen receptor inactivation which follows translocation and nuclear binding to the acceptor site has been proposed to be due to dephosphorylation of the receptor secondary to the action of nuclear phosphatase (80). Auriccio's group has also hypothesized that the inactive receptor may be reactivated through phosphorylation by an ATP-dependent enzyme in the cytoplasm (81). These findings suggest that the receptor is cycled by the dephosphorylation process in the nucleus, followed by a rephosphorylation event in the cytoplasm, contributing in this manner to cytoplasmic replenishment.

The process of activation will be discussed and studied in detail in Chapter 5.

1.9 STEROID HORMONE-RECEPTOR RELATIONSHIPS WITH SPECIAL REFERENCE TO THE OESTROGEN AND PROGESTERONE RECEPTORS :

The oestrogen receptor system is only part of a complex hormonal mechanism which influences breast cancer growth and function. Many other hormone receptors have been identified in cultured breast cancer cells, such as progesterone,

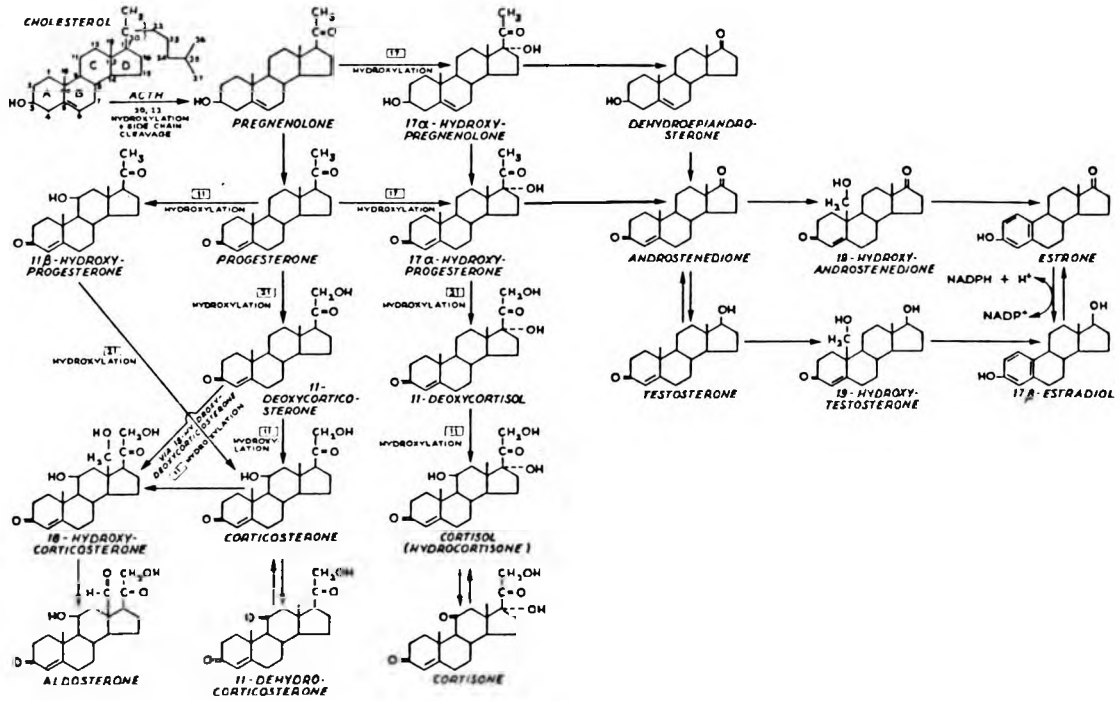


Figure 1.2 : Biosynthesis of adrenal corticosteroids, androgens and oestrogens (From Harper 1980).

androgen, insulin, glucocorticoid, retinoid, prolactin, transferrin, epidermal growth factor, calcitonin, and Vitamin D receptors, but the unique physiological role of each has not been fully elucidated. Tumours may contain a heterogeneous population of cells with a mixture of various receptor positivities, as well as completely autonomous cell types, a situation that profoundly influences the effectiveness of hormonal therapy. The presence of PR in ER-positive tumours suggests that these tumours are capable of synthesizing at least one oestrogen-regulated product, but it does not imply that all the oestrogen-induced responses will necessarily be intact. In many systems in vivo or in vitro, the administration of oestradiol to an oestrogen-responsive cell causes an 8 to 20-fold rise in PR, which can be prevented by the inhibition of protein or RNA synthesis (82 - 83). The measurement of progesterone receptors was hampered initially by the lack of a suitable ligand, since progesterone binds readily to other plasma proteins such as transcortin (84). R-5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione) which is synthetic progestin, shows high affinity for progesterone receptor, lack of tight plasma binding, a slow dissociation rate, and negligible interference with other hormone receptors (85).

The PR has been studied extensively in the chick oviduct system by Schrader and O'Malley who have purified the receptor into an A and B components (86-87). The A subunit binds tightly to naked DNA, while the B component binds selectively to non-histone protein-DNA complexes (88) and has little affinity for DNA. When present as an AB dimer, the DNA binding site of the A subunit is masked. It is hypothesized that initial binding of the receptor to chromatin takes place exclusively through the B subunit, but that the A subunit subsequently interacts directly with DNA and stimulates messenger RNA synthesis (89). Progesterone therapy in systems in vivo and in vitro may mediate either growth, regression, or no growth change in mammary cancer, and the speci-

fic mechanisms involved are not fully understood. Huggins and Young(90) showed that both pregnancy and the administration of progesterone promoted the growth of DMBA-induced rat mammary tumours. In contrast to it stimulatory effects, progesterone can cause regression of animal and human breast tumours, and the response appears even more significant if an oestrogen progesterone combination is used (91-92). Shafie et al (93) using MCF-7 human mammary hetero-transplanted tumours in nude mice, demonstrated that progesterone given alone did not change either the latent period, growth rate, nor frequency of tumour appearance.

1.10 STEROID RECEPTORS IN NORMAL, HYPERPLASTIC AND MALIGNANT HUMAN UTERUS

Human endometrium has been subjected to biochemical analysis by many investigators. The majority of these have confined their attention either to premenopausal samples or to neoplastic tissue from postmenopausal women (114). A few publications refer to analyses performed on endometria of normal histology from postmenopausal women receiving various oestrogen and progestin combinations (96,115).

The endometrium is composed of several different cell types which can be divided into epithelium and stroma. The epithelium is mainly glandular together with some luminal epithelium. The stroma is more heterogeneous, the major constituent bring stromal cells together with variable numbers of erythrocytes, leukocytes and endothelial cells. Stromal morphology varies depending on the hormonal status of the woman (116). Despite the complex cellular composition of the human endometrium, almost all receptor studies have reported on whole endometrium and have largely been interpreted as though it was a homogeneous cell population. Few workers (117) demonstrated that when epithelium and stroma from premenopausal endometria were separately analysed, the stromal cells may contain less oestrogen receptors than epi-

thelium.

Human endometrium is a tissue in which the female sex steroids exert their actions during a woman's reproductive life. Endometrial carcinoma is at present the commonest malignancy of the female genital tract (118) and together with breast carcinoma it is the most important target for endocrine cancer therapy. While surgery and regional irradiation are the primary treatments for patients with endometrial carcinoma, hormone therapy is widely used for advanced and recurrent disease, and is also used prophylactically after the initial therapeutic measures. There is a striking similarity between patients with endometrial and breast carcinoma in their response rate to endocrine therapy (118).

Normal endometrium

There are great inter-individual variations in endometrial ER and PR levels, which cannot be explained by methodological reasons. Moreover, the receptor levels measured in the uteri of premenopausal women fluctuate during the menstrual cycle. In normally cycling women, cytosol endometrial ER levels are maximum during the proliferative phase around the midcycle, and the lowest levels are seen near the end of the menstrual cycle (118)(100). The maximal nuclear ER concentration is seen later during the proliferative phase and extends to the early luteal phase (118). The highest levels of cytosol PR are present during the preovulatory period of the cycle, and then rapidly become depleted subsequent to ovulation (98). Tsibris et al measured ER and PR receptors in human endometrium during the menstrual cycle to determine their hormonal regulation and establish their usefulness in understanding normal reproduction and in managing gynaecologic disorders (106). However, the clinical use of such receptor data, obtained mainly from tissues removed by cure-

tage or aspiration, may be impeded by the observations that the normal uterus has an uneven distribution of these receptors along its longitudinal axis(88,107). Tribis et al (106) tried to map possible variations in the distribution of endometrial cytoplasmic and nuclear receptors during the menstrual cycle and used accurately placed endometrial samples obtained from 45 uteri removed for benign diseases. A number of other investigators (98, 107-109) have measured ER and PR levels in unspecified regions of human endometrium and in cervical epithelium. There are a few facts where there is agreement among most of the investigators :

1) Dissociation constants do not vary significantly from the different sections of endometrium, at different times during the menstrual cycle nor after menopause.
2) Endometrial levels of ER and PR may vary up to 5-fold among women on the same day of their cycle. This finding is not surprising considering that inter-individual differences have been reported for plasma and endometrial tissue concentrations of ER and PR.
3) there is an "asymmetry" in human endometrium in the distribution of cytoplasmic and nuclear receptors for ER and PR.

There are two potentially important considerations which are dictated by the uneven distribution of endometrial ER and PR : Whereas ER and PR assays have proven useful in the management of patients with breast cancer, similar attempts to correlate endometrial ER-PR with the stage of endometrial cancer or make a prognosis of the patient's response to progestin therapy have not been as successful (110). On this point though some authors expressed different opinions (118) The difficulty in establishing these correlations is probably due to the technique of endometrial biopsy (use of curette or

aspirator) the existence of receptor "gradients" along the endometrial cavity and the normal fluctuation of receptor levels during the cycle which may or may not fluctuate in the diseased tissue. Uteroscopic directed endometrial biopsies of diseased and adjacent normal tissue are expected to diminish the above difficulties and also provide meaningful receptor data for the management of infertile patients. Each hysterectomy specimen could become an excellent model to test whether certain endometrial enzymes are regulated via ER or PR because, in addition to the normal receptor oscillations during the menstrual cycle, the uneven distribution of endometrial ER and PR could provide an "internal control" in such studies. For example, if we assume that endometrial carcinogen activating enzymes (111) or plasminogen activator (112) are regulated through the ER, then these enzyme levels along the long endometrial axis ought to parallel the levels of cytoplasmic or nuclear ER, in addition to the expected decrease in enzyme activity in the secretory phase. The intracellular concentration of oestrogen in human endometrium has been considered to be regulated by the circulating oestrogen and the extent of the intracellular oestrogen metabolism, i.e. conversion of potent oestrogen (E_2) to less potent oestrogen (E_1) or conjugate oestrogens which have no oestrogenic potency (113).

These data, together with other observations about the interrelationship between oestrogens and progestins, and sex steroid receptor levels in normal human endometrium (119-120) and in animal tissues (121-122) support the idea that oestradiol stimulates de novo synthesis of ER and PR in human endometrium, and that progesterone leads to the depletion of the target cell content of these receptors.

1.10.2 Hyperplastic endometrium

ER and PR concentrations vary greatly in different reports, at least partly because of methodological variations. The

marked interindividual variation of the receptor levels even in tissues with the same histological appearance makes comparisons with normal tissue difficult. The development of endometrial hyperplasia is characterized by an unopposed oestrogen action, which suggests that the receptor levels should be raised, or at least not decreased. The data available for ER partially support this assumption. Gurpide and Tseng (121) found the cytosol ER levels in the endometrial hyperplasia to be similar to those of proliferative endometrium, and cytosol ER tends to be high in hyperplastic endometrium, although not significantly higher than in the normal endometrial tissue (119). By contrast, it has also been reported that the cytosol ER concentration of hyperplastic endometrium is lower than in normal tissue (99). The concentration of cytosol PR in hyperplastic endometrium has been reported not to differ significantly from that in normal tissue (119).

1.10.3 Malignant endometrium

It is well known that in patients with elevated oestrone production in peripheral tissues or in their ovaries show an increased risk of developing endometrial cancer (94). Epidemiologic studies have established an association between oestrogen use during menopause and occurrence of endometrial cancer (95). Some workers have reported that oestrogens used in combination with progesterone reduce the incidence of endometrial abnormalities markedly (86-97). Several investigations have demonstrated the presence of oestradiol and progesterone receptors in normal and neoplastic human endometrium (98-101). Attempts to quantitate receptor concentrations and variations give conflicting results. Differences in analytical techniques and insufficient histological grading of the endometrium may have caused these variations in receptor values (102).

In endometrial carcinoma, the measurement of cytosol oestro-

gen and progestin receptors can be recommended for clinical use (103). The main application at present is in the categorization of patients with advanced disease into groups which will most likely respond to endocrine treatment or are most likely to benefit from combination cytotoxic chemotherapy. For this categorization of cytosol progestin receptors seems to be most suitable. The importance of measurements of nuclear receptors and progestin-regulated proteins (104-105) and the application of endocrine challenge tests (105) needs further clarification. ER and PR have associations with known prognostic risk indicators of endometrial carcinoma. However, in each indicator category there are individual patients with receptor data diverging from the general pattern. It is therefore likely that receptor measurements give information which is additive to that obtainable by conventional risk analysis. The role of female sex steroid receptors in ovarian carcinoma is obscure. Their assays may be useful in predicting the clinical behaviour of ovarian carcinoma. They may also find applications in the characterization of the potential hormone dependency of such tumours.

1.11 STEROID HORMONE RECEPTORS IN THE TARGET ORGANS OF NON-HUMAN PRIMATES:

There has not been an increasing interest over recent years in the application of non-human primates as models for studies on human reproduction. Despite limited data many primates have been proposed as suitable models for man for studies on steroid endocrinology. The rhesus monkey has up to now been the best-studied non-human primate in the investigation of the factors regulating normal reproductive function in man (124-125).

It was decided to study the molecular properties of receptors of a different monkey - the Vervet monkey (*Cercopithecus aethiops pygerythrus*). While this species is not widely

employed in biomedical research, the limited data presently available (126-129) suggest that C. aethiops pygerythrus C.a.p. might be a suitable alternative for the rhesus monkey in studies of reproductive function including embryology and teratology. The reproductive cycle of the Vervet monkey, has been found to be 30 days, indicating a remarkably close similarity to that of the human female especially with respect to progesterone and oestrogen metabolism and excretion (130). Initial studies of steroid excretion by the Vervet monkey proved a much closer endocrinological similarity to man than any other species of the Old World monkeys previously studied.

Setchell et al (130) found two phenolic compounds which he designated as Compound 180/410 and Compound 180/442 that have been shown to exhibit significant quantitative changes during the menstrual cycle of the monkey. The chromatographic behaviour of these compounds resemble oestrogen steroids but preliminary studies suggest their structures may be non-steroidal. Both compounds have been detected in human urine.

The Vervet monkey's name is borrowed from the French Vernacular name of this species, cercopithèque vervet (131). This monkey has a wide distribution in Africa from Southern Ethiopia and Somalia to the Cape Province. The Vervet monkey is predominately a Savanna woodland species, being generally absent from open grassland and open scrub. They are diurnal and gregarious, occurring in small troops up to 15 or 20. These troops may join others without aggression at watering places or at preferred feeding sites, forming aggregations of up to about 100. Within the troops there is a clear order of dominance. Vervet monkeys are predominately vegetarians living on wild fruits, flowers, leaves, seeds and seed pods. Other monkeys' menstrual cycle and steroid receptors have also been studied. Chrousos et al (132) investigated the oestrogen and progesterone receptors in an oestrogen - and

progesterone "resistant" primate the squirrel monkey. This monkey which is a New World primate, has elevated plasma oestradiol and progesterone concentrations compared to those in the cynomolgus macaque, an Old World primate (133-136). Uterine progesterone receptor concentrations examined in ovariectomized squirrel monkeys 2 days after oestrogen treatment were about one eighth those in identically treated cynomolgus macaques. McMillin et al (137) studied the effect of oestramustine phosphate on pituitary, gonadal and adrenal function in the green monkey. Their results are consistent with a pure oestrogen effect produced by hydrolysis of the carbamate-ester bond at a site distant from oestrogen target tissues and could explain most of the reported in vivo effects of this compound. Baulieu et al (138) investigated hormonal and immunological aspects of sex steroid-binding plasma protein of different primates and their cross-reactivity with antibodies against human steroid binding SBP plasma proteins. Their results agree with others (139-141), that human and the chimpanzee steroid binding proteins appear to be very similar and their concentrations are identical. Although showing total cross-reactivity with human SBP, gorilla SBP binds oestrone as do the SBPs of other primates species, but not those of man and chimpanzee. Brenner et al (142) studied the cyclic changes in oviductal morphology and residual cytoplasmic oestradiol binding capacity induced by sequential oestradiol-progesterone treatment of spayed Rhesus monkey.

CHAPTER 2.

Optimization of conditions for quantitative and qualitative determination of ER and PR VMU.

METHODS OF RECEPTOR ASSAY

INTRODUCTION.

A number of methods have been used to detect the presence of steroid hormone receptors. In practice, the best assay for receptor detection should be simple, sensitive, rapid, reliable, and economical.An 'ideal' assay exhibiting all these features does not yet exist.The advantages and disadvantages of currently used and newer promising assays should be carefully considered and evaluated. The large array of assays presently available can be functionally grouped by the following classification:

I. Removal of the unbound hormone
   1. Dextran-coated charcoal (DCC) assay
   2. Gel filtration
   3. Immobilized antisteroid antibodies

II. Removal of the bound receptor from free hormone
   1. Sucrose density gradient ultracentrifugation
   2. Protamine sulphate precipitation
   3. Hydroxylapatite assay
   4. DEAE chromatography
   5. Controlled pore glass beads
   6. Agar gel electrophoresis
   7. Isoelectric focussing (IEF)
   8. High-performance liquid chromatography (HPLC)

III. Exchange assays:
   A. Temperature-dependent methods:
      1. Protamine sulphate exchange assay

2. Hydroxylapatite exchange procedure

B. Temperature-independent methods:

1. Silver nitrate dithiothreitol(DTT) method
2. Mersalyl assay method
3. NaSCN method

IV. Histochemical methods

1. Antihormone antibody methods
   a. Immunofluorescent techniques
   b. Immunoperoxidase techniques
2. Steroid-fluoresceinated conjugates
3. Antireceptor antibody methods

Prior to giving details on the assay methods, some factors regarding specimen collection and preparation should be considered.

2.2 COLLECTION AND PREPARATION OF BIOPSIES

Hormone receptors are heat-labile proteins. Immediately after surgical removal of the specimen, the tissue should be chilled and kept at 4°C to ensure receptor stability. Freshly excised tissue should be rinsed in isotonic saline to remove blood, but all contact with formalin should be strictly avoided. The tumour should be trimmed from adjacent fat and normal tissue, preferably in a container with iced saline. The specimen should be examined by a pathologist to ensure that a representative sample of tumour has been chosen.

If there is a delay of more than an hour before the selected receptor assay is performed, the specimen should be frozen, preferably in liquid nitrogen, but at least on dry ice. Results with several human breast tumour samples examined at intervals of two months indicate that ER showed no significant loss in binding capacity over several months in tissues that were quick frozen at -86°C(143). Preliminary studies

have shown that lyophilization of cytosol followed by storage at 4°C may be a possible method for conveniently preserving samples of ER assay, but PR may be too unstable for this technique(143-145). The receptor stability is markedly influenced by pH, with maximum stability at approximately pH 7.4(146) to pH 7.8(147). High salt concentrations can cause changes in receptor form without a loss of binding properties(148-149). When using the DCC assay, low protein concentrations in the assayed nuclear or cytosol fraction may influence the estimation of binding capacity and binding affinity, particularly with receptor-poor tumours(150). The lowest acceptable limit of protein for most standard assays is 1 mg/mℓ in tumour cytosols with high receptor activity and 3 mg/mℓ in cytosols with low receptor values.

Miller et al (151) found that receptor integrity is promoted if 10 to 20 mM molybdate is included in the extraction buffer. It was proposed that molybdate may provide protection against specific proteolytic cleavage by stabilization of a phosphate group. In a study by Anderson et al(152) the addition of molybdate to the buffer increased both the percentage of PR-positive breast tumour samples and the absolute receptor levels. The effect of molybdate on ER assays was less evident, although in some samples all increases in ER level occurred. The effect of molybdate will be studied in detail in Chapter 3.

Some workers have found that buffers containing DTT also exert a stabilizing action of receptor(11) through protection of sulphydryl groups(153), while others have found no effect on receptor binding sites(155).

The addition of glycerol to the buffer during progesterone assays markedly improves the stability of the receptor(156).

A tumour with a receptor concentration of 3 fmol/mg(157-158)

to 10fmol/mg(159-160) or higher is usually considered oestrogen receptor positive, although the definition of 'positive' and 'negative' may vary slightly according to the laboratory and type of assay.

Quality control of receptor assays is mandatory for their reliable use in the clinical management of breast cancer patients. Several groups, such as the EORTC Breast Co-operative Group(1980) and NCI Sponsored Consensus Meeting on Steroid Receptors in Breast Cancer(1979), have recommended an interchange of duplicate tumour specimens or lyophilized calf uterus homogenates to ensure standardization and quality control(161). Specific guidelines have been established by these groups to help avoid pitfalls in performance and interpretation of receptor assays(162-163).

2.3 METHODS WHICH REMOVE UNBOUND HORMONE

2.3.1 Dextran-coated charcoal assay:

The DCC assay relies on the capacity of charcoal to adsorb free hormone, while the addition of dextran limits the adsorption of the hormone-receptor complex to the charcoal (164). By using increasing concentrations of radiolabelled hormone and a concurrent range of unlabelled hormone competitor, titration of the receptor to a saturation endpoint occurs. This titration procedure provides an accurate measure of the binding capacity and affinity of the receptor. Scatchard(61) analysis or some other technique of data transformation are performed to quantify the number of binding sites, usually expressed as femtomoles per mg protein, and to obtain the K_d or dissociation constant, which is equal to the negative reciprocal of the slope of the line. Several groups have demonstrated excellent quantitative correlation regarding ER analysis between the DCC assay and sucrose gradient methods(148,152,165), although Chester et al (166) found the DCC assay for ER more sensitive than

the sucrose gradient or gel electrophoresis methods. Anderson et al(152) found the DCC assay significantly more sensitive for PR than sucrose gradient analysis, and this difference was more pronounced when molybdate was absent.

The DCC assay is currently the most popular method for evaluating ER and PR receptor because it is relatively easy and inexpensive, as well as sensitive and accurate. Several samples can be processed simultaneously.This method does not allow identification of receptor form, and when performed at 0 to 4°C only unoccupied receptors are measured.

If only a single concentration of labelled hormone and competitor is used in a 'one-point assay' the potential for error in quantifying the amount of receptor is increased, and the K_d cannot be obtained.

2.3.2 Gel filtration.

Gel filtration is an infrequently used method which offers no advantages over more practical assays. In a typical gel filtration assay, cytosol is incubated with labelled hormone, then applied to a Sephadex column, a filtration column containing a suspension of packed porous gel beads. The receptor-bound radiolabelled hormones are excluded from the gel beads by reason of their geometry and mass, and are eluted most rapidly(170). The free and non-specifically bound hormone is retained by the accessible space within the gel interstices(171). Since Sephadex itself has considerable affinity for oestradiol, about 15 per cent of the receptor complex can be dissociated by this procedure, leading to false decrease in the amount of measured receptor(165).

When thin-layer gel filtration procedures are performed, which are a variation of the column method, the gel can be cut into strips for quantitative recovery and for elucidation of receptor forms(172).

2.3.3 Immobilized antisteroid antibodies.

Specific antisteroid antibodies can be used to separate free hormone from hormone-receptor complexes and non-receptor proteins. The antibodies, usually bound to Sepharose(174), polyacrylamide beads(175) or polyvinylidene removing free steroid from solution after incubation with receptor-containing cytosol (176-177). There have been some problems with quantitation and some risk of cross-reaction with extraneous antigens, but the method is fast and small samples can be used.

2.4 METHODS WHICH REMOVE BOUND RECEPTOR FROM FREE HORMONE

2.4.1 Sucrose density gradient ultracentrifugation.

Sucrose gradient ultracentrifugation gives information regarding the sedimentation velocity of proteins as a function of their molecular weight and density(178-23). In the usual method, cytosol is first incubated with radiolabelled ligand, with or without competitor, and then layered on prepared sucrose gradients. The gradients are centrifuged for about 16 hours if swinging buckets are used, and for a shorter time if a fixed-angle vertical-tube rotor is used (179). Fractionated samples are collected and counted. Standards are run in the gradient tubes for comparison with known markers, such as ^{14}C-labelled bovine serum albumin (4.6 S) or human IgG (approximately 6.8 S). In the past when molybdate was not usually used most tumour cytosols displayed the presence of an 8S oestrogen and progesterone receptor form, while some contained an additional 4S form(45) for both receptors. Steroid receptor complex in the absence of molybdate sediments in the 8S region of the gradient(180), but with molybdate the density is increased, and the absolute amounts of receptor are markedly increased(181). Since only one concentration of steroid is usually used, no information is obtained regarding affinity of the ligand for the receptor. The method

gives useful information regarding molecular forms of the receptor, although it is a slow and expensive way to quantify steroid receptor binding.

2.4.2 Protamine sulphate precipitation.

In this method developed by Steggles and King(182) the receptor is precipitated with protamine, after which the protamine-receptor complex is incubated with labelled hormone. At 4°C unoccupied receptor sites bind to radiolabelled steroid, leaving unbound hormone in solution. This assay has several variations and modifications.

2.4.3 Hydroxylapatite assay.

In the hydroxylapatite assay (HAP) the hormone-receptor complex is absorbed to hydroxylapatite either in 'batch' or in column form, and the free steroid and plasma contaminants are removed by multiple washes (183,144). Although this procedure is tedious, the assay is more reliable than the DCC assay at low protein concentrations and can be performed on very small tumour samples(184).

2.4.4 DEAE chromatography.

Ion-exchange chromatography separates proteins by taking advantage of net charge differences(185).Steroid receptors are acidic proteins which can be adsorbed to a DEAE-cellulose column, which is an anion exchanger(186). The unbound free steroid can be washed through the column using increasing concentrations of low ionic strength buffers. The method is reproducible and allows the measurement of receptor in dilute samples. The major problems of this method include the tendency of free oestradiol and testosterone to bind to DEAE at low ionic strength, and the inability of receptors to bind to the column at high ionic strength. Analysis of molecular forms of the receptor and separation from non-

receptor binding proteins is easily achieved.

2.4.5 Controlled pore glass beads.

This promising new method, based on the ability of the receptor to bind tightly to controlled pore glass beads, may greatly simplify the commercial receptor assays(54-55). Cytosol or nuclear extract is poured over a column of the beads where the receptors, retained in the crevices of the porous beads, are saturated by labelled hormone. Excess steroid is washed away, after which the receptor-bound steroid is eluted with ethanol for counting.

2.4.6 Agar gel electrophoresis.

Agar gel electrophoresis separates the hormone-receptor complex from free steroid on the basis of electrophoretic mobility(189). After electrophoresis, the labelled hormone-receptor complex is found on the anodal side of the well, while two peaks representing steroid hormone-binding globulin and free steroid are bound on the cathodic side. Agar gel electrophoresis compares fabourably with other quantitative receptor assays, especially if the cytosol is treated briefly with DCC before electrophoresis to remove most of the free and albumin-bound steroid(190).

Polyacrylamide gel electrophoresis has shown some promise in the measurement of ER in breast tumours(191), although receptor aggregation often poses a problem in this method, owing to the small pore size of the gel.

2.4.7 Isoelectric focussing.

The technique of isoelectric focussing, using slabs of polyacrylamide gel or other stabilizing matrix, allows the separation of free and non-specifically bound steroid hormones from hormone-receptor complexes on the basis of their charge

properties(170-192). This procedure, often combined with limited proteolysis, may be used for oestrogen or progesterone receptor analysis, or the two different receptors can be analysed concomitantly on the same gel(193). In comparison with other methods,isoelectric focusing is more sensitive and rapid(1.5 to 2 hours /gel) than other commonly used methods(193,144). Several samples can be analysed per gel, only one incubation with labelled steroid is needed, and fractionated macromolecules can be recovered. The special problems inherent in this method are analogous to those encountered with other types of electrophoresis, such as receptor aggregation and damage from potential reactants in the gel(170-192).

2.4.8 High-performance liquid chromatography.

High-performance liquid chromatography(HPLC) has exciting potential as a method for the analysis and characterization of steroid receptors based on parameters such as molecular weight. Qualitative relationships and multiple forms of the receptor can be maintained by this rapid gel-exclusion system, and contaminants can be identified easily(194). In addition to rapid analysis time and minimal receptor modification, this approach represents improved resolution and high reproducibility. The analysis are economical, after the initial investment in equipment has been made.

2.5 EXCHANGE ASSAYS

In clinical samples, the relative quantities of unbound nuclear and cytoplasmic receptors vary according to the hormonal milieu at the time of tumour excision. In a premenopausal woman or a postmenopausal tamoxifen-treated patient, for example, one would predict that unbound cytoplasmic receptors would be lowest and nuclear receptors highest, while the opposite would be true in postmenopausal women. In order to evaluate both occupied and unoccupied nuclear recep-

tor sites, various direct and indirect exhange methods have been designed.

2.5.1 Protamine sulphate temperature-dependent assay

In a variation of the protamine sulphate assay incubation of a KCℓ-extracted nuclear fraction at 25 to 30°C for several hours permits exchange of any previously bound hormone, while at 4°C only unoccupied receptor sites bind to radiolabelled hormones (195). This is a temperature-dependent phenomenon, and optimum time, temperature, and concentration of steroid must be established. A major disadvantage of this method is degradation of the receptor due to the proteolytic activity found in human tumour nuclear extracts.

2.5.2 Hydroxylapatite temperature-dependent exchange procedure.

Both bound and unbound receptor bind to hydroxylapatite(HAP) which makes it an ideal tool for an exchange assay. After HAP-bound receptors from the KCℓ-extracted nuclear fraction are pelleted by centrifugation, they are incubated with labelled hormone in a temperature-dependent exchange assay. Unbound steroid is removed in several wash-centrafugation steps, after which the bound hormone is extracted with ethanol(196). This method, which is only minimally affected by monovalent ions, appears to be superior to the protamine sulphate exchange procedure.

2.5.3 $AgNO_3$/DTT temperature-independent exchange method.

In this novel method developed by Hospelhorn et al(187) exchange is carried out at 0 to 4°C without raising the temperature as in previously described exchange assays. This exchange assay is based on modification of the methods using controlled pore glass beads. Bound and unbound receptor is

extracted with KNO_3 and bound oestradiol is released by reaction with $AgNO_3$. The sulphydryl bonds are then reduced by DTT to allow the binding of labelled oestradiol to nuclear receptors(184,188). The potential of this interesting assay has not been fully explored.

2.5.4 Mersalyl temperature-independent exchange method.

In this assay, developed by Traish, Muller and Wotiz(197) exchange of bound nuclear receptor complexes may be achieved at 0 to 4°C in the presence of molybdate and mercurial reagent, mersalyl acid. Specific binding is subsequently measured by the hydroxylapatite procedure. Mersalyl does not appear to alter binding affinity or sedimentation characterises of the receptor. This assay circumvents problems such as receptor degradation or incomplete exchange that occur more commonly with temperature-dependent exchange assays.

2.5.5 NaSCN temperature-independent exchange assay.

Bresciani et al(198) have developed a precise temperature-independent exchange assay using NaSCN, which solubilizes receptor from the nuclear pellet more efficiently than KCl extraction. The nuclear fraction is incubated for 16 hours at 4°C in the presence of excess labelled hormone and NaSCN, and virtually complete exchange of bound oestradiol is achieved. In this assay quantitative extraction of receptor from nuclei and exchange is achieved in a single step. This method allows the release of even the KCl extraction-resistant receptor, thus permitting reliable and accurate quantitation of receptor.

2.6 HISTOCHEMICAL METHODS

The histological detection of steroid hormone and specific receptors in morphologically intact tissue allows the investigator to visualize the uptake, transport and distribution

of hormones, and to understand more fully hormone-receptor interactions under varying physiological and pathophysiological conditions.

2.6.1 Antihormone antibody methods.

Several studies(199,200) have used anti-oestradiol antibody or antibody involving a polymeric oestradiol(201,202) to localize oestrogen receptor in human breast tumours. The tumour tissue is evaluated with a double antibody technique using anti-oestradiol antiserum followed by a fluorescein conjugated anti-immunoglobulin antiserum. Indirect immunofluorescence techniques using sheep anti-oestradiol and rabbit anti-progesterone antiserum plus fluorescein isothiocyanate (FITC)-conjugated antiserum have been used by Mercer et al(200) in an attempt to detect simultaneously the presence of both these hormones in human breast cancer cell lines and biopsies.

The results of these studies using antihormone antibody suggest the presence of hormone-specific binding, but problems with non-specific binding yield less than satisfactory results in some of the studies(201).

Various immunoperoxidase techniques, each using anti-steroid antibody plus the enzymatic activity of horseradish peroxidase with a chromogenic substrate, have been used to localize oestrogen in breast cancer cells(203). The peroxidase anti-peroxidase(PAP) method can enhance sensitivity 100 to 1000 times compared with immunofluorescence methods, and can be performed on fixed rather than frozen specimens(77). The principal disadvantage of this method, as in other histochemical methods, is the difficulty in quantitation of receptor.

2.6.2 Steroid-fluoresceinated conjugates.

Another cytochemical approach to trace steroid receptors using the fluorescence microscope utilizes steroid analogues that carry a visual tracer and retain binding affinity for steroid receptors. For the most part, these steroid derivatives consist of steroid molecules covalently linked to a protein carrier that has been previously substituted with FITC(202-205). These macromolecular derivatives, as is also true of polymeric oestradiol, cannot penetrate the membrane of intact cells, but the fluorescein tag can mark receptor positive cells. Pertschuk et al(205), using 17β-oestradiol and 11α-hydroxyprogesterone conjugated to fluoresceinated BSA, found a 92 per cent correlation with a biochemical assay in 314 breast cancer specimens assayed for ER and 86 per cent correlation in 86 specimens assayed histochemically for PR. Pertschuk et al also found a similar correlation with the DCC assay using androgen-ligand conjugates of fluoresceinated BSA in a double-blind study of 108 prostatic cancer specimens.

In another method, a fluorescein-labelled oestrogen (17FE) with a fluorescein moiety coupled to the 17 position of oestradiol can enter the cell membrane and can trace receptor sites in target tissues in a manner reflecting the interaction of native oestrogen(202). Another group found no direct quantitative relationship between this cytochemical method utilizing 17FE and the DCC assay.They did find, however,that the cytochemical technique was useful in measuring the proportion of ER-positive cells in a tumour sample, and that 81 per cent of tumours with ER values of 10 to 99 fmol measured by DCC assay were associated with greater than 10 per cent marker-positive cells. Fluorescence of the entire receptor-positive cell occurs most commonly, but sometimes only the nucleus or the cytoplasm is stained selectively. While further refining of this cytochemical technique is necessary, the assay is simple, rapid, inexpensive and deserving of

more thorough evaluation. The same 17FE that is used for the histological assay may also be used in separation methods such as the DCC assay. This fluorometric DCC assay is similar to the radiometric assay, except that fluoroscent-labelled hormones are utilized and measured, instead of employing radiolabelled hormones(206).

Unfortunately, none of the histochemical assays has been evaluated independently in a prospective and blinded fashion. While obviously appealing, their routine use cannot be considered appropriate at the present time. Further studies will be required before more traditional assay methods are abandoned.

2.6.3 Receptor antibodies.

The recent development of specific monoclonal antibodies to the oestrogen receptor protein allow detection of the receptor independent of the binding of labelled hormone by the receptor(207). Part of the difficulty in obtaining specific antibody to receptor is related to the very low concentration of oestrogen receptor, even in target tissues. Antibodies to calf uterine oestrogen receptor generated in rabbits(208) or goats(209) crossreact with oestrogen receptor from many species, while those obtained from immunization of the Lewis rat react only with calf oestrogen receptor (208). Splenic lymphocytes from a rat immunized with partially purified receptor from human breast cells were fused with three different mouse myeloma cell lines, yielding several different hybridoma cell lines(210-211). These hybridoma cells secreted specific monoclonal antibody against human ER that cross-reacts with ER of several other mammalian species (207). Recent work from the laboratory of DeSombre, Greene and Jensen (209-211) has been directed toward optimizing a plastic bead radioimmunoassay, using two of the monoclonal antibodies. This assay, as well as others using immunoperoxidase or immunofluorescence methods, may provide a basis

for simple, efficient detection and localization of oestrogen receptor in clinical samples. Monoclonal antibodies also provide useful reagents in the purification of oestrogen receptor.

2.7 QUANTITATIVE ASSAYS FOR OESTROGEN AND PROGESTERONE RECEPTORS IN VERVET MONKEY UTERI

2.7.1 REAGENTS:

(a) Steroids:

The radioligands [2,4,6,7^{3}H(N)]-17β-oestradiol(^{3}H-E_2; specispecific activity:92Ci mmole), [17α-methyl-^{3}H]promogesterone (^{3}H-R5020;specific activity:85Ci/mmole) and [1,2,6,7,21^{3}H(N)]-progesterone (^{3}H-P; specific activity : 195.4 Ci/mmole) were obtained from New England Nuclear. Unlabelled 17β-oestradiol (E_2),oestriol(E_3),oestrone(E_1), testosterone(T), dihydrotestosterone(DHT), dehydroepiandrosterone(DHEA), diethylstilbestrol(DES), Norethindrone(NE) and hydrocortisone (cortisol; HC) were all purchased from Sigma while progesterone (P) and promogestone (R5020) were bought from New England Nuclear. Ethinyloestradiol (EE_2) was obtained from Merck Chemicals. Radioligands were initially checked for purity by thin layer chromatography in a benzene : ethylacetate (9:1) system, or by high performance liquid chromatography (HPLC) on a reverse phase column (μBondapak C_{18}; Waters Associates).

Scintillation counting was performed in a Beckman LS 9000 counter using Ready-Solv HP (Beckman Instruments) as cocktail.

(b) Buffers:

The main buffer ($TEDAG_{10}$) contained 10mM Tris HCl, 1.5mM EDTA, 1mM dithiothreitol (DTT, Sigma) 1mM sodium azide and 10 % (m/v) glycerol (Merck Chemicals) and the pH was adjus-

ted to 7.4 at 4°C.

A second incubation buffer ($TEDAG_{60}$), employed in the assay of PR contained the same ingredients as the $TEDAG_{10}$ buffer, except that the glycerol content was 60 % (m/v).

A third buffer (TEDAG-MO) was prepared from $TEDAG_{10}$ by the addition of sodium molybdate to a final concentration of 10mM.

A very concentrated solution of KCl(2M) was prepared in $TEDAG_{10}$ buffer.

(c) Dextran coated charcoal (DCC):

The dextran coated charcoal suspension consisted of 0.5 % (m/v) pre-washed,activated charcoal (Merck Chemicals), 0.1 % (m/v) gelatine (Carrageenan Type 1; Sigma) 0.5 % dextran 170 (Pharmacia Fine Chemicals) in $TEDAG_{10}$ buffer. Fresh DCC solutions were prepared every fortnight.

(d) Ligands and competitors:

The [^{3}H]-17β-oestradiol was received, dissolved in ethanol. A series of concentrations ranging from 0.25 to 8nM was prepared in buffer ($TEDAG_{10}$) as well as an identical range of the ^{3}H-E_2,containing a 100 fold excess of either unlabelled E_2, EE_2 or DES.

The same procedure was followed for the ^{3}H-R5020 as described above. A series of concentrations ranging from 0.25 to 32nM was prepared with or without a hundred fold excess unlabelled promogestone (R5020).

Solutions (1mM) of the following unlabelled competitors were prepared in absolute ethanol: 17β-oestradiol(E_2),oestriol(E_1) oestrone(E_1), testosterone(T), dihydrotestosterone(DHT), de-

hydroepiandrosterone(DHEA), progesterone(P), promogestone (R5020), DES, EE_2, norethindrone (NE) and hydrocortisone (HC). From these solutions were prepared a series of 8 concentrations of each ranging from 0.675nM to 10^{-5}M in $TEDAG_{10}$ buffer.

2.8 STANDARD ASSAY FOR CYTOSOLIC PROTEIN:

A modification of Lowry's method (240) for protein determination was employed in the assay for cytosolic protein concentration.

(a) Procedure:

0.2 mℓ of cytosol was diluted 1:20 with distilled water. To 1 mℓ of this solution, 1 mℓ of alkaline buffer (1 M $NaCO_3$, 0.25 M NaOH) was added. 0.4 mℓ of copper reagent (0.1 % ($CuSO_4.5H_2O$,0.2 % NaK tartarate) was added and the mixture was allowed to stand at room temperature 10 min.

After that 0.75 mℓ of Folin Ciocateau reagent (diluted 1:4) was added to the incubation mixture and it was allowed to stand another 10 min at room temperature. In this time, a blue colour was developed which was read at 700 nm. A standard curve was drawn with 13 different dilutions of BSA.

(b) Results:

Fig 2.1 depicts the standard curve for the modified Lowry assay, employing bovine serum albumin (BSA) as standard and it seems that there is a straight correlation between the concentration of BSA and the absorbance of 700 nm.

2.9 COLLECTION OF VERVET MONKEY UTERI (VMU):

Uteri were obtained from the National Institute of Virology, Edenvale, South Africa on a regular basis from animals sa-

crificed for vaccine production. Monkeys were kept in quarantine for six weeks after being tested for tuberculosis. The estimated age of the monkeys varied between 2 and 3 years and they weighed between 3 and 3.5 kg. On the day of bilateral nephrectomy they were washed and the abdominal areas shaved, following sedation with ketamine (10mg/kg). Intravenous infusion of a 6 % saline solution of sodium nembutal (1 - 1.5 ml) followed until a level of surgical anaesthesia was attained. Uteri were quickly excised following the removal of the kidneys, placed into sterile sample bottles and then packed into crushed ice before being transferred to a - 70°C deepfreeze.

2.10 PREPARATION OF CYTOSOL:

(a) Preparation of cytosols from uterine slices:

Uteri were trimmed of fat and connective tissues and cut longitudinally into "identical" halves. The uterine tissue (packed in powdered dry ice on a petri dish) was cut into thin slices with a very sharp scalpel, taking care never to let the tissue thaw. The slices were then suspended in 5 volumes of $TEDAG_{10}$ buffer kept on crushed ice. Often individual uteri or groups of uteri were totally sliced up and the slices mixed thoroughly, prior to homogenization and preparation of cytosols. Homogenization was executed by 3 bursts of 10 seconds duration, alternated with 50 seconds cooling periods (crushed ice) using a TP Ultra Turrax (Janke and Kunkel). The collagen rich homogenate was centrifuged in a SW 50.1 rotor at 105000xg' for 30 min at 4°C (Beckman L8-80 centrifuge). The thin layer of fat was removed and clear cytosol drawn off with a pre-cooled syringe. Protein concentration was quantified by means of the modified Lowry procedure (240). Cytosols, not immediately processed, were divided into 2.5mℓ aliquots and then stored at -70°C until assays could be performed.

Cytosols employed in sucrose density gradient assays in the presence of 10mM sodium molybdate were prepared as described above, except that the tissue was homogenised in TEDAG-MO buffer.

(b) Preparation of cytosols from tissue powders:

Uteri were pooled (5 - 35) and then cut into thin slices in powdered, dry ice. A large amount of powdered dry ice was added to and mixed with the tissue slices and the mixture then poured into a pre-cooled (-86°C) mortar. The tissue slices were then ground to a fine tissue powder with a porcelain pestle ensuring that the temperature of the powder was kept at -86°C. The powder was then divided into smaller batches of ∿ 5 g quantities and stored at -70°C in a deep-freeze.

Whenever tissue powder was needed for an experiment, an amount of the powder was weighed out, taking care that spatulas and vials are pre-cooled to -70°C (or 86°C) before handling the powder. Tris-buffer (0-4°C) was added to the tissue powder prior to homogenization. Homogenization and preparation of the cytosols were executed in the same way as described in Section 2.10(a) above.

2.11 RECEPTOR ASSAYS:

(a) Oestrogen Receptor Assays:

Aliqouts (100µℓ) of cytosol were thoroughly mixed and then incubated with 100 µℓ of ^{3}H-E_2 at 5 different concentrations (0.25-8nM) for 30 min at 20°C. Non-specific binding was assayed as described above, but the ^{3}H-ligand solutions also contained a 100 fold excess of unlabelled or DES. The reaction was terminated with the addition of 500µℓ DCC. The suspension was vortexed, left on ice for 10 min and centrifuged at 2000xg' for 15 min. Aliquots

(500μℓ) of the supernatants were assayed for radioactivity, following the addition of 4 mℓ scintillation cocktail.

(b) Progesterone Receptor Assays:

These assays were carried out simultaneously with the ER assay on aliquots of the same cytosols. Standard conditions used were the following:
Cytosols were incubated on crushed ice for 2 h, following the addition of 100μℓ of the radioligand (0.25-32nM). After the first 2 h, 100μℓ of $TEDAG_{10}$ was added to the incubation mixture and thorough vortexing was followed by a reincubation in crushed ice for another 2 h. The incubation was terminated by the addition of 500μl of DCC. The reaction mixture was vortexed and the charcoal pelleted at 2000xg' for 15 min (0°-4°C) and the supernatants monitored for radioactivity as described above.

(c) Results:

A typical Scatchard plot of the titration analysis data appear in Fig. 2.2, and 2.3.

In the course of this investigation well over a hundred uteri were employed in numerous experiments, including titration assays, sucrose density gradient analyses and studies on the molecular properties of VMU ER and PR. In a large number of experiments cytosols derived from tissue powders were employed in order to obtain batches of cytosols consistent in the levels of ER and PR. Problems of experimental reproducibility were often experienced when groups of experiments were executed on cytosols produced from individual uteri and it was therefore deemed necessary to prepare batches of cytosols prior to a set of related experiments.

Table 2.1 summarizes the binding data for a group of

21 individual uteri, while Table 2.2 represents binding data obtained on tissue powders.

Values for the ER range from 151 and 822 femtomoles per mg protein (fmp) with a mean of 327±165 fmp while PR values range from 444 to 2267 fmp (mean : 1285±511). Mean dissociation constants found for this batch of VMU were $3.15\pm1.4\times10^{-10}$M and $2.38\pm0.2\times^{-9}$ for the ER and PR respectively. A mean ratio for PR to ER levels of 4.5±2.4 per uterus was observed. Mean correlation coefficients (r) obtained, following regression analyses on binding data were 0.967 for the ER and 0.985 for the PR assays. A weak, positive correlation (r = 0.31) was found to exist between the levels of ER and PR in the VMU.

For the tissue powders a means of 224±61 fmp and 742±288 fmp were obtained for ER and PR respectively. Mean K_d values were found to be $3.8\pm0.9\times 10^{-10}$M and $2.9\pm0.7\times10^{-9}$M respectively for ER and PR. The mean ratio for PR/ER was 3.3 and a correlation coefficient of 0.95 between the levels of ER and PR could be illustrated.

2.12 OPTIMIZING CONDITIONS FOR ASSAYS OF CYTOSOLIC ER AND PR IN VMU:

2.12.1 Effect of temperature on the stability of the VMU ER and PR:

(a) Experimental

A group of four VMU were cut longitudinally into two "identical" halves. The one half was immediately processed and analysed for ER and PR, while the other part was first stored in Medium 199 for 60 min at room temperature prior to analysis.

(b) Results:

A loss of specific binding 16 - 35 % (ER) and 10 - 25 % (PR) was observed when uteri were left at room temperature in Medium 199 (Fig. 2.4). This finding indicated that posthysterectomy treatment of the assay material was very important in preserving the labile macromolecular components in the intact tissue. Although a significant decrease in binding capacity (B_{max}) could be observed for both receptors, only the progesterone receptor exhibited a drop in binding affinity (increase in K_d) following the incubation of the uteri in Medium 199 at room temperature. Binding data is summarised in Tables 2.3 and 2.4.

2.12.2 Effect of freezing and thawing on the ER and PR Receptors.

(a) Experimental:

A small number of VMU were cut into small pieces; the pieces were thoroughly mixed and subsequently homogenized. The protein concentration of the cytosol was adjusted to ∿ 2 mg/mℓ. One batch of cytosol was immediately analysed for ER and PR, while another was stored in the deepfreeze at -70°C and analysed 24 hours later.

A batch of cytosol was subjected to repeated freezing and thawing (3 cycles) and the process followed on sucrose density gradient centrifugation (10-35%) by centrifugation at 520000xg' for 2 h in a vertical rotor (VTi-890; Beckman Instruments). This procedure is described in detail in Section 2.5.1.

(b) Results:

Scatchard plots and binding data are represented in Table 2.5 and 2.6 and Fig 2.5.

Freezing and thawing caused a slight, but significant ($p=0.001$) decrease in the binding capacity of the cyto-

sol. The difference in B_{max} varied from 2.8 to 17.6 % for ER (mean difference = 10.4±8.3 between groups) and -5.0 to 13.1 % for the PR (mean 1.1±4.5 %). A significant difference ($P<0.001$) was observed for the K_d values: 17.0±13.8% for the ER and 108.3±11.3 % for the PR. Thus freezing and thawing tend to damage receptors and in the case of the PR a vast effects was observed on receptor affinity (K_d). Corroboration for the fact that freezing and thawing physically affected the receptors could be seen in 10-35 % SDG profiles as depicted in Fig. 2.6.

2.12.3 Effect of temperature and duration of incubation of incubation on Scatchard plots and binding data:

(a) Experimental:

A batch of cytosol was prepared as described in Section 2.10. Cytosol protein concentration was adjusted to ∿ 2 mg/mℓ and binding assays were executed employing several variations in incubation conditions:

(a) 0-4°C x 30 min.
(b) 0-4°C x 4 h
(c) 0-4°C x 18 h
(d) 10°C x 30 min.
(e) 20°C x 30 min.
(f) 37°C x 30 min.
(g) 50°C x 30 min.

(b) Results:

A temperature optimum graph is presented in Fig 2.7(A). A summary of the binding data is presented in Table 2.7 while the effect of different incubation conditions is depicted in Fig. 2.7 (B)

At 37° C, receptors exhibited some denaturation and, al-

though no vast effect on B_{max} is noted, the receptor affinity (K_d) is influenced. At low temperatures, times had to be extended to more than 4 h in order to obtain sufficient exchange of cold and radioligands for reliable K_d values. It would therefore seem appropriate to incubate for 30 min at 20°C or for 18 - 24 h at 0-4°C during the assay for the ER. Because the PR exhibited susceptibility to thermodynamic conditions, we preferred to incubate at 0-4°C or at least 4 h.

2.12.4 Effect of different temperatures of incubation on the association and stability of VMU ER and PR:

(a) Experimental:

Cytosol (1 mℓ) was incubated with 1 mℓ 10 nM tritiated ligand (^{3}H-E_2 or ^{3}H-R5020) with and without a 250 fold excess level of the appropriate unlabelled competitor at either 0°C, 20°C or 37°C. Immediately, following the addition of the radioligands, aliquots (100µℓ) of the incubations mixtures were taken at regular time intervals (2 min intervals for the first 10 min; 10 min intervals for the next 60 min and 30 min interval for up to 3 h) and added to a DCC suspension (1 mℓ) on ice. Following thorough vortexing, the DCC suspensions were left on the ice for 15 min and then spun at 2000xg' . Aliquots (500µℓ) of the supernatants were monitored for radio-activity, following the addition of 2 mℓ cocktail to the sample. Vials were counted in a Beckman LS 9000 counter at an efficiency of 25 - 30 %.

(b) Results:

Association curves for both receptors are presented in Fig. 2.8. From these curves it is clear that the formation of the hormone-receptor complexes was completed within 15 min (ER) and 30 min (PR) at 0°C. At 20°C,

however association proceeded much faster and plateaux were reached within 7 min (ER) at 15 min (PR) but a drastic inactivation could be observed, soon after maximum saturation was reached (< 5 min for both ER and PR). After 40 min at 37°C the PR was totally inactivated while the ER was still 50 % active.The ER was completely inactivated after 90 min at 37°C.

In contrast to the rapid inactivation of both receptors at 37°C, they seemed to be very stable at 0°C and 20°C for at least 3 h and even after 24 h at 0°C there seemed to be no significant loss of activity.

From these observations and for practical purposes it was decided to incubate at 20°C for 30 min for the ER while a total of 4 h of incubation at 0°-4°C was employed for the PR assay.

2.12.5 Effect of Protein concentration of cytosol on the B_{max} and K_d.

(a) Experimental

Tissue was homogenized and cytosols were prepared as previously described. The protein concentration was found to be approximately 5.0 mg/mℓ.

Two variations of this experiment were executed:

(i) Binding assays were performed on cytosols serially diluted with plain Tris HCℓ buffer ($TEDAG_{10}$) thus changing the total protein content as well as the specific binding protein for ER and PR.

(ii) binding assays were performed on cytosols serially diluted with a solution of 5 mg/mℓ bovine serum albumin (BSA) in $TEDAG_{10}$ buffer, keeping the total protein of the cytosol constant at ∿ 5 mg/mℓ while

the specific binding proteins were diluted as in (i) above.

(b) Results

Binding data for both experimental variations are depicted in Fig. 2.9 and Table 2.8.

When the cytosol was only diluted with buffer, a clear linear relationship could be established between cytosol protein concentration (0.29-5.0 mg/mℓ) and the amount of ligand specifically bound (Fig 2.9). Dilution of cytosols with the ∿ 5mg/mℓ BSA solution rendered a fairly linear graph with the non-linearity ensuing at 1.1 mg/mℓ cytosolic protein (Fig. 2.9).

Although the concentration of receptors (B_{max} in fmol/mℓ) vary proportionally with the buffer dilution (assay 1-5) and the actual receptor dilution (assay 1-5), the actual receptor content per mg cytosolic protein (B_{max} in fmol/mg protein) is not affected. Also the K_d seemed to be constant over a wide area of protein concentration. As soon as the ratio of non-specific binding proteins (BSA) relative to specific binding proteins (ER) increased to between 4.5 and 8.2 the linearity of the Scatchard plots were significantly ($p<0.001$) affected.

2.12.6 Effect of the type of unlabelled competitors employed in competitive protein binding assays for ER:

(a) Experimental.

Cytosol (30mℓ) was prepared from 2 VMU (5 g) in $TEDAG_{10}$ buffer and stored in 1mℓ aliquots at -70°C until needed. Aliquots (100µℓ) of cytosol were incubated with 100µℓ of 5 different concentrations (0.25-8nM) of labelled ligand

for 30 min at 20°C.

Non-specific binding was assayed as described above, but the ^{3}H-ligand solutions also contained a 100 fold excess of either unlabelled E_2, EE_2 or DES. The reaction was terminated with the addition of 500µℓ DCC. The suspensions were vortexed, left on ice for 10 min and centrifuged at 2000g' for 15 min. Aliquots (500µℓ) of the supernatants were assayed for total and non-specific binding.

A range of ^{3}H-E_2 concentrations containing different excess levels (10^{-1}, 1, 10, 10^2, 10^3, times of either unlabelled DES, EE_2 or E_2, relative to the radioligand concentrations was prepared. Multipointed competitive protein binding assays were performed on the cytosols, employing the different non-specific binding reagents prepared.

(b) Results:

Scatchard plots of titration analysis data revealed that at a 100 fold excess level no significant difference could be observed in both the binding capacity B_{max} or dissociation constants (K_d) when either DES, EE_2 or E_2 were employed in assessing the level of non-specific binding in the cytosols (Fig. 2.10). Some differences could however be observed when the excess levels of the unlabelled competitors were varied (Fig 2.10-2.12 and Table 2.9.

2.12.7 Effect of a proteolytic enzyme (Trypsin) on affinity (K_d) binding capacity (B_{max}) and SDG profiles for ER in VMU:

(a) Experimental:

Cytosol was prepared from a pool of 5 small VMU as

described in Section 2.10. The cytosol was divided into two parts. Multipoint titration assays and sucrose density gradient (10-35%) analyses (see description of methodology in section 2.10 and 2.11) were performed on the one part of the cytosol which the same experiments were carried out on the other part of two cytosols except that a post-incubation step of partial trypsinization was included.

Partial trypsinization was based on a method published by Wrange et al (304): Following the incubation of aliquots of the cytosols with the appropriate concentration of radio-ligand (specific and non-specific binding) at 20°C for 30 min, a trypsin solution (13.6 µg/mℓ was added and a second incubation at 10°C for 30 min followed. The reactions were terminated by the addition of soybean trypsin inhibitor (304) and DCC, the charcoal was pelleted at 2000xg' (4°C) whereafter the supernatants were monitored for specific and non-specific binding or subjected to density gradient (10-35%) centrifugation.

(b) Results:

The effect of partial trypsinization on receptor ligand binding capacity and affinity is depicted in Fig. 2.14 and 2.15 and Table 2.10.

2.13 OPTIMIZING CONDITIONS FOR QUALITATIVE DETERMINATION OF ER AND PR BY SDG:

2.13.1 SDG centrifugation - experimental procedure

(a) Low ionic strength conditions

Freshly prepared cytosols (6 - 8 mg/mℓ protein) were incubated with ^{3}H-E_2 or ^{3}H-R5020 at a final concentration

of 5 nM with or without a 10^3 times excess of unlabelled DES or R 5020 respectively. Following a 30 min incubation at 20°C, the incubation mixture was added to a pellet of DCC, prepared by centrifuging 4 mℓ of DCC suspension at 2000xg' for 15 min. The suspension was vortexed and then centrifuged for 15 min at 2000xg' (0°-4°C). The supernatant was, immediately drawn off from the charcoal and stored in polypropylene tubes on ice prior to layering onto the gradients.

Gradients were prepared for centrifugation in a vertical rotor in either Quickseal or nitrocellulose tubes (Beckman Instruments) by handlayering of four different concentrations of sucrose in $TEDAG_{10}$ buffer (10%, 18.3%, 26.7% and 35% sucrose). A minimum of 2 h of conditioning at room temperature was necessary to create continuous linear gradients (10-35%). Gradients containing a final concentration of 10mM sodium molybdate, were prepared separately.

Linearity of gradients were checked regularly by refractometry (Fig 2.16) and the gradients were calibrated using four unlabelled marker proteins myoglobin (2.0S), ovalbumin (3.6S), bovine serum albumin (4.6S) and aldolase (7.3S). The markers were run separately and in duplicate in continous gradients and not more than 1 mg of each marker was layered onto the gradients in 200µℓ of $TEDAG_{10}$ buffer. The marker gradients were fractionated and the fractions scanned spectrophotometrically at 280 nm. (Fig 2.17). With each experimental set of cytosols run on sucrose gradients a duplicate set containing ^{14}C-methylated bovine serum albumin (NEN) was also included.

Cytosols (200µℓ) were run in duplicate for both total and non-specific binding at a maximum gravitational

force of between 365 000xg' and 520 000xg' for 2 to 20 h (2°C) depending on the rotor used. Rotors employed were either the VTi-80 SW 50.1 or SW 05 (Beckman Instruments) in a Beckman L8-80 ultracentrifuge. Following the completion of centrifugation, the gradients were fractionated from the top by displacement (Pharmacia P-3 peristaltic pump) with a 50 % m/v of sucrose containing Trypan Blue. Five drop fractions were collected manually into mini vials (miniPoly-Q; Beckman Instruments), 2 mℓ of scintillation cocktail was added and the vials were than counted, using a fraction plot digital integration program.

(b) High ionic strength conditions:

The same methodology described in (a) above was employed. None of the gradients prepared, however contained molybdate. Prior to layering onto the gradients, tubes containing the incubation mixtures were cooled in crushed ice and an ice cold solution of 2 M KCl in $TEDAG_{10}$ buffer was added to the solutions to a final concentration of 0.4 MKCl. The tubes were vortexed, their total contents transferred onto prepared DCC and vortexing repeated. Following centrifugation at 2000xg' (4°C) the supernatants (200μℓ) were immediately layered onto gradients containing 0.4 M KCl and processed as described in (a).

Before deciding on which optimal conditions for sucrose density gradient centrifugation to employ several different conditions of centrifugation were screened (Table 2.11).

(c) Results

The linearity of a representative gradient is depicted in Fig. 2.16. A typical calibration curve appears in

Fig. 2.17 and the calibration data is summarized in Table 2.12. Sucrose density gradient (SDG) profiles obtained with the various methods of centrifugation, outlined in Table 2.11 appear in Fig. 2.18.

It was subsequently decided to employ routinely the vertical rotor (VTi-80) at maximum speed (80 000 r.p.m.) for a duration of 120 min or to a constant $\omega^2 t$ of 5.3×10^{11}.

TABLE 2.1

Summary of Receptor Data obtained for ER and PR in 21 VMU :

	B_{max} *(fm/mg protein)		K_d ($\times 10^{-9}$M)		Sedimentation coefficient (svedbergs)	
	Range	Mean (± SD)	Range	Mean (± SD)	Range	Mean (± SD)
ER	151-822	327±165	0.15-0.798	0.315±0.14	7.2-7.9	7.6±0.5
PR	444-2267	1285±511	2.3 -3.1	2.38 ±0.2	6.7-7.7	7.2±0.7
PR/ER	1.1-13.1	4.5±2.4	-	-	-	

p(*fm/mg protein = femtomoles per milligram cytosolic protein).

TABLE 2.2

Summary of binding data obtained, following the execution of assays on VMU tissue powders.

Group	No. of uteri	No. of Assays		Binding data				
				ER		PR		PR/ER
		ER	PR	B_{max} (fmol/mg prot)	K_d ($\times 10^{-10}$ M)	B_{max} (fmol/mg prot)	K_d ($\times 10^{-9}$ M)	
A	30	4	4	176±14	4.0±0.53	457±18.7	4.0±2.0	2.6±1.2
B	20	21	11	373±46	5.0±2.1	1168±300	3.0±1.2	4.3±2.1
C	20	13	9	160±20	2.6±1.1	554±40.4	2.6±1.6	3.5±0.9
D	15	13	12	213±22	3.3±0.8	660±36	2.8±2.0	3.0±0.8
E	10	26	8	300±29	3.9±1.5	871±97	2.2±1.3	2.9±0.9
TOTAL	95	77	44					
MEAN				224±61	3.8±0.9	742±283	2.9±0.7	3.3±0.95

(B_{max}, K_d are the mean values for the group of assays executed on a particular tissue powder).

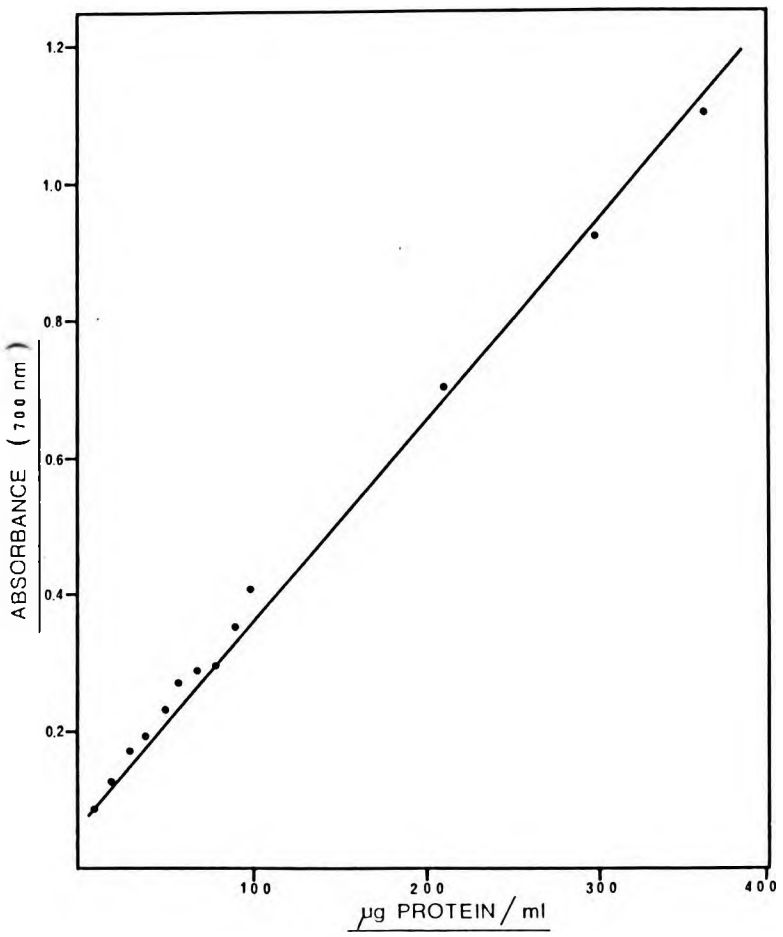


Figure 2.1 : Standard curve for the determination of protein concentration in VMU cytosols with the modified Lowry assay (240). Bovine serum albumin (BSA) was used as protein standard.

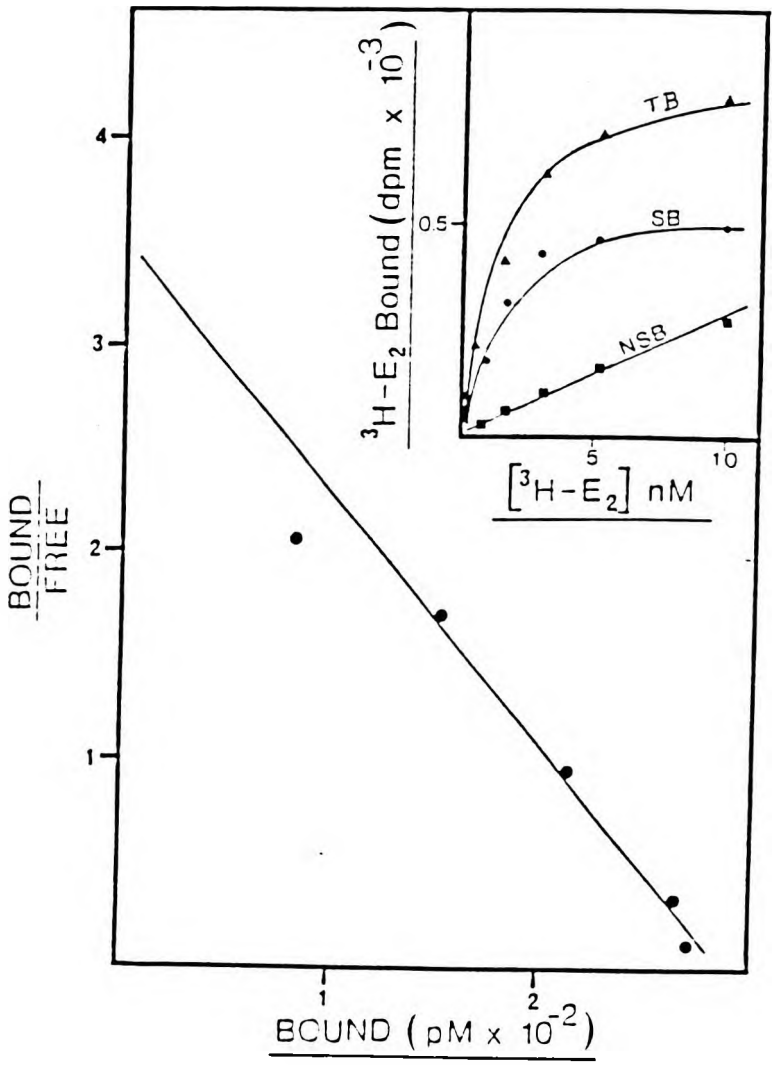


Figure 2.2 :Competitive protein binding assays for cytosolic VMU ER; saturation curves and Scatchard plots. Aliquots (100 µℓ) of cytosol were incubated with 100 µℓ of ^{3}H-E_2 at 5 different were concentrations (0.25-8mM)(total binding, TB). Non-specific binding NSB) was assayed when the ^{3}H-ligand solutions contained a 100 fold excess of unlabelled DES. The reaction was terminated with the addition of 500µl) DCC solution. After centrifugation (2000xg'x15 min), aliquots (500µl) of the supernatants were assayed for total (TB) and non-specific binding (NSB). Specific binding (SB) was calculated by subtracting NSB from TB.

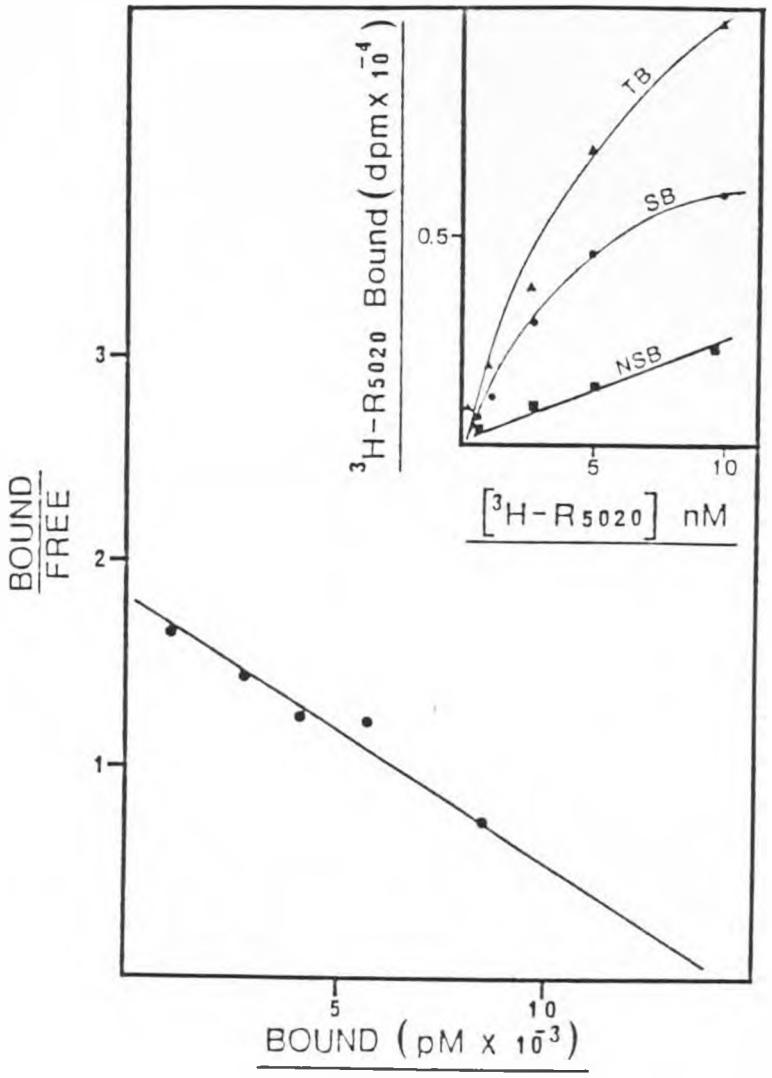


Figure 2.3 :Competitive protein binding assays for cytosolic VMU PR; saturation curves and Scatchard plots. Aliquots (100 µℓ) of cytosol were incubated with 100 µℓ of ^{3}H-E_2 at 5 different were concentrations (0.25-8mM)(total binding, TB). Non-specific binding NSB) was assayed when the ^{3}H-ligand solutions contained a 100 fold excess of unlabelled DES. The reaction was terminated with the addition of 500µl) DCC solution. After centrifugation (2000xg'x15 min), aliquots (500µl) of the supernatants were assayed for total (TB) and non-specific binding (NSB). Specific binding (SB) was calculated by subtracting NSB from TB.

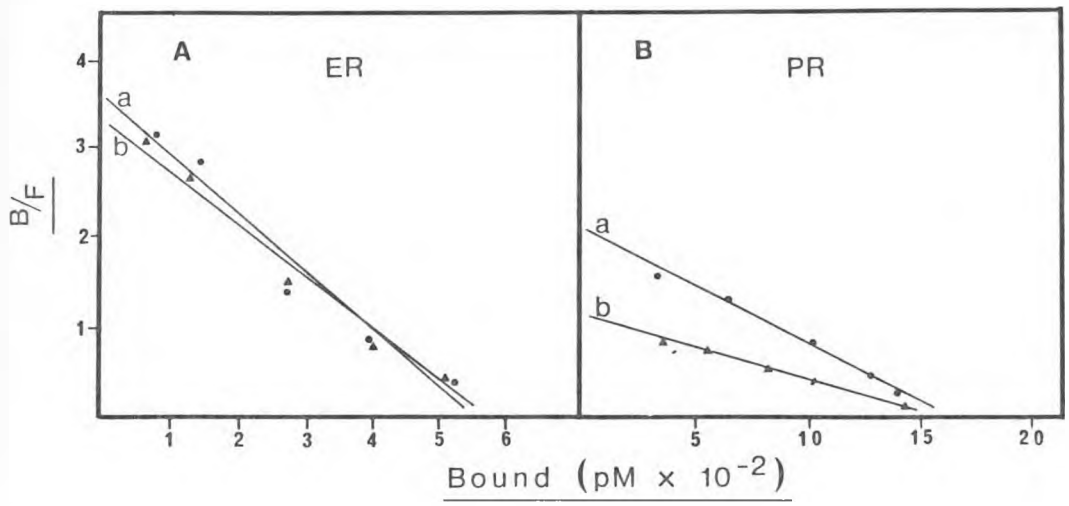


Figure 2.4 : Effect of the temperature on the receptor binding capacity and affinity in intact VMU; (A) ER and (B) PR (●) uteri were immediately processed following hysterectomy and analysed for ER and PR while (▲) uteri were left at room temperature in Medium 199 for 60 min prior to analysis.

TABLE 2.3

Binding data obtained for the ER following (a) immediate post-hysterectomy assaying, (b) assaying following incubation of intact uteri at room temperature (60 min).

uterus No	B_{max} (f mol/mg protein)	K_d ($\times 10^{-10}$ M)	%Δ between a & b
1a	471	2,32	
			+26,7
1b	345	1,08	
2a	395	1,28	
			+10,9
2b	352	2,17	
3a	409	3,19	
			+33,2
3b	273	2,7	
4a	277	3,0	
			+19,4
4b	304	2,75	
Mean of group a	388±81	2,45±0.86	
Mean of group b	318±37	1,98±0.82	
%Δ group a versus b	18±54	19±4.6	

TABLE 2.4

Binding data obtained for the PR following (a) immediate post-hysterectomy assaying, (b) assaying following incubation of intact uteri at room temperature (60 min).

uterus No	B_{max} (fmol/mg protein)	K_d ($x10^{-9}$M)	%Δ
1a	2491	0,8	+25
1b	1868	1,56	
2a	1625	1,95	+16
2b	1350	1,73	
3a	2638	2,77	+32
3b	1003	4,63	
4a	1554	3,37	+35
4b	1003	2,53	
Mean of group a	2077±450	2,23±1,1	
Mean of group b	1306±408	2,61±1,4	
%Δ group a versus b	37 ±16.7	+17,6±13,4	

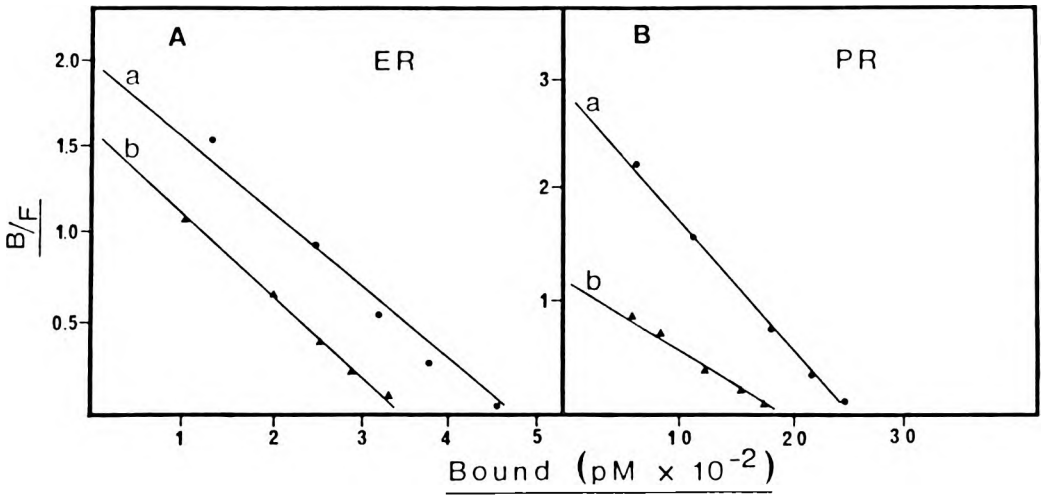


Figure 2.5 : Effect of freezing and thawing on VMU (A) ER and (B) PR receptors.
Representative Scatchard plots of the binding data obtained from assays on two batches of cytosols, where one batch (●) of cytosol was immediately analysed for ER and PR while another (▲) was stored in the deepfreeze at -70°C prior to analysis 24 hours later.

TABLE 2.5

Effect of freezing and thawing of cytosols on binding properties of the VMU ER.

Uterus No	B_{max} (fmol/mg protein)	K_d ($\times 10^{-10}$ M)	%Δ between a & b
1a	1079	3,10	
			17,6
1b	889	3,26	
2a	570	1,54	
			+2,8
2b	554	1,71	
3a	847	3,8	
			+7,4
3b	784	2,44	
5a	471	2,32	
			+13,1
5b	409	3,19	
6a	345	1,08	
			6,9
6b	321	1,51	
7a	395	1,262	
			4,5
7b	377	3,02	
8a	352	2,17	
			13,6
8b	304	2,75	
Mean of group a	579.9±281	2.18±1.0	
Mean of group b	519.7±2.33	2.55±0.7	
% Δ group a versus b	10.4±8.3	17,0±13.8	

TABLE 2.6

Effect of freezing and thawing of cytosols on binding properties of the VMU PR.

Uterus No	B_{max} (fmol/mg protein)	K_d ($\times 10^{-9}$ M)	%Δ between a & b
1a	2491	0,9	
			-5,0
2b	2638	2,775	
3a	1868	1,56	
			+2,8
4b	1781	4,638	
5a	1625	1,95	
			+4,3
6b	1554	3,378	
5a	471	2,32	
			+13,1
5b	409	3,19	
Mean of group a	1613.8±844.6	1.68±0.61	
Mean of group b	1595.5±918.5	3.50±0.80	
% Δ group a versus b	1.1±4.5	108.3±11.3	

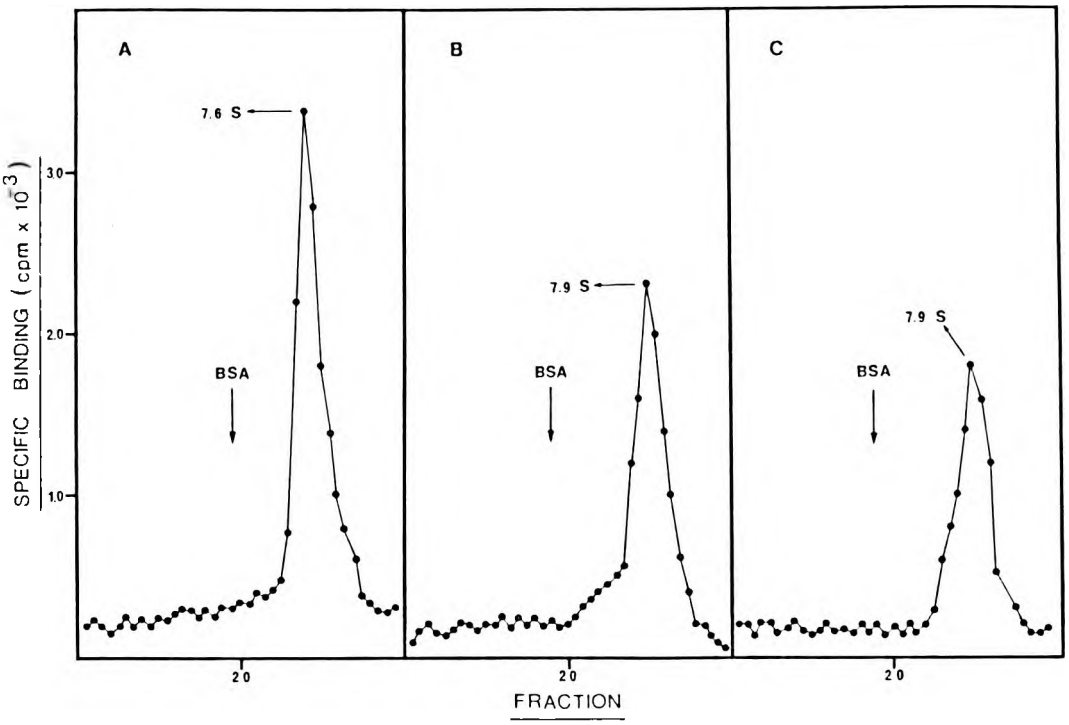


Figure 2.6 : Effect of freezing and thawing on VMU cytosols SDG profiles for VMU ER receptors. Cytosol prepared from a batch (15) of uteri was subjected and the process followed by SDG centrifugation on 10-35% gradients (520000xg' for 2 h in a VTi-80 rotor):(A) Control cytosol was analysed immediately by SDG; (B) Cytosol analysed on 10-35% SDG following one cycle of freezing and thawing (24 h);(C) cytosol analysed following two cycles of freezing and thawing.

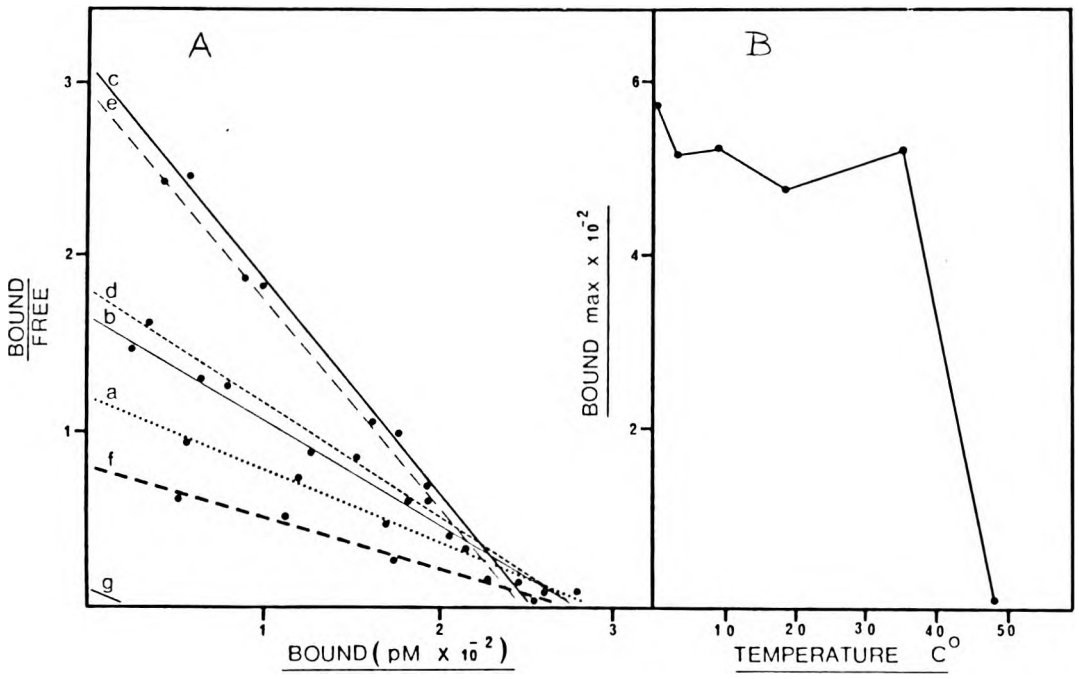


Figure 2.7 : Effect of different incubation conditions in the assay of ER in cytosols prepared from VMU. A batch of of cytosol was prepared and binding assays were executed employing several variations in incubation conditions : (A) representative Scatchard plots of binding data obtained with varying incubation conditions: (a) 0-4°Cx30 min; (b) 0-4°Cx4 h; (c) 0-4°Cx18 h; (d) 10°Cx30 min; 20°Cx30 min; (e) 37°Cx30 min; (f) 50°Cx30 min. (B) temperature optimum curve for the ER.

TABLE 2.7

Effect of different incubation conditions on binding parameters for cytosolic ER in VMU.

Incubation Temp. (C°)	Incubation Time	B_{max} (fmol/mg protein)	K_d ($x10^{-10}$M)
0	30 min	586	3.94
4	4 h	549	2.4
4	18 h	545	-
10	30 min	548	1.53
20	30 min	467	0.8
37	30 min	539	3.9
50	30 min	~20	-

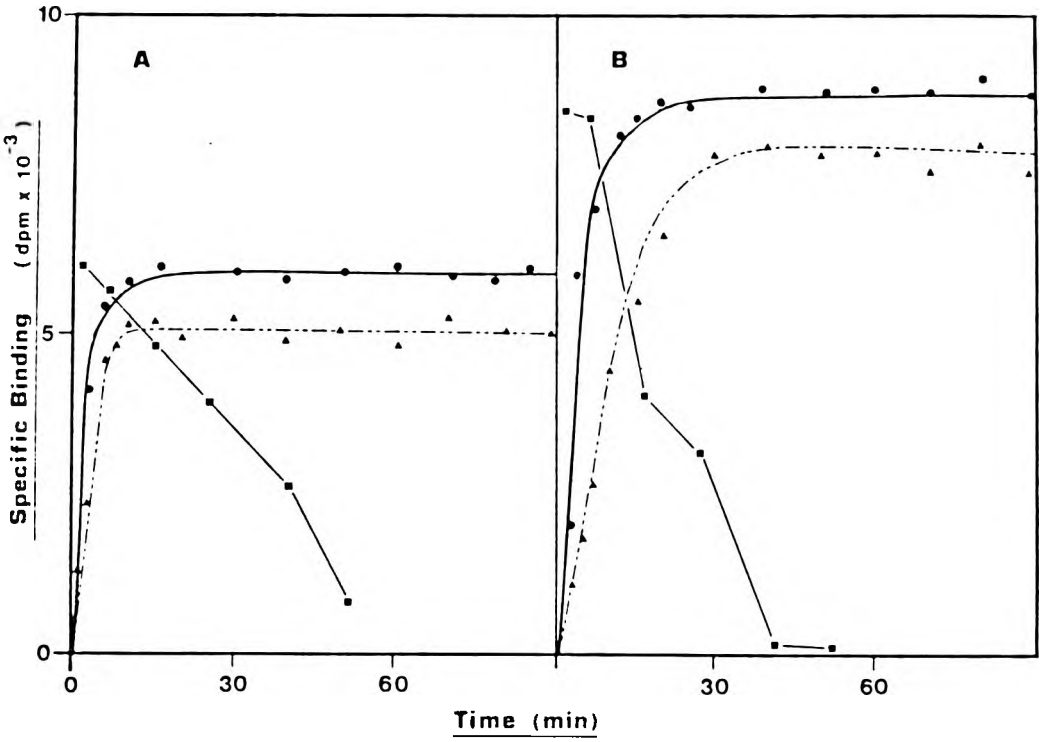


Figure 2.8 : Receptor-ligand kinetics of the VMU ER(A) and PR(B) association.
Cytosol (1 mℓ) was incubated with 1 mℓ of 10nM tritiated ligand (^{3}H-E_2 or ^{3}H-R5020) in the presence or absence of a 250 fold excess of the appropriate unlabelled competitor at either 0°C(▲), 20°C(●) or 37°C (■). Immediately, following the addition of the radioligands, aliquots (100µℓ) of the incubation mixtures were taken at determined time intervals and added to a DCC suspension (1 mℓ) at 0°-4°C. The DCC suspensions were left of the ice for 15 min and then spun at 2000xg' for another 15 min. Aliquots (500µℓ) of the supernatants were monitored for total and non-specific binding.

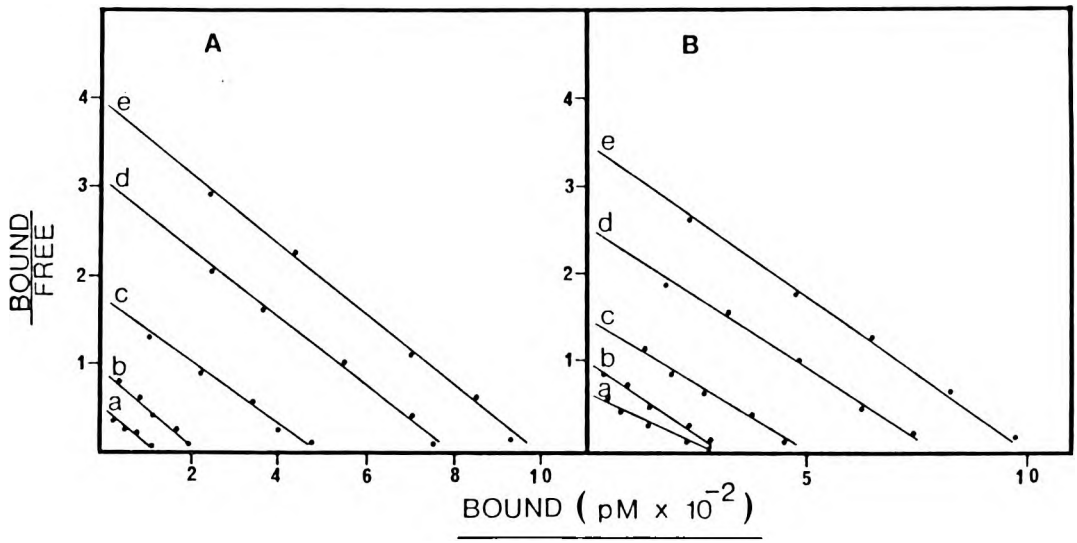


Figure 2.9 : The effect of the cytosolic protein concentration on Scatchard plots derived from binding data obtained on the VMU ER : (A) Binding assays were performed on cytosols, serially diluted with plain Tris-HCl buffer ($TEDAG_{10}$): (B) binding assays were performed on cytosols serially diluted with a solution of 5mg/mℓ bovine serum albumin (BSA) in $TEDAG_{10}$ buffer.

TABLE 2.8

Effect of the protein concentration of the cytosol on binding parameters for ER in VMU

Means of Dilution	Assay No	Cytosolic protein Concentration (mg/mℓ)	Total protein Concentration (mg/mℓ)	B_{max} (fmol/mℓ)	B_{max} (fmol/mg protein)	K_d ($\times 10^{-10}$ M)
	1	4.9	4.9	980	200	2.90
Dilution	2	3.9	3.9	775	199	2.87
with	3	2.4	2.4	483	201	2.96
buffer	4	1.1	1.1	225	205	3.00
	5	0.6	0.6	115	192	3.07
Dilution	6	4.9	4.9	990	200	2.83
with	7	3.9	4.9	775	158	3.10
∿5mg/mℓ	8	2.4	4.9	477	97	3.18
BSA in	9	1.1	4.9	285	58	4.38
buffer	10	0.6	4.9	260	53	8.67

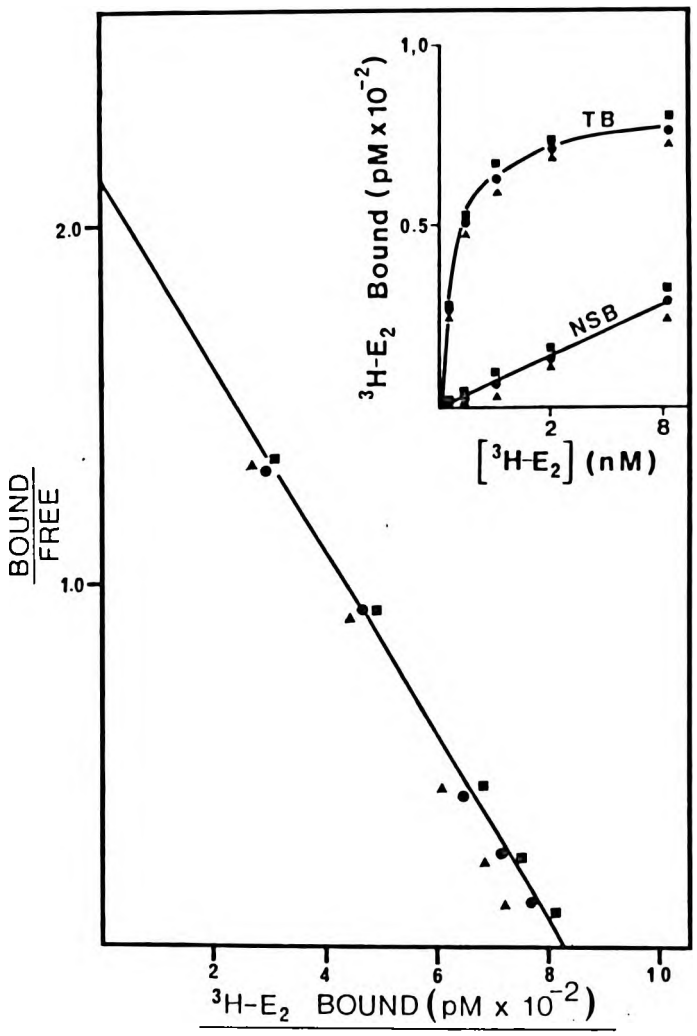


Figure 2.10: Effect of different unlabelled competitors employed in the assessment of non-specific binding on the quantitative assay of VMU ER.
Aliqouts (100µℓ) of cytosol were incubated with 100µℓ of ^{3}H-E_2 at 5 different concentrations (0.25-8mM) for 30 min at 20°C. Non-specific binding was assayed with ^{3}H-ligand solutions containing 100 fold excess of either unlabelled E_2, (●), EE_2 (■) or DES (▲). The reaction was terminated with the addition of 500µℓ DCC. After centrifugation at 2000xg' for 15 min, aliquots (500µℓ) of the supernatants were assayed for total and non-specific binding.

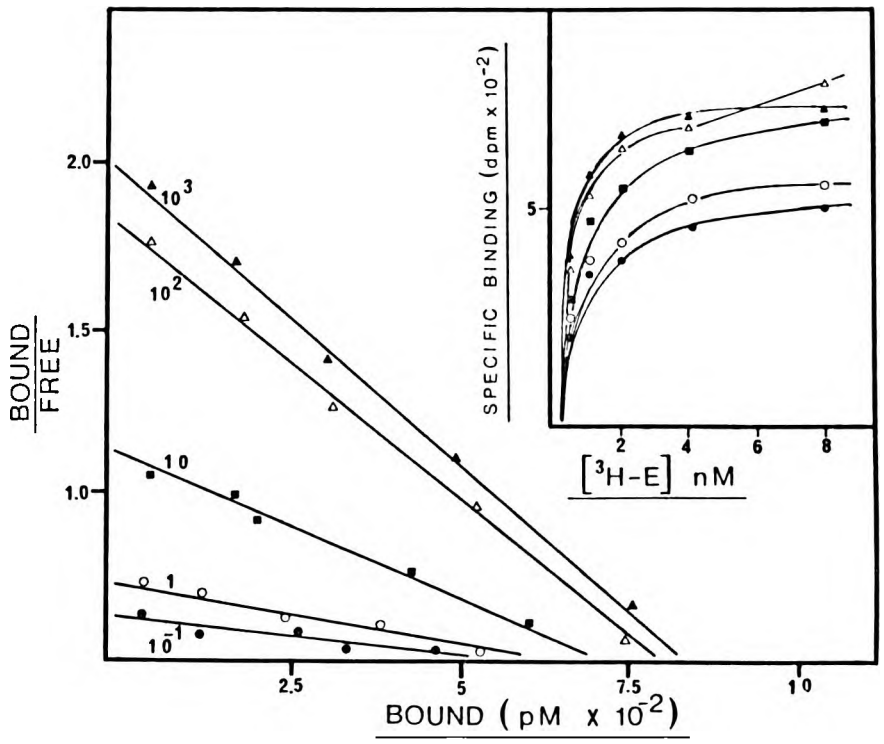


Figure 2.11: Effect of different excess levels of unlabelled competitors on Scatchard plots and saturation curves derived from titration analysis data for the cytosolic VMU ER employing E_2 as unlabelled competitors.
A range of ^{3}H-E_2 concentration containing different excess levels(10^{-1}, 1, 10, 10^2, 10^3 times) of E_2 relative to the radioligand concentrations was prepared. Multipoint competitive protein binding assay was performed on the cytosols, employing the different non-specific binding reagents prepared.

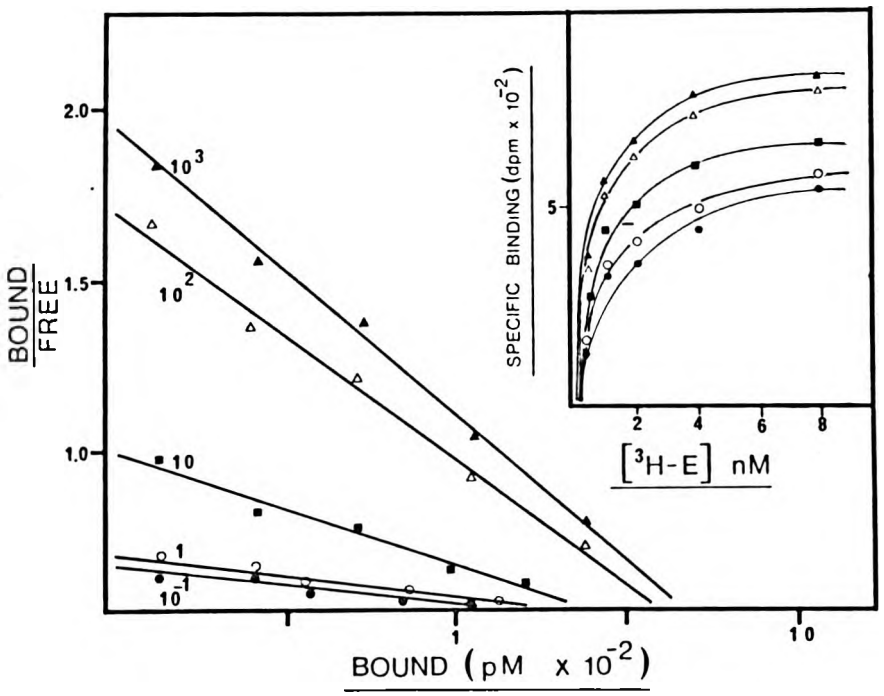


Figure 2.12: Effect of different excess levels of unlabelled competitors on Scatchard plots and saturation curves derived from titration analysis data for the cytosolic VMU ER employing EE_2 as unlabelled competitors.
A range of ^3H-E_2 concentration containing different excess levels(10^{-1}, 1, 10, 10^2, 10^3 times) of EE_2 relative to the radioligand concentrations was prepared. Multipoint competitive protein binding assay was performed on the cytosols, employing the different non-specific binding reagents prepared.

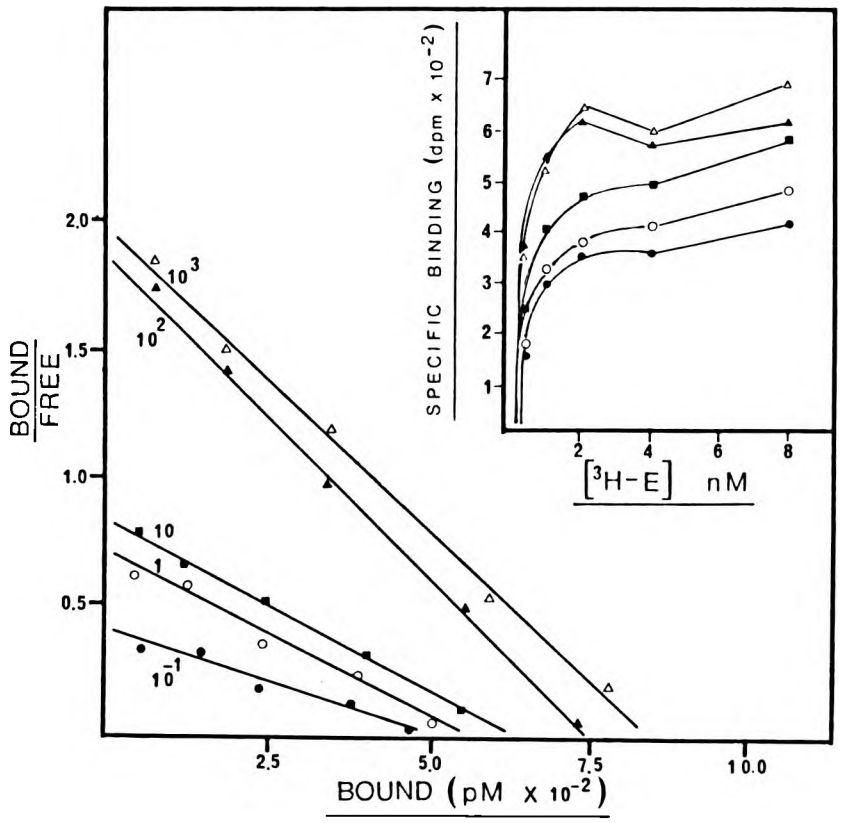


Figure 2.13: Effect of different excess levels of unlabelled competitors on Scatchard plots and saturation curves derived from titration analysis data for the cytosolic VMU ER employing DES as unlabelled competitors.
A range of ^{3}H-E_2 concentration containing different excess levels(10^{-1}, 1, 10, 10^2, 10^3 times) of DES relative to the radioligand concentrations was prepared. Multipoint competitive protein binding assay was performed on the cytosols, employing the different non-specific binding reagents prepared.

TABLE 2.9

Effect of different excess levels of unlabelled competitors on B_{max} (fmol/mg protein) and K_d($\times 10^{-10}$M) for ER in VMU cytosols.

Excess level (10^x)	Unlabelled competitor					
	E_2		EE_2		DES	
	B_{max}	K_d	B_{max}	K_d	B_{max}	K_d
x = -1	122	9.18	199	9.76	106	1.7
x = 0	129	8.5	133	1.09	105	6.42
x = 1	152	6.4	149	7.09	134	7.70
x = 2	170	4.5	171	4.76	149	4.18
x = 3	175	4.19	177	4.25	163	4.19

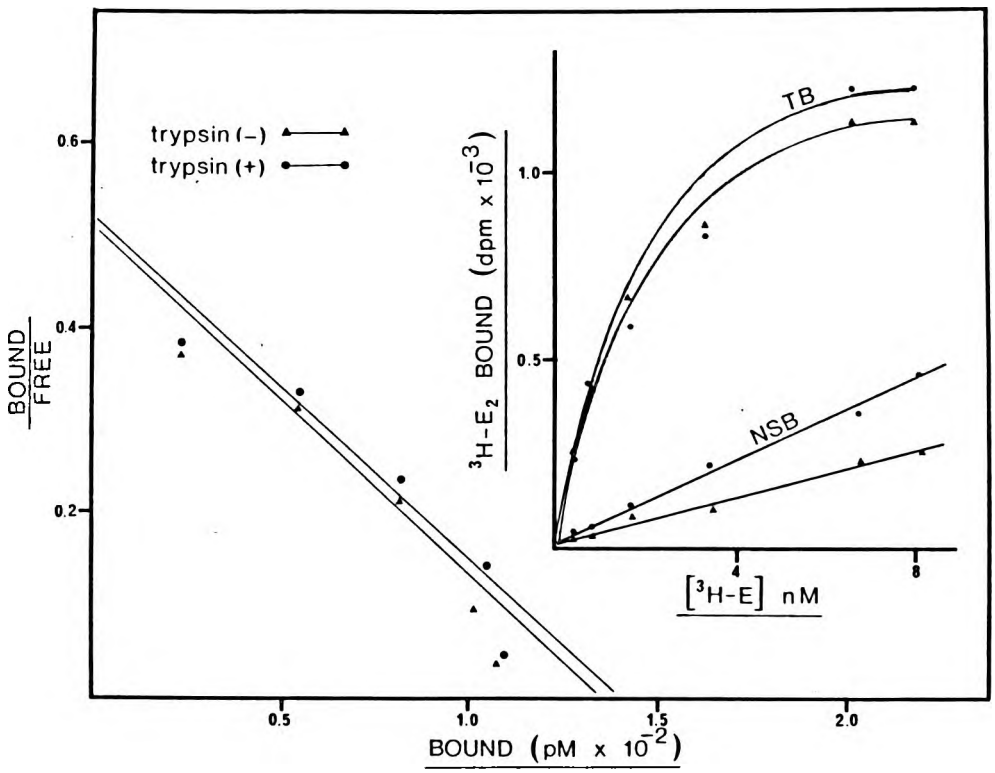


Figure 2.14: Effect of partial trypsinization of VMU cytosol on the titration analysis of ER.
Aliquots of cytosol, (100µℓ) prepared from a small pool of 3-4 uteri, was incubated with ^{3}H-E_2 (0.2-8nM) for 30 min at 20°C in the absence (total binding) or presence (non-specific binding) of a 100 fold excess DES. Following incubation the tubes were put on ice, 500 µℓ of a trypsin solution added (1 mg/mℓ) to the incubation mixture and the reaction mixture reincubated for 30 min at 10°C to effect partial proteolysis. This reaction was stopped by the addition of soybean trypsin inhibitor (2 mg/mℓ), followed by DCC (500µℓ) and the 2000xg' supernatant monitored for total and non-specific binding. A set of controls was run on the same cytosol, excluding the post-incubation trypsinizition.

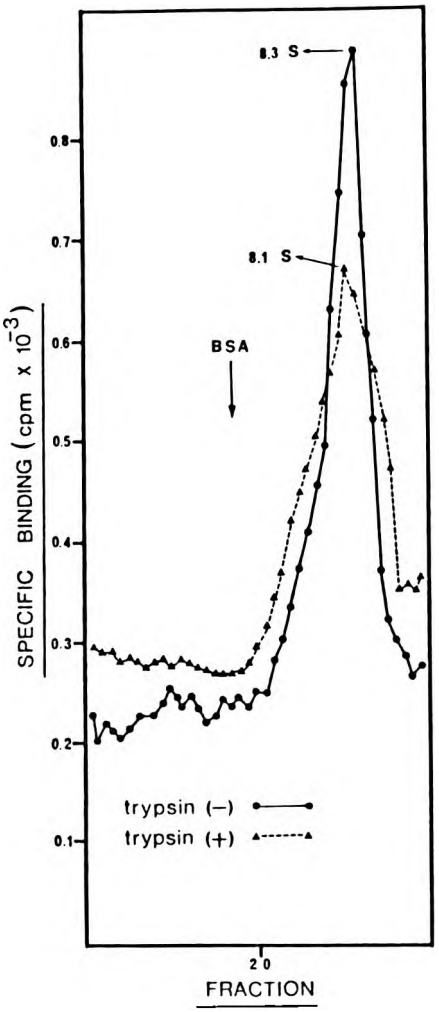


Figure 2.15: Effect of partial trypsinization of VMH cytosols on SDG profiles.
Aliquots of cytosol (100µℓ), prepared from a pool (15 uteri) of vervet monkey uteri were incubated (20°Cx30 min) with ^{3}H-E_2 (final concentration, 5nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess of DES. Following the termination of incubation the tubes were all transferred to ice, 500µℓ trypsin (1mg/mℓ cytosol in $TEDAG_{10}$ buffer) added and reincubated at 10°C for 30 min. Trypsinization was terminated by the addition of 100µℓ soybean trypsin inhibitor (2 mg/mℓ buffer) and the reaction mixture then transferred to tubes containing DCC pellets. The 2000xg' supernatants (200µℓ) were layered onto 10-35 % sucrose density gradients, subjected to ultra-centrifugation (VTi-80 rotor,520000 xg'x120 min) and then fractionated into 40 five drop fractions. The fractions were monitored for total and non-specific binding following the addition of scintillation cocktail (2 mℓ) and counting in a Beckman LS 5800 counter (25-30 % efficiency). In a control experiment, SDG profiles of non-trypsinized cytosols were obtained in the same way as described above.

TABLE 2.10

Effect of partial trypsinization on ligand binding capacity (B_{max}) and affinity (K_d) of VMU ER.

Assay No	Trypsinization	B_{max} (fmol/mg protein)	K_d ($\times 10^{-10}$ M)
1	-	138	2.75
2	+	133	2.75
3	+	125	2.91
4	+	130	2.65

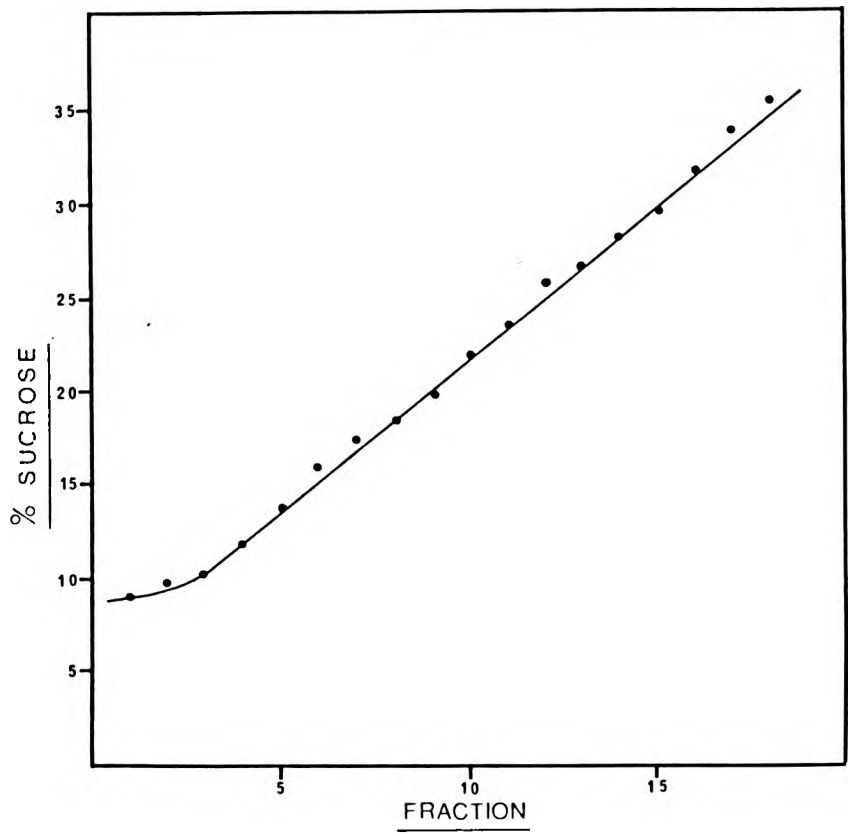


Figure 2.16: Linearity of continuous 10-35 % sucrose density gradients (SDG) as monitored by refractometry. Gradients were prepared by the hand layering of 1.15 mℓ each of four different concentrations (10 % 18.3%, 26.7 % 35 %) of sucrose in $TEDAG_{10}$ buffer, ranging from 10 to 35 %. A minimum of 2 h of conditioning at room temperature was required to create continuous linear gradients. Gradients were then manually fractionated into 40, five drop fractions and the sucrose concentration monitored in a refractometer (Atago, Japan).

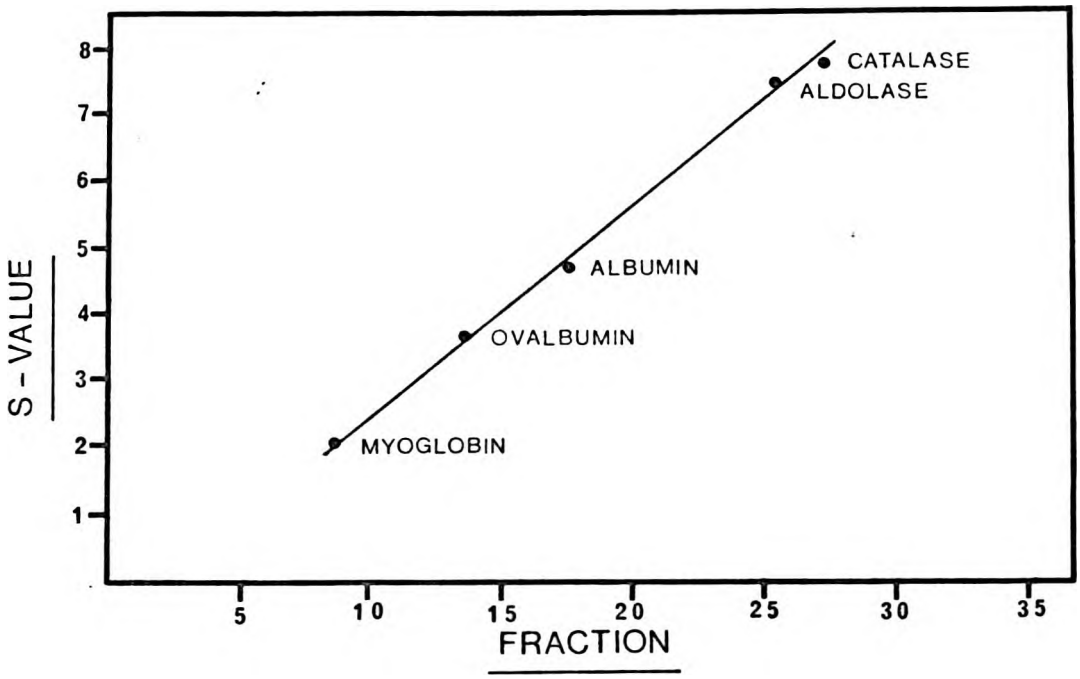


Figure 2.17: Calibration of sucrose density gradients. Gradients were prepared by the hand layering of four different concentrations of sucrose (10 %, 18.3 %, 26.7 % and 35 %) in $TEDAG_{10}$ buffer. After conditioning at room temperature for 2 h, 200µℓ samples of 4 different standard solutions of 1 mg/mℓ were layered, each of them on a separate gradient. The proteins employed were myoglobin (2.04 S), ovalbumin (3.5 S), bovine serum albumin (4.6 S) and aldolase (7.35 S). and catalase (7.5-7.8 S). Centrifugation was executed as described in the legends of Fig. 2.15 and 2.16 above. Following the completion of centrifugation (520000xg'x2 h), the gradients of the standard proteins were fractionated into 40, five drop fractions. A small volume of distilled water (200µℓ) was added to each fraction and the absorbance at 280 nm determined in a spectrophotometer (Beckman Model 35).

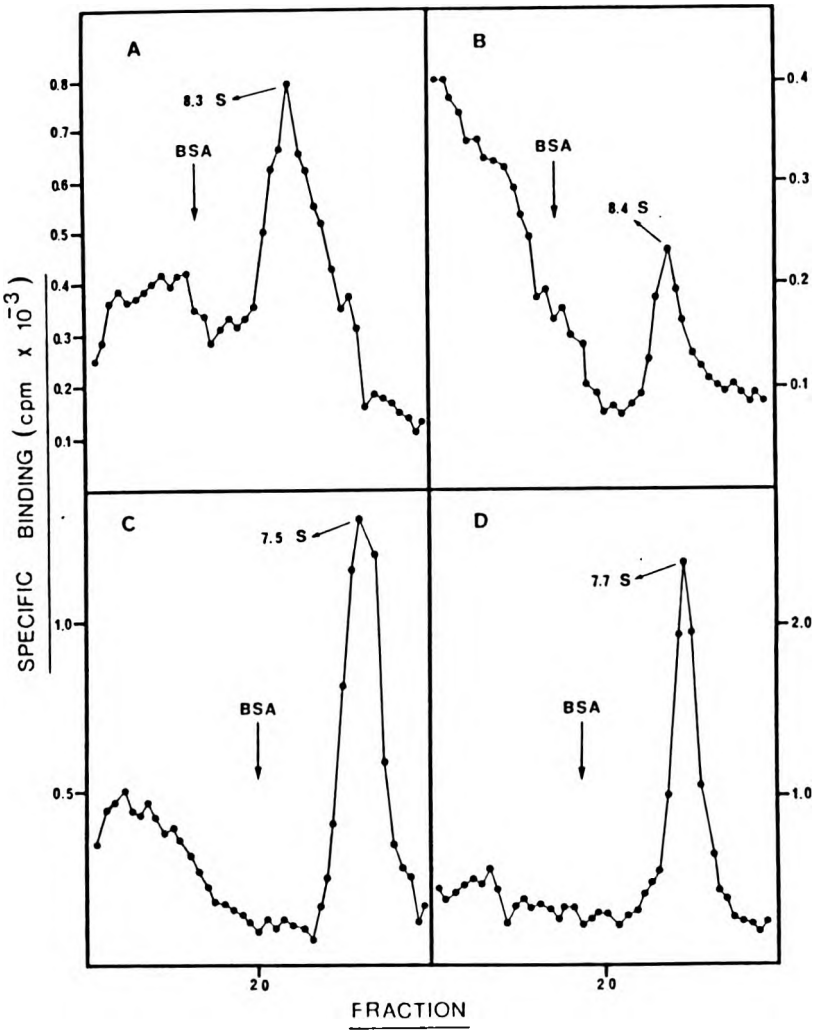


Table 2.18: Comparison of typical SDG profiles obtained following different centrifugation conditions. Cytosols were incubated at 20°C for 30 min and DCC treated aliquots (200µℓ) layered on top of 10-35 % linear SDG. Centrifugation was executed, employing the following conditions : (A) SW 50.1 rotor at 45000 rpm for 16 h, (B) SW 65 rotor 60000 rpm for 16 h, (C) VTi 80 rotor at 75000 rpm for 3 h and (D) VTi 80 rotor at 80000 rpm for 2 h.

TABLE 2.11

Comparison of various conditions of ultracentrifugation for the qualitative analysis of VMU ER and PR on continuous sucrose density gradients.

Type Rotor	Speed RPMxg	Duration of centrifugation	Duration of incubation	Incubation Temp.
1. SW 50.1	45000	16 h	4 h	0°-4°C
2. SW.65	60000	16 h	4 h	0°-4°C
3. VTi80	65000	16 h	30 min	20°C
4. VTi80	65000	20 h	30 min	20°C
5. VTi80	75000	3 h	30 min	20°C
6. VTi80	80000	2 h	30 min	20°C

TABLE 2.12

Calibration of 10-35 % continuous linear sucrose density gradients using unlabelled standard proteins.

PROTEIN	MW (daltons)	S-VALUE (Svedbergs)	FRACTION NO
Myoglobin	17800	2.04	9
Ovalbumin	43000	3.55	14
Albumin	67500	4.6	18
Aldolase	158000	7.35	26
Catalase	232000	7.5 - 7.8	28
Thyroglobulin	669000	19.2	Bottom

CHAPTER 3.

MOLECULAR PROPERTIES OF THE VMU ER AND PR AND THE EFFECT OF SODIUM MOLYBDATE ON THE PROPERTIES OF THESE MACROMOLECULES.

3.1 HETEROGENEITY OF STEROID HORMONE RECEPTORS AND THE SODIUM MOLYBDATE EFFECT.

The oestrogen receptor from different tissues exhibits a complex variety of molecular forms depending on the molecular environment (*in vitro* or *in vivo*) like the presence or absence of structural modifiers, and the type of isolation or separation methodology employed (212).

Human breast cancer cells were found to contain, in addition to the 8 S and 4 S forms, (discussed before in Chapter I), a high molecular weight species (9-10 S), a 2-3 S form meroreceptor, as well as some other intermediate forms (223-214). Some of these have been demonstrated to have some functional significance: e.g. a 5.5 S species appears to interact with the nucleus (215). Bovine uterine oestrogen receptors, which is perhaps the best documented system, exhibit the greatest variety of molecular forms. Murayama's laboratory reported the presence of 2.9 S, 4 S, 5 S, 6 S, 7 S and 8 S forms (216). The activation of uterine oestrogen receptors as investigated by Milgrom *et al* (217) and by Notides *et al* (63) led to a $\sim$ 4 S $\rightarrow$ 5 S conformational shift demonstrating a distinct polymorphism. Milgrom demonstrated that in the rat uterus, activation involved a conversion of the 4 S form to an altered 4 S form followed by the association of this altered 4 S with another moiety (itself, another form of itself, or a different macromolecule) to yield a 5 S form (218). Notides concluded that the activated 5 S species is a homodimer (63). The multiplicity of receptor forms detected, so far, has led to the active pursuit of their origin and interrelationships. There are a number of mechanisms which could explain this diversity.

Puca et al (219) were the first to demonstrate a Ca^{+2}-activated factor in calf uterine cytosol, capable of transforming a larger receptor moiety (8.6 S to 5.3 S), to a smaller species (4.5 S). This receptor transforming factor (RTF), exhibited protease activity, (not trypsin) and could not be inhibited by diisopropylfluorophosphate (DFP) or phenylmethylsulfonyl fluoride (PMSF) (219). The protease activity was caseinolytic while N-benzoylarginine methyl ester seemed to inhibit RTF activity, N-benzoyltyrosinamide and L-leucylglycylglycine did not (216). Puca et al suggested that the RTF might be essential for nuclear translocation of the receptor (216).

Sherman et al (220) investigated the Ca^{+2}-activated, leupeptin -sensitive, protease activity, capable of converting the large cytosolic receptor species (8.6-9.2 S) to the "meroreceptor". This would imply that receptor heterogeneity, as detected, could be the result of controlled or uncontrolled protease action during incubation, centrifugation or gel filtration. Murayama et al (216) and Puca et al (218-219) also suggested a possibility of multiple 4 S species arising by protease action. They reported that the 4.5 S oestrogen receptor exists as two different forms, both sedimenting at 4.5 S, but differing in molecular weight (MW = 82,000 or 65,000 daltons) and stokes radius (44 A and 35 A). Notides et al (244) was concerned about the possibility that multiple proteases may act on the receptor. He reported that the general proteolytic activity of human, calf and rat uterine cytosol can vary considerably. Using the protease inhibitors DEP, EDTA and p-chloromercuribenzoate and hemoglobin as substrate, he determined that the proteases from the three tissues exhibited different biochemical properties. It would seem that the molecular heterogeneity of the receptors could be the result of the cell's own capacity to synthesize and modify the oestrogen receptor in controlled fashion.

Wittliff et al (212, 221-222) observed that patients bearing

tumours exhibiting predominantly the 4 S form of the receptor were less likely to respond to hormonal therapy, whereas those with tumours containing the 8 S or a combination of 4 S and 8 S forms were more likely to give an objective response. They proposed that the "basic" subunits were heterogeneous; that is that there were at least two different types of the 4 S receptor. This heterogeneity could arise in any of several ways: differences in either primary sequence of the protein, conformational changes, covalent or non-covalent modifications of protein structures. The multiple isoforms could combine as homodimers or heterodimers to give rise to the "activated" or 5.5 S moiety. The 8 S form could represent a tetramer consisting of various combinations of heterogeneous 4 S subunits. The SDG profiles of tumours with ER, exhibiting both 8 S and 4 S forms, may represent cases where there was an overproduction of one of the two 4 S species.

A number of exogeneous modifiers of receptor structure and function been studied recently (223-233). Sodium molybdate has been used in relatively low concentrations (5-20 mM) by many workers to "stabilize" the various steroid hormone receptors against inactivation and degradation (223-224). Molybdate was also found to prevent receptor "activation" (225-227). It has been suggested that the mechanism of action could involve : the inhibition of phosphatases (228-230) (implicating a phosphorylation-dephosphorylation mechanism), inhibition of proteases (151) or RNase activity (151) or by the direct interaction with the receptor protein (231,225).

Some of the most important effects of the molybdate anion, reported in theliterature seem to be rendering higher yields of receptors obtained in cytosols, prepared in the presence of sodium molybdate (232).Although a host of evidence would ascribe the effect of sodium molybdate to its possible inhibitive action on either proteases or phosphatases, the

real physicochemical role of this compound remains unsolved. Some researchers claim the effect of molybdate to be completely reversible (230, 151).

In the light of the uncertainty as to what the molecular mechanism of the molybdate effect was, it was decided to investigate the effect of sodium molybdate on the molecular properties of the VMU ER and PR.

Chromatography has played a prominent role in the development of biochemistry. The introduction of paper, thin-layer, gas, gel permeation, ion exchange and affinity chromatography into biochemical research enabled researchers to gain a lot of structural information on macromolecules like proteins and nucleic acids.

The rapid chromatographic separation of proteins require that mobile phases be forced through microparticulate columns under pressure. This requires mechanical stability at high mobile phase velocities which cannot be achieved with most classical gel type and resin like polymer support materials. Suitable column packing materials for HPLC columns must be semirigid to rigid. Additionally, the material must be macroporous to allow sufficient molecular penetration for molecular permeation and interaction with stationary phase functional groups and have adequate surface area to enable relatively high loading capacity in the case of ion exchange, reverse phase and affinity chromatography columns.

Theoretically, gel permeation chromatography (GPC) is the simplest and most predictable chromatographic method. Solutes are separated by size with the large excluded molecules eluting first and the small totally included molecules eluting last. In practice, however, many extraneous mechanisms such as adsorptive, hydropholic, and ionic effects may affect the retention of a solute. Since

the physical and chemical properties of proteins vary so widely, it is inevitable that some will interact with any given support.

Isoelectric focussing differs from all the other separation methods in that it is an equilibrium method. A pH gradient is set up in the separation channel, and solutes migrate from any initial position in the channel to form a sharp Gausian zone at a pH corresponding to their isoelectric (or more strictly isoionic point) pI. Equilibrium methods possess two important advantages over the more usual kinetic methods. In the first place, after equilibrium has been attained, the process is time-independent, so that the zone definition does not deteriorate with the further passage of time. Secondly, since solutes migrate from all positions in the channel towards the final equilibrium position, no definite starting zone is required, and the initial solute mixture may indeed occupy the entire volume of the separation channel.The only notable disadvantage of the isoelectric focussing method arises from the concentration of the solutes in very sharp zones. Under these conditions the solubilities of the solutes may be exceeded locally, and precipitation may occur, particularly as most ampholytes are least soluble in aqueous media near their isoelectric points.

There are five methods of separation using isoelectric focussing:

a) Free solution
b) Density gradients
c) Gels
d) Porous materials
e) Continuous flow methods

The potential advantages of agarose have prompted researchers to investigate it as a medium for IEF. The gels are

easy to prepare, it is optically clear for scanning purposes and it is chemically inert. Also agarose has a relatively large pore size, making it suitable for the focussing of large molecules.

The separation in ion exchange chromatography (anion or cation exchanges) is obtained by reversible adsorbion of ionic species onto an inert matrix (e.g. cellulose), containing defined covalently linked functional groups like $-OCH_2CH_2N(C_2H_5)_2$ (diethylaminoethyl, DEAE) or $-OCH_2COO^-$ (carboxymethyl,CM)-. Separation of macromolecules (proteins) is obtained on the basis of differences existing in the net ionic charge which affects the overall pKa values of the different protein molecules. Elution from the column can be effected by a variation in either the concentration of a competing ion (ionic strength) or the pH of the mobile phase. The differences in charge properties of biological compounds are considerable. Ion exchange chromatography is a powerful technique enabling researchers in separating macromolecular species with only minor differences in ionic properties (e.g. proteins differing by only one amino acid).

Recently, Pharmacia introduced the chromatofocussing technique for the separation of proteins based on differences in iso-electric points (pI). A pH-gradient is created over an anion exchange column with the aid of polybuffers, containing ampholytes within a particular pH-range. Proteins are then eluted in order of high to low pI values from the column, allowing separations within 0.01 pH unit. This principle was also recently introduced in HPLC (309).

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Most of the reagents employed were the same as those described in Chapter 2 (section 2.7.1). Other reagents used in

the course of this investigation were:

16a-[^{125}I]Iodooestradiol (^{125}I-E_2; specific activity > 1500 Ci/mmol) was bought from Amersham International. The Blue Dextran 2000 was bought from Pharmacia. The polybuffers (96 and 74) were bought from Pharmacia.

The following compounds were all purchased from Sigma Chemical Company : N-ethylmaleimide (NEM), iodoacetic acid (IAA), O-Phenanthroline (O-Phen), dithionitrobenzene (DTNB), leupeptin (Leu), phenylmethylsulfonyl fluoride (PMSF) and N-α-p-tosyl-L-chloromethylketone (TLCK).

Ultrogel AcA-22 gel was supplied by LKB. Other columns, employed in this investigation were the Waters I-250 (Millipore), as well as the AX-1000 and AX-500 (Synchropak).

The most important materials used in isoelectric focussing are the carrier ampholytes which provide the stable pH gradient. At the present time the synthetic materials supplied by LKB products under the trade name of Ampholines are quite satisfactory.

LKB Ampholines are currently available as one wide-range pH 3-10 (LKB 8141), three medium-range, pH 3-6, 5-8 and 7-10 (LKB 8142-8144) and six narrow-range grades. The later cover only 2 pH units and include the regions 3-5, 4-6, 5-7, 6-8, 7-9 and 8-10.

3.2.2 Collection of Vervet monkey uteri (VMU) and preparation of cytosols:

Collection and storage of uteri have been described in detail in Section 2.9. Preparation of cytosols from either tissue slices, or tissue powders have been outlined in Section 2,10. All preparations were executed in the cold (0-4°C) unless stated otherwise.

3.2.3 Binding capacity (B_{max}) and dissociation constants (K_d) of ER and PR in VMU: Molybdate effect.

(a) Oestrogen receptors assay:

Aliquots (100µℓ) of cytosol (CYT(+) and CYT(-)) were thoroughly mixed and then incubated with 100µℓ of [^{3}H]-oestradiol at 5 different concentrations (0.25-8nM) for 30 min at 20°C. Non-specific binding was assayed in parallel with 100 fold excess of unlabeled DES. The reaction was terminated with the addition of 500µℓ DCC. The suspension was vortexed, left on ice for 15 min. Aliquots (500 µℓ) of the supernatants were assayed for radioactivity following the addition of 4 mℓ scintillation cocktail.

(b) Progesterone receptor assay:

The assays were carried out simultaneously with the ER assay on aliquots of the same cytosols. Standard conditions used were the following: Cytosols (CYT(+) and CYT(-)) were incubated on crushed ice for 2 h, following the addition of 100µℓ of the radioligand. After the first 2 h, 100µℓ of $TEDAG_{60}$ was added to the incubation mixture and thorough vortexing was followed by a reincubation in crushed ice for another 2 h. The reaction was stopped by the addition of DCC (500µℓ), the reaction mixture vortexed and then pelleted at 2000xg for 15 min (0-4°C) and the supernatants monitored for radioactivity.

3.2.4 Effect of sodium molybdate on the association of VMU ER and PR with their respective ligands and the stability of the receptor-ligand complexes at 0°C, 20°C and 37°C:

Cytosols (CYT(+) and CYT(-)) were incubated with tritiated ligand ([^{3}H]-E_2 or [^{3}H]-R-5020 to a final concentration of 5 nM, with and without a 250 fold excess level of the appropriate unlabelled competitor (DES or R5020) at either 0°C,

20°C or 37°C. Immediately, following the addition of the radioligands, aliquots (100µℓ) of the incubation mixtures were taken at regular time intervals (2 min intervals for the first 10 min; 10 min intervals for the next 60 min and 30 min intervals for up to 3 h) and added to a DCC suspension (1 mℓ) on ice. Following thorough vortexing, the DCC suspensions were left on the ice for 15 min after which they were centrifuged at 2000 xg' for another 15 min. Aliquots (500 µℓ) of the supernatants were monitored for radioactivity following the addition of 4 mℓ cocktail to the sample. Vials were counted in a Beckman LS 900 counter to an efficiency of 25-30 %.

3.2.5 Ligand specificity of VMU ER and PR :

Aliquots (100µℓ) of freshly prepared uterine cytosols were incubated with 50µℓ of either labelled oestradiol or progesterone (8nM) in the presence of 50µℓ of increasing concentrations (0.169nM-2.6µM) of a group of competitors (E_2, E_3, E_1, EE_2, DES, P, R 5020, NE, T, DHT, DHEA, HC) for 30 min at 20°C. Reactions were terminated by the addition of cold DCC (500µℓ) and subsequent incubation on ice for 10 min. Charcoal was pelleted by centrifugation (2000xg' x 15 min) and aliquots (500 µℓ) of the resulting supernatants counted for radio-activity.

3.2.6 Effect of sodium molybdate on the dissociation of VMU ER and PR.

Cytosols (CYT(+) and CYT(-)) were equilibrated with 5nM [^{3}H]oestradiol or [^{3}H]R-5020 for 1 h at 0°C.

The [^{3}H]-oestradiol and [^{3}H] R-5020 dissociation were assayed at 20°C after the addition of 5nM (1000 fold excess) of unlabelled oestradiol. Receptors inactivation (control) was measured in a parallel incubation with the addition of buffer without the unlabelled oestradiol or promogestone.

After time intervals at 20°C (1 min at the first 15 min and 15-30 min for 130 min) 100µℓ aliquots were vortexed with DCC solution (1 mℓ) at 0°C and radioactivity was counted of 500 µℓ of the 2000xg'x10 min supernatant. Each determination has been corrected for non-specific binding.

3.2.7 Reversibility of the effect of sodium molybdate on the VMU ER and PR.

a. Cytosols (CYT(-) and CYT(+) were incubated at 20°C for 30 min with either ^{3}H-E_2 or ^{3}H-R5020 (final ligand concentration of 5nM). Parallel incubation mixtures also contained a 100 fold excess of either DES or R5020 respectively. Incubations were terminated by transfer of the incubation mixtures to DCC pellets, vortexing and subsequent centrifugation at 2000xg' (15 min x 4°C). The resulting supernatants (200µℓ) were then layered onto the sucrose density gradients, prepared in Quickseal tubes (Beckman Instruments) by handlayering 4 different concentrations (10 %, 18.3 %, 26.7 % and 35 %) of sucrose in $TEDAG_{10}$ or TEDAG-MO buffers. Prior to layering of the samples the gradients were left to condition at room temperature for at least 2 h in order to obtain continuous 10 - 35 % gradients. Centrifugation was then executed, employing the VTi-80 rotor in a Beckman L8-80 ultra-centrifuge for 2 h at a maximum gravitational force of 520000xg' (2°C) to a constant $\omega^2 t$ of 5.3 x 10".

Gradients were prepared, calibrated and processed as described in section 2.13. Altogether four different combinations of runs were executed : cytosols containing molybdate (CYT(+))) were either run on gradients containing (GRAD(+)) or not containing (GRAD(-)) molybdate and the same for cytosols not containing (CYT(-)) molybdate. Under the influence of the gravitational force the receptor molecules are being displaced from an environment containing molybdate to one not containing molybdate and vice versa. Therefor this technique is excellent in studying the reversibility of

the "molybdate effect".

(b) Dialysis studies monitored by SDG:

CYT(-) and CYT(+) cytosols (2 mℓ) were dialysed in cellulose bags against either $TEDAG_{10}$ or TEDAG-MO buffer (500 mℓ) over a period of 4 h with a total of 4 buffer exchanges. Aliquots of the cytosols were taken before and after dialysis and then charged by incubating with the radio ligands (^{3}H-E_2 or ^{3}H-R5020; 5nM) at 20°C for 30 min with or without an excess (250 fold) of either DES or R 5020 respectively. Following the incubation, unbound ligands were adsorbed on a DCC pellet and 200µℓ of the resulting supernatants layered onto sucrose density gradients, prepared and calibrated as described above. Aliquots (100µℓ) of the supernatants were also assayed for radioactivity in order to calculate the specific binding for the different steps in the experiment. Gradient centrifugation was performed and the individual gradients processed as described in (a). The gradients were selected to have the same constitution irrelevant to molybdate content as that of the buffer against which dialysis was performed.

3.2.8 Influence of proteolytic inhibitors and thiol reactive agents on VMU ER and PR.

(a) Influence of Phenylmethylsulfonylfluoride (PMSF; Sigma):

Uteri were cut into thin slices and the slices thoroughly mixed, prior to homogenization. Aliquots (~400-500 mg) of uterine tissue then homogenized in $TEDAG_{10}$ or TEDAG-MO buffers containing 1mM PMSF in order to "neutralize" the effect of proteolytic enzymes in the changes in the gradient profiles. Subsequent to incubation with the appropriate ligands (^{3}H-E_2 or ^{3}H-R5020; 5nM) the charged cytosols were processed as described in section (a) of the previous experiment.

In another set of experiments aliquots of the tissue slices were homogenized in $TEDAG_{10}$ buffer, containing 5mM of the PMSF. The 105000xg' cytosols were then incubated with the ligands (^3H-E_2 or ^3H-R5020, 8nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess of either DES or R5020 respectively. Following DCC treatment, the 2000xg' supernatants were monitored for specific binding (single point saturation assay) and then subjected to SDG and IEF analysis (sections 2.13 and 3.2.10).

(b) Influence of leupeptin (Sigma):

The experiments were performed exactly as described for the PMSF, except that homogenization buffers contained 2mM leupeptin.

(c) Effect of N-ethylmaleimide (NEM) on VMU ER and PR.

Vervet monkey uteri were sliced up and the thin tissue slices thoroughly mixed before portions of the batch (~400 mg) were homogenised in a small volume (1.5 mℓ) of $TEDAG_{10}$ buffer containing the inhibitor at a specific concentration. The NEM was used in the buffer at a final concentration of 5mM. Cytosolic protein concentration was determined by the method of Lowry (240).

A single point saturation assay was conducted (8nM final concentration of ^3H-E_2 or ^3H-R5020) on the 105000xg' cytosol for both ER and PR, to assay total and non-specific binding in the presence or absence of the inhibitor. Ligand-charged, DCC treated, 2000xg' cytosolic supernatants were subjected to SDG and IEF analysis as described in Sections 2.13 and 3.2.10.

(d) Effect of iodoacetic acid (IAA) on VMU ER and PR:

Experiments were executed exactly as described in Section 3.2.8.(c) above, except that the inhibitor IAA (20mM) was included in the homogenization buffer.

(e) Effect of O-phenanthroline (O-Phen) on VMU ER and PR :

Experiments were executed exactly as described in Section 3.2.8(c) above, except that the inhibitor O-Phen (10mM) was included in the homogenization buffer.

(f) Effect of dithionitrobenzene (DTNB) on VMU ER and PR :

Experiments were executed exactly as described in Section 3.2.8(c) above, except that the inhibitor DTNB (10mM) was included in the homogenization buffer.

(g) Effect of N-α-p-tosyl-L-lysine-chloromethylketone (TLCK) on VMU ER and PR :

Experiments were executed exactly as described in Section 3.2.8(c) above, except that the inhibitor TLCK (5mM) was included in the homogenization buffer.

3.2.9 Chromatography of VMU ER and PR on size exclusion, ion exchange and chromatofocussing columns

3.2.9.1 Size exclusion chromotography of the VMU ER:

It was decided to investigate the effect of molybdate on receptor heterogeneity with the aid of gel permeation columns:

(a) Size exclusion chromatography on a Ultrogel AcA-22 polyacrylamide gel :

All experiments were run in parallel on "identical" columns to obtain the results of total and non-specific binding at

the same time. Both columns were packed with LKB Ultrogel AcA-22, at a flow rate of 9.6 mℓ/hour and 0.96 mℓ fractions were collected. The wet bed diameter of the gel beads was 60-140 microns. The effective fractionation range for globular proteins was between 10^5 and 10^6 daltons. The dimentions of the packed columns were 16mmx570mm. Columns were equilibrated with Tris-EDTA containing dithiothreitol (1mM) at pH 7.4. Sodium azide (3mM) was added to all the buffers to prevent bacterial growth. The linear flow rate of the columns was 4.77cm/h for both of the columns.

Columns were calibrated prior to use. The total volume (V_t) was determined with ^{3}H-leucine (0.1μCi). Blue dextran 2000 (Pharmacia) was employed to calculate the void volume (V_o) of the column: The following standard proteins were used :

(1) catalase (M.W.=232,000 daltons)
(2) Thyroglobulin (M.W.=669,000 daltons)
(3) aldolase (M.W.=158,000 daltons)
(4) ferritin (M.W.=444,000 daltons)

Elution of the standard proteins was monitored by measuring the optical densities at 280 nm. The distribution coefficient (K_D) of each binding component and standard was calculated from its elution volume (V_e):

$$K_D = \frac{V_e - V_o}{V_t - V_o}$$

The K_D was plotted against log molecular mass (M.W.) and $(K_D)0{,}333$ against the Stokes radius(Rs).

Cytosols were prepared in the presence or absence of 20mM sodium molybdate as described in Section 2.10. Columns were also run with $TEDAG_{10}$ buffer containing 0.154 M or 0.4 M KCl. The incubation was executed at 20°C for 30 min during

which time the cytosol was incubated with 10nM ^{3}H-E_2 to a final incubation concentration of 5nM. Incubation with 250 fold excess of unlabelled ligand (for NSB) was performed simultaneously. The incubation was terminated by mixing with a DCC pellet and 500µl of the 2000xg' supernatant was layered on the column. Five hundred microliter fractions were collected (LKB 7000 Ultrarac fraction collector) directly into counting vials. All procedures were conducted at 0-4°C in a cold room. Four mℓ of Beckman Ready Solv CP cocktail was added and samples were monitored for radioactivity.

(b) Gel permeation chromatography on a Waters I-250 gel column.

Cytosol was prepared and labelled with ^{3}H-E_2 as previously described in Section 2.10 and protein assayed by the method of Lowry et al (240). Cytosol protein concentrations were usually in the range of 6-8 mg/mℓ. The column employed was a Waters I-250 column containing a hydrophilic gel, linked to silicon beads of uniform size (5 µm). The column length was 25 cm. and the flow rate was 1 mℓ/min. Total volume (V_t), determined with a mixture of polar and non-polar labelled amino acids was approximately 12.5 mℓ. The void volume (V_o) of the column was determined with Blue Dextran (MW ~ $2x10^6$ daltons) and it was found to be 5.91-6.71 mℓ (depending on the ionic strength). The column was eluted isocratically with 25 mM phosphate buffer containing 1.5 mM EDTA and 10 % glycerol (PEG buffer). The column was calibrated by a set of marker proteins employing the following mobile phases:

(a) PEG buffer,
(b) PEG buffer containing 100 mM KCl ($PEGK_{100}$) or
(c) PEG buffer containing 400 mM KCl ($PEGK_{400}$).

Experiments were performed in the following way:

(1) Cytosols prepared without sodium molybdate, were run on columns eluted with PEG buffer.
(2) Cytosols, prepared without molybdate, were run on columns eluted with $PEGK_{100}$ buffer.
(3) Cytosols, prepared with or without molybdate, were run on columns eluted with $PEGK_{400}$ buffer following re-incubation by charged cytosols in 0.4 MKCl containing $TEDAG_{10}$ at various time-intervals (t-zero and 30 min) at 0-4 °C.

In the first case the incubation was immediately stopped by the addition of the charged cytosols to a DCC pellet prepared from 2 mℓ of DCC and the 2000xg' supernatants were then run on the column, employing $PEGK_{400}$ buffer as mobile phase. In the other experiment a second incubation was prolonged for 30 min at 0-4°C and the DCC treated cytosol then fractionated on the column employing $PEGK_{400}$ buffer as the mobile phase.

3.2.9.2 HPLC chromatography of VMU ER and PR on an AX-1000 anion exchange column:

Cytsols were prepared in the presence and absence of sodium molybdate (CYT(+) and CYT(-)). The different cytsols were charged by incubation with $^{125}I\text{-}E_2$ for 18-24 h at 0-4°C in the presence or absence of a 250 fold excess DES. Unbound ligand was removed by DCC pellet and the 2000xg' supernatants applied to the columns. Columns were equilibrated with 25 mM Tris buffer (pH 8.2 prior to sample application and developed with a limiting buffer containing 500 mM disodium hydrogen phosphate (Na_2HPO_4) at a flow rate of 1 mℓ/min. Fractions of 1 mℓ were collected straight into counting vials.

3.2.9.3 Chromatofocussing of VMU ER and PR on an AX-500 polyamine column:

Cytosols were either prepared in the presence (CYT(+)) or absence (CYT(-)) of sodium molybdate (10mM). Aliquots of cytosols were incubated with ^{125}I-E_2 with or without unlabelled DES (250 fold excess) for 18-24 h at 0-4°C. Reactions were terminated by DCC treatment and the 2000xg' supernatants injected onto the AX-500 column. Prior to sample application, the columns were equilibrated with a 25mM Tris buffer (pH 8.4) until the column was pH 8.4. It was then eluated with a 30:70 mixture of Polybuffers 96 and 74 (Pharmacia) respectively (dilution 1:15 with 20% glycerol). The pH of the polybuffer mixture was adjusted to 4.5. The flow rate was 1 mℓ/min and 1 mℓ fractions were collected straight into counting vials. The radioactivity of the fractions was monitored in each fraction and the pH was determined for every second fraction.

3.2.10 Isoelectric focussing (IEF) of VMU ER and PR on agarose gels:

3.2.10.1 Experimental procedure:

a. Gel preparation:

Agarose gels were prepared containing a concentration of 1 % agarose, 12 % sorbitol and the appropriate pharmalyte range at a final dilution of 1 to 16. A typical gel contained :

(1) 0.35 g agarose
(2) 4.2 g sorbitol
(3) 31.5 mℓ water
(4) 2.2 mℓ pharmalyte

Gels were handled by casting the gel upon a hydrophilic plastic sheet (Gel Bond - Pharmacia). The agarose and the sorbitol were first dissolved in boiling water, the solution cooled down to 75°C and the Pharmalyte carrier ampholyte added prior to casting of the gel. Gels were left overnight at 0-4°C to set. As the anode solution, 0.05 M H_2SO_4, was used, while the cathode solution consisted of 1 M NaOH.

A Pharmacia, Constant Power Supply (ECPS 3000/150) was used for the focussing at the following settings :

Mode:	Constant power.
Set Voltage:	1500 V
Set current:	maximum
Set power:	15 watts
Time:	2 hours
Temperature:	10°C approx.

Gels were generally prefocussed for 30 min prior to the application of the cytosols. Once the gels were focussed, they were fixed, (5 % sulfosalicylic acid plus 10 % trichloroacetic acid) washed and then dried before staining and destaining. Gels were then cut into slices of 1-3 mm width and the slices dissolved in 2 mℓ of scintillation cocktail, prior to counting radioactivity.

The pH gradient of each individual gel was calibrated, employing the standard proteins : lactoglobulin (pI = 5.5), horse myoglobin (pI = 7.4 and 7.0), carbonic anhydrase (pI = 6.1) and whale myoglobin (pI = 7.7 and 8.2).

(b) Preparation and incubation of cytosols :

Cytosols were prepared as described before in section 2.10. Incubations were conducted in parallel with CYT(-) and CYT(+) cytosols. Aliquots (100µℓ) of cytosol were incubated with 100 µℓ of ^{125}I-E_2 (final ligand concentration of 5 nM) at 20°C for 30 min without (total binding) or with (non-specific binding) a 100 fold excess of DES. Incubations were terminated by transfering the incubation mixtures to DCC pellets, vortexing and subsequent centrifugation at 2000 x g' for 15 min at 4°C. The resulting supernatants (40µℓ) were applied to the gels on small rectangles of filter paper, or in a specially designed plastic sample applicator.

3.3 RESULTS :

Table 3.1 shows the values for B_{max} and K_d for VMU ER and PR receptors. The results represent mean values for about 30 VMU, which were carried out in groups of 7-8 uteri at a time. There was a significant difference between B_{max} values obtained for cytosols, homogenized in the presence (CYT(+)) or absence (CYT(-)) of sodium molybdate. In the presence of sodium molybdate the B_{max} values were found to be consistently higher (20-30 %) for both ER and PR. No difference in the binding affinity (K_d) could, however, be illustrated for both receptors, either in the presence or absence of sodium molybdate. Representative Scatchard and saturation plots appear in Fig. 3.1. The effect of molybdate appeared to be very specifically directed towards the high affinity binding sites (Fig 3.1 panel inserts) while the non-specific binding levels were not significantly different.

Association curves for both ER and PR (CYT(+) and CYT(-) cytosols) are represented in Fig. 3.2. It is clear that the formation of the hormone receptor complexes was complete within 15 min (ER) to 30 min (PR) at 0°C. At 20°C, however, association proceeded much faster and plateaus were reached within 7 min (ER) to 16 min (PR) for both CYT(+) and CYT(-) cytosolic receptors. At 37°C the receptors were rapidly inactivated, while molybdate provided some protection against the process of inactivation and denaturation. At this temperature, without molybdate, a very rapid association occurred at 37°C (<2 min), but in the presence of molybdate the receptors remained stable for 15 min (ER) and 12 min (PR) respectively, before being inactivated. After 40 min at 37°C the PR was totally inactivated in both CYT(+) and CYT(-) cytosols while the ER appeared to be more stable without molybdate : 30 % active in the absence of sodium molybdate and 40 % active in the presence of molybdate. After 90 min at 37 °C the ER was totally inactivated, even in the presence of sodium molybdate. Both receptors appeared to be very stable at 20°C for up to 3h, at 0°C for up to 24h.

Ligand specificity for the two receptors is represented in Fig 3.3 and 3.4. Relative binding affinities (RBA) and apparent K_d values for the ligands (mean of 2 to 4 assays) have been calculated according to Rodbard et al (233) and the data are summarized in Table 3.2.

These results indicated a much narrower specificity for the ER as compared to the PR. For the ER the ligand preference was of the order : DES > EE_2 > E_2 > E_3 > E_1 while no affinity was observed for any of the other steroid hormone categories. The result also seemed largely in agreement with literature reports (305-306). The PR, on the other hand, exhibited a much lower level of ligand specificity, a finding partially in agreement with other investigators (224,307). The most remarkable characteristic was the high affinity of the PR for EE_2 (91.2 %) and the relative high

affinity for other oestrogens : $E_2 > E_1 > E_3$ (see Table 3.2). It would seem that the VMU progesterone receptor displays a unique property in comparison with other progesterone binding proteins. Published values for the relative binding affinities of oestrogens for the PR were found to be much lower than our values (221,308).

The dissociation of receptor-ligand complexes exhibited a biphasic pattern (Fig 3.5) for both ER and PR. Molybdate increased the rate of dissociation of the fast dissociating complexes (FDC), but slowed down the dissociation of the slow dissociating complexes (SDC) (see Table 3.3). The half lives of the ER and PR complexes at 20°C was found to be 35 min for CYT(+) and 43 min for CYT(-) for ER and 22 min for CYT(+) and 25 min for PR.

The most marked effect of the molybdate on the receptor yield and stability can be appreciated in Fig. 3.6 - 3.8 and Table 3.4. Once the receptors are "protected" during homogenization (CYT(+)), they appear to be more stable, even under conditions where no molybdate is present (GRAD(-)). Receptors, not "protected" during the homogenization process can only be partially regenerated to a higher binding capacity by molybdate, present in the gradient solution (GRAD(+)). This sedimentation dialysis experiment (Fig. 3.7 and 3.8) implies that the "protective" effect of molybdate is not fully reversible, an observation which applies to both ER and PR in the VMU.

The sucrose density gradient profiles appearing in Fig. 3.9 depict the results obtained, when CYT(+) and CYT(-) cytosols were dialysed in cellulose tubing against different compinations of $TEDAG_{10}$ or TEDAG-MO buffers, over a period of 4 h in the cold prior to labelling and centrifugation. Much better reversibility has been obtained for both ER and PR in this experiment. The partial loss in ligand binding capacity obtained cannot be attributed to receptor inacti-

vation during the experiment, since low temperatures (0-4°C) prevailed and no significant changes in the binding capacities were observed in control cytosols (CYT(+) or CYT(-)) subjected to the same conditions. A quantitative analysis of the binding data appear in Table 3.5.

The effect of the proteolytic inhibitors Leu (2mM) and PMSF (1mM) in the presence or absence of molybdate is depicted in Fig. 3.10 and 3.11, while the relevant binding data appear in Table 3.6. The action of molybdate does not seem to be affected by the presence of Leu or PMSF. Receptor inactivation still occurs in molybdate deficient media, varying between 14 % for the PR and 61.4 % for the ER in the presence of Leu. In molybdate containing cytosols (CYT(+)) a higher yield of receptors (ER and PR) was found when these cytosols were prepared in the presence of Leu when compared to that found in control conditions (CYT(+) and GRAD(+)). The ER proved to be exceptionally prone to inactivation in the absence of molybdate even though Leu was present in the homogenization buffer (61.4 % inactivation). No stabilizing effect on ER and PR was observed when molybdate and PMSF were present in the homogenization buffer.

Sedimentation coefficients for both ER and PR did not change significantly. Only in the case of the PR, another specific binding component, sedimenting at 5.2 S was observed.

At a higher concentration of PMSF (5mM) and in the absence of molybdate (CYT(-)) ligand binding capacity of the receptors was inhibited by 86.1 % (PR) to 89.2 % (ER) (Table 3.7) and this result was strongly corroborated by the SDG profiles and IEF patterns obtained (Fig. 3.12 and to Fig. 3.15).

A loss in the ligand binding capacity of the ER (44.1 %) and PR (22.5 %) was observed in this batch of experiments in the presence of 2mM Leu, a result which is somewhat contradic-

tory to that, illustrated in Fig. 3.10 and 3.11. However we found that differences in the apparent stability of the receptors did exist between individual uteri, a finding which might relate to the levels of endogenous receptor inactivating factors (e.g. proteolytic enzymes) existing in the uterine tissue at different stages of development or the hormonal status of the female, prior to removal of the uterus. For this reason, most of the experiments were executed on batches of uteri. Curiously enough, the ER appeared to be more labile in the presence or absence of Leu relative to the PR (Table 3.7) and this was also observed in the first batch of experiments (Fig. 3.10-3.11).

Ligand binding capacity of both receptors (Table 3.7) was severely inhibited in the presence of the sulfhydryl reactive agents NEM (77.0-81.5 %), IAA (93.8-94.9 %) and DTNB (94.1 - 95.4 %). These results were all corroborated by the SDG profiles and IEF patterns (Fig. 3.12 to Fig. 3.14).

O-Phen did not cause such a great loss in receptor ligand capacity (ER : 29.2 %; PR : 51.4 %) than TLCK (ER : 82.1 ; PR : 66.7 %) or the other inhibitors (Fig 3.12 to Fig. 3.14 and Table 3.7). IEF patterns and SDG profiles, however, indicated a much greater effect of this compound on the integrity of the ER and PR. As for TLCK, although SDG profiles confirmed the finding that the binding capacities of both ER and PR were severely inhibited, some specific binding components could still be observed in IEF gels. Whereas TLCK tended to effect a shift in pI values to a higher pH range (6.5 → 7.2 : ER ; 6.5 → 7.0 : PR), O-Phen had the opposite effect, shifting the peaks to lower pH ranges (Table 3.8). Only Leu caused significant shifts in the sedimentation coefficients of ER (8.2 → 9.9) and PR (8.4 → 9.5).

The calibration curve for the Ultrogel AcA-22 column appears in Fig.3.16. A representative column elution profile of the

size exclusion chromatography of VMU ER is depicted in Fig. 3.17.

To facilitate the description of the various forms of the VMU oestrogen receptor, it was decided to use the nomenclature proposed by Sherman et al (213) and to number the forms from class I to class V in order of decreasing molecular size as measured by gel filtration. Table 3.9 summarizes the results obtained for the molecular masses and Stokes radii(r^*) calculated from the column data while Fig. 3.17 depicts typical column elution profiles obtained for the VMU ER. A highly aggregated complex (MW = 3.9 - 4.7 $x10^5$ daltons) appeared in the void volume of the column, when the cytosols, prepared in hypotonic buffers ($TEDAG_{10}$) were fractionated on Ultrogel AcA-22 employing hypotonic buffer as the mobile phase.

Receptor aggregation tended to be slightly more prominent when fractionation was executed in the absence of sodium molybdate (MW = $4.68x10^5$daltons; $r^* \approx 63.2$ Å) as compared to that obtained in the presence of sodium molybdate (MW = $3.87x10^5$ daltons; $r^* \approx 61.8$ Å). Only Class I receptors were noticed under these conditions. Fractionation of cytosols (CYT(-)) on the same column, employing a hypertonic mobile phase (0.4 MKCl) yielded a Class III type of receptor (MW = $9.2x10^4$ daltons; $r^* \approx 42.3$ Å), while an isotonic mobile phase (0.15 MKCl) yielded a similar type of receptor molecule (MW = $8.6x10^4$ daltons, $r^* \approx 41.8$ Å). A summary of the molecular masses and Stokes radii appears in Table 3.9.

Calibration curves of the Waters I-250 gel exclusion columns are depicted in Fig 3.18. The ionic strength of the mobile phase affected the elution volumes of the standard proteins and molecular masses were subsequently calculated, employing the proper calibration curves. Typical, representative column elution profiles for VMU ER on the Waters 1-250 gel column appear in Fig. 3.19, while the calculated molecular

size data are summarized in Table 3.10. The molecular size for the ER, as determined with a hypotonic mobile phase (PEG, PEGM) was 2.006×10^5 daltons, both in the presence or absence of sodium molybdate (Table 3.10). Extensive receptor aggregation (MW = $7.2\text{-}8.6 \times 10^5$ daltons) was observed in the absence of molybdate, following an extended incubation in 0.4 MKCl, prior to size exclusion chromatography (hypertonic mobile phase). However, this aggregation effect was not observed with cytosols prepared in the presence of sodium molybdate (MW = $1.1 - 1.5 \times 10^5$ daltons).

The effect of sodium molybdate on the subunit integrity of the VMU ER is obvious when Fig. 3.20 and Fig. 3.21 are compared. In the case of CYT(-) cytosols (Fig. 3.20) two well resolved high affinity ligand binding molecular species were obtained. One species eluted at 90mM and the other at 230mM salt (HPO_4^{-2}). There appeared to be a 1:1 ratio (51 % : 49 %) for the surface areas representing the two subunit peaks. When molybdate was present, only one peak was observed, eluting at 189 mM HPO_4^{-2}. Receptor yields for CYT(-) cytosols were only 30-50 % of the values obtained with CYT(+) cytosols.

Data obtained with the AX-500 column, following HPLC chromatofocussing is depicted in Fig. 3.22 and 3.23 as well as in Table 3.11. The apparent receptor heterogeneity observed with isoelectric focussing on Agarol gels was underlined with the results of this experiment. At least 7 high affinity binding components were observed (Table 3.11) spanning the pH range 8.0 to 4.0 in cytosols not prepared in the presence of sodium molybdate (CYT(-)), while only 3 components were detected in (CYT(+) cytosols. It would appear that the molybdate oxyanion affected the ionic properties of the high affinity bindings components to such an extent, that they eluted at lower pI values. In a way this finding was also corroborated by data obtained on anion exchange columns, as well as isoelectric focussing.

Typical, representative iso-electric profiles for the VMU ER appear in Fig. 3.24 and 3.25. More than 90x1 mm fractions were derived from each gel, with the result that well resolved peaks were obtained. Iso-electric points and receptor yields in individual peaks are summarised in Table 3.12.

The effect of molybdate on the receptor species was clearly observed upon inspection of the iso-electric focussing pattern : In the absence of molybdate (CYT(-)) the pattern revealed clearly defined peaks with pI's of 5.9 (28.9 %), 6.2 (30.3 %) and 6.8 (40.7 %) respectively. Molybdate containing cytosols (CYT(+)) yielded one major high affinity binding species at pI = 5.9 (39.9 %), with two minor entities, appearing at pI = 5.6 (13 %) and 6.8 (16.6 %). A broad, undefined area, containing some specific binding components appeared in the pH region 5.0-5.5 (30.5 %).

As a rule, receptor yield was much lower in the absence of molybdate, yielding between 30 % and 50 %, (>10 assays) lower specific binding, compared to IEF assays on CYT(+) cytosols.

TABLE 3.1

The effect of molybdate on the binding capacity and affinity of ER and PR in the VMU.

Receptor	B_{max} (fmol /mg protein)		K_d ($\times 10^{-9}$ M)		%I*
	CYT(+)	CYT(-)	CYT(+)	CYT(-)	%I*
ER	191 ± 51	135 ± 62	0.32 ± 0.1	0.3 ± 0.1	29.3
PR	566 ±184	356 ± 57	7.7 ± 1.5	7.4 ± 1.5	37.1

(*%I = % inactivation of ligand binding capacity; CYT(+) = 100 % value)

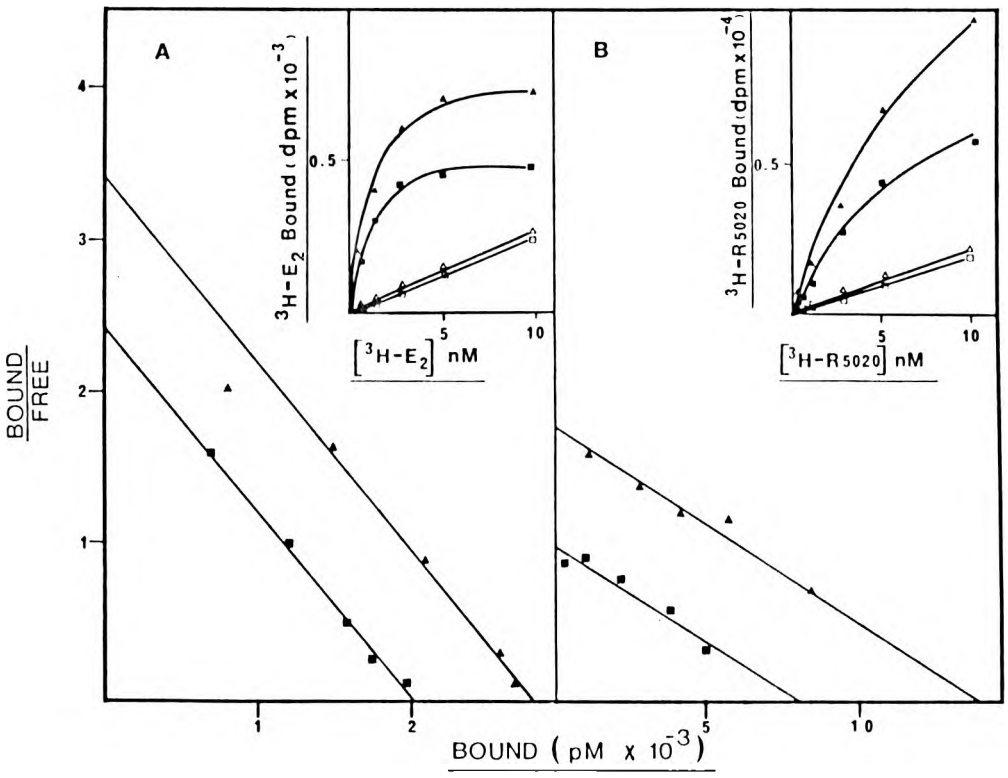


Figure 3.1 : Scatchard and saturation curves for VMU ER and PR. Cytosols were prepared in buffer containing (▲) or not containing (■) 20mM sodium molybdate. Aliquots (100µℓ) of cytosols were incubated with the appropriate ligand (^{3}H-E_2 or ^{3}H-R5020) in the absence (total binding) or presence (non-specific binding) of a 100 fold excess of DES or R5020 respectively, at 5 different concentrations (0.25-8nM) : 30 min at 20°C for the ER and 4 h on ice for PR. The reactions were terminated with the addition of DCC (500µℓ) and aliquots (500µℓ) of the 2000xg' supernatants monitored for total and non-specific binding : (A) oestrogen receptor; (B) progesterone receptor.

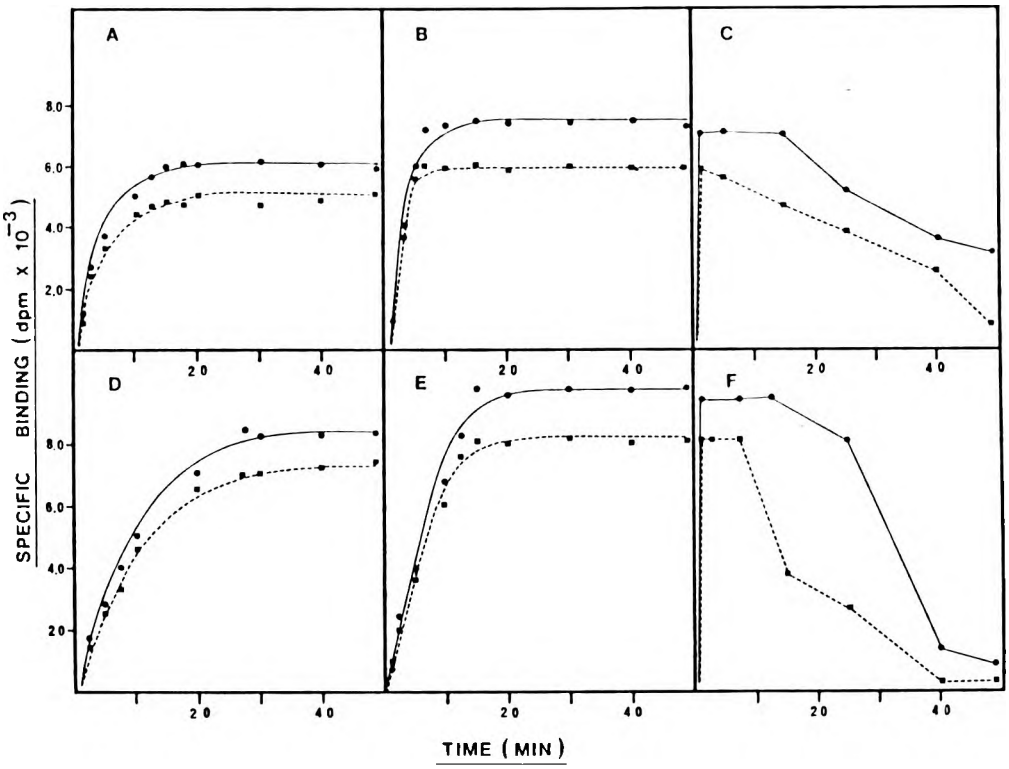


Figure 3.2 : Receptor ligand association as a function of time and temperature : the effect of sodium molybdate. Cytosols were prepared in the presence (●) or absence (■) of sodium molybdate, and incubated with tritiated ligand ($|^3H|$-E_2 or ^{3}H-R5020) to a final concentration of 5 nM with and without a 250 fold excess level of the appropriate unlabelled competitor (DES or R5020) at three different temperatures (0°C, 20°C and 37°C). Following the addition of the radioligands, aliquots (100µℓ) of the incubation mixtures were taken at determined time intervals and added to a DCC suspension (1 mℓ) on ice. Aliquots of 500µℓ of the supernatant of 2000xg'x10^3 were monitored for total and non-specific binding : (A,B,C) association of the VMU ER with ^{3}H-E_2 in the presence (CYT(+),●) or absence (CYT(-),■) of 10 mM sodium molybdate at 0°C(A), 20°C(B) and 37°C(C); (D,E,F) association of the VMU PR with ^{3}H-R5020 in the presence ((CYT(+),●) or absence ((CYT(-),■) of 10mM sodium molybdate at 0°C(D), 20°(E) and 37°C(F).

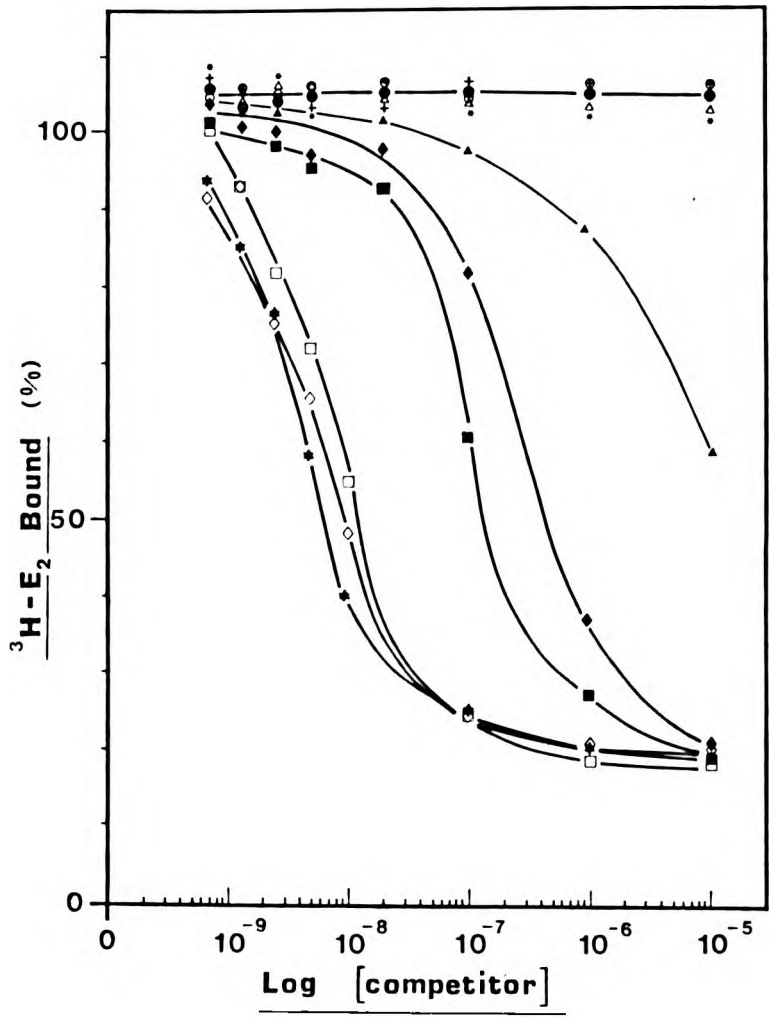


Figure 3.3 : Competition of various ligands for binding to the VMU ER. Aliquots (100µℓ) of freshly prepared cytosols were incubated with 50µℓ of labelled oestradiol (final concentration, 8 nM) in the presence of 50µℓ of a range of 8 different concentrations (0.169 nM -2.6µM) of a group of competitors : oestradiol (E_2,□), oestriol (E_3, ■), oestrone (E_1,◆) ethynyloestradiol (EE_2,◇), diethylstilbestrol (DES,✱), progesterone (P, +), promogestone (R5020,△), norethindrone (NE,▲), testosterone (T,○), dehydrotestosterone (DHT,●), dehydroepiandrosterone (DHEA),*) and hydrocortisone (HC,•) for 30 min at 20°C. Reactions were terminated by the addition of cold DCC (500µℓ) and subsequent incubation on ice for 10 min. Charcoal was pelleted by centrifugation (2000xg'x15 min) and aliquots (500µℓ) of the resulting supernatants counted for radio-activity.

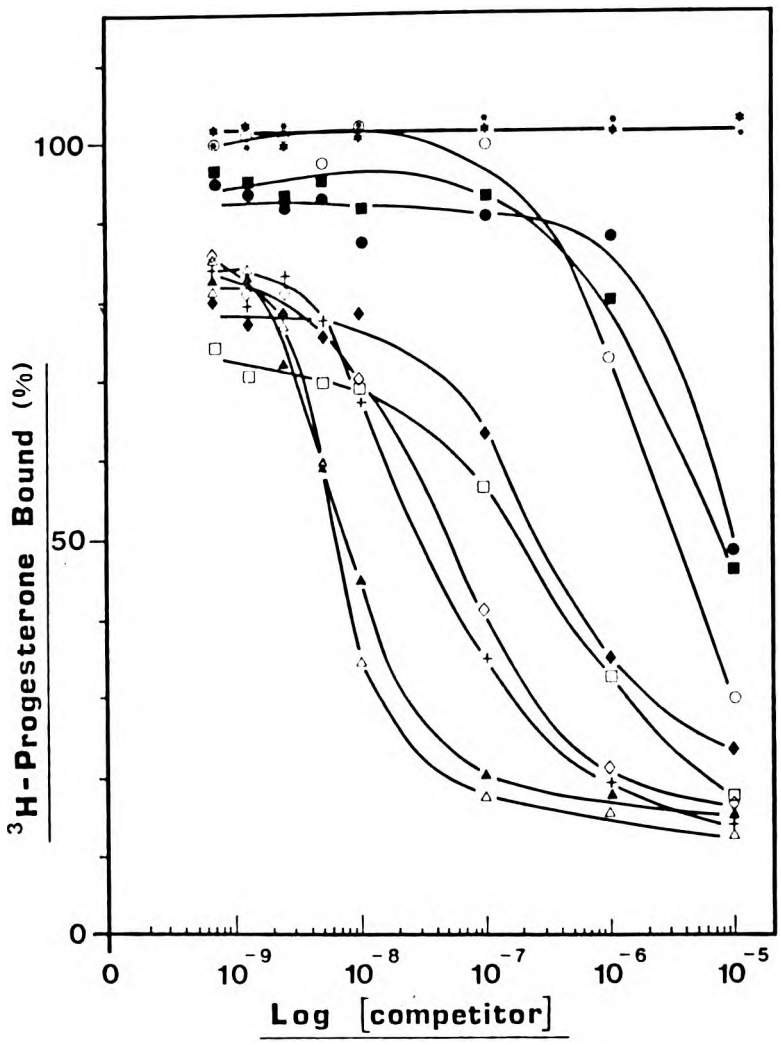


Figure 3.4 : Competition of various ligands for binding to the VMU PR. Aliquots (100µℓ) of freshly prepared cytosols were incubated with 50µℓ of labelled progesterone (final concentration, 8 nM) in the presence of 50µℓ of a increasing concentrations (0.169 nM -2.6µM) of a group of competitors : oestradiol (E_2,□), oestriol (E_3,■), oestrone (E_1,◆), ethynyloestradiol (EE_2,◇), diethylstilbestrol (DES,✦), progesterone (P, +), promogestone R5020,△), norethindrone (NE,▲), testosterone (T,O), dehydrotestosterone (DHT,●), dehydroepiandrosterone (DHEA),*) and hydrocortisone (HC,*) for 30 min at 20°C. Reactions were terminated by the addition of cold DCC (500µℓ) and subsequent incubation on ice for 10 min. Charcoal was pelleted by centrifugation (2000xg'x15 min) and aliquots (500µℓ) of the resulting supernatants counted for radioactivity.

TABLE 3.2

Relative Binding Affinities and Apparent Dissociation Constants(K_d) of various Ligands for ER and PR:

Ligand	ER		PR	
	RBA (%)	K_d (M)	RBA (%)	K_d (M)
DES	174.2	1.8×10^{-10}	< 0.1	-
EE_2	133.3	2.4×10^{-10}	99.4	2.6×10^{-9}
E_2	100.0	3.2×10^{-10}	15.9	2.0×10^{-8}
E_3	7.2	7.4×10^{-9}	0.7	4.0×10^{-7}
E_1	3.4	1.1×10^{-8}	13.0	1.5×10^{-8}
NE	0.1	2.6×10^{-6}	623.0	4.2×10^{-10}
P	< 0.1	-	100.0	2.4×10^{-9}
R5020	< 0.1	-	596.2	4.0×10^{-10}
T	< 0.1	-	1.9	1.6×10^{-7}
DHT	< 0.1	-	0.6	4.7×10^{-7}
DHEA	< 0.1	-	< 0.1	-
HC	< 0.1	-	< 0.1	-

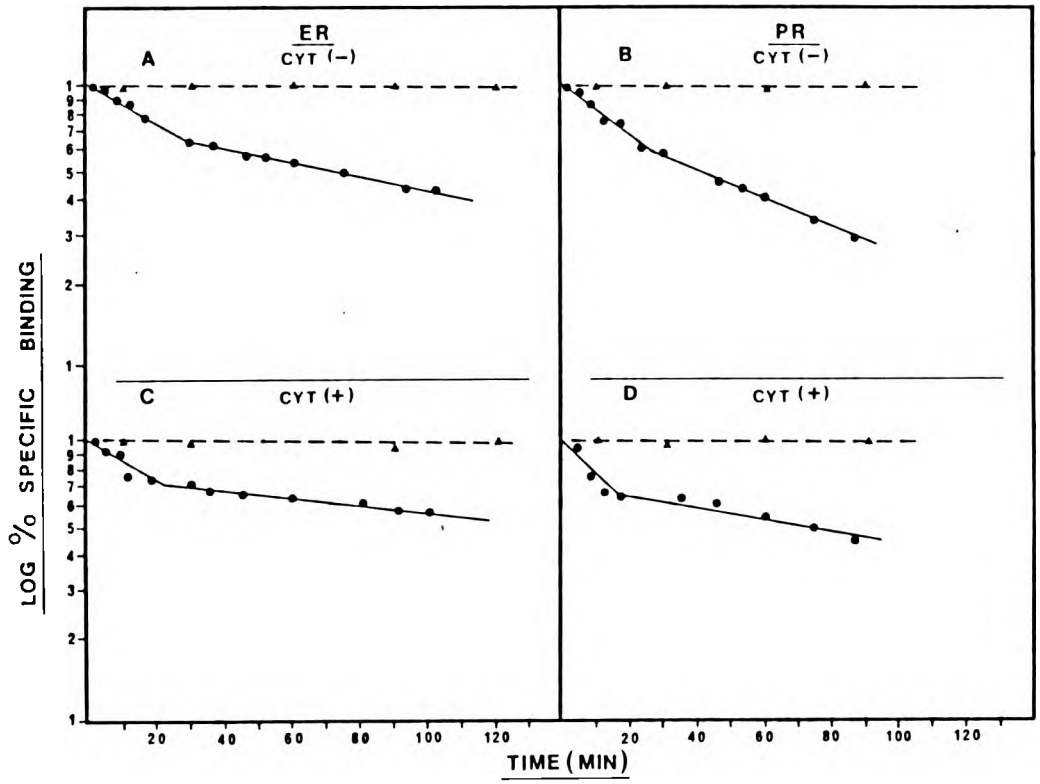


Figure 3.5 : Effect of sodium molybdate on the dissociation of VMU ER and PR.
Cytosols (CYT(+) and CYT(-)) were equilibrated with 5nM of the proper radioligand (^{3}H-E_2 or ^{3}H-5020). The dissociation was assayed at 20°C following the addition of 5µM(1000 fold excess) of unlabelled oestradiol or progesterone(●). Receptor inactivation was checked in a parallel incubation at 10°C with the addition of buffer containing no unlabelled oestradiol or progesterone(▲). At determined time intervals at 20°C, 100µℓ aliquots were removed and added to a DCC suspension (1 mℓ) on ice. Aliquots (500µℓ) of the resulting 2000xg' min supernatants were monitored for radioactivity. Each determination has been corrected for non-specific binding : dissociation of ER and PR in molybdate-free cytosols (panels A and B) or in cytosols prepared in the presence of 20mM molybdate (panels C and D).

TABLE 3.3 :

Effect of sodium molybdate on the dissociation of VMU ER and PR at 20°C.

Receptor	Rate constants of dissociation ($\times 10^{-3}$ min^{-1})			
	CYT(-)		CYT(+)	
	FDC	SDC	FDC	SDC
ER	7.02	2.08	8.55	1.41
PR	10.31	5.39	12.91	2.24

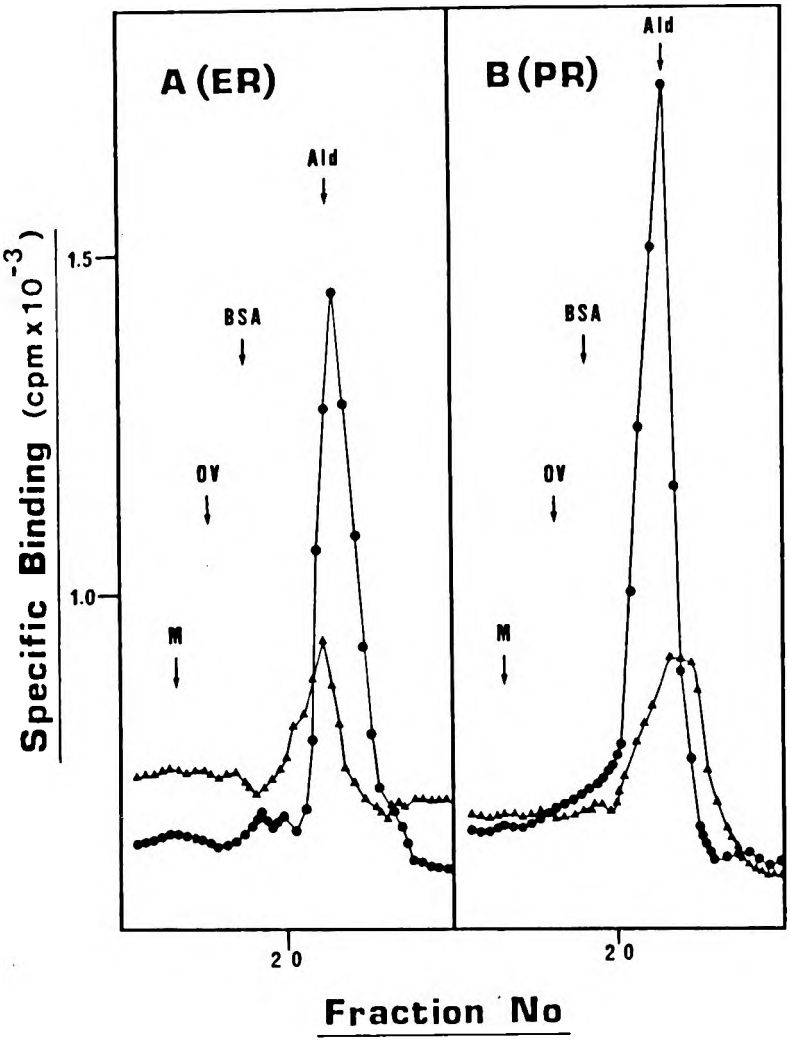


Figure 3.6 : Effect of sodium molybdate on the VMU ER (A) and PR(B) SDG profiles.
Cytosols containing (●) and non containing (▲) sodium molybdate were incubated at 20°C for 30 min with either ^{3}H-E_2 of ^{3}H-R5020. Parallel incubation mixtures also contained a 100 fold excess of either DES or R5020 respectively. Reactions were terminated by transferring the incubation mixtures to DCC pellets. The 2000xg' supernatants (200µℓ) were then layered onto hypotonic 10-35% sucrose density gradients. The cytosols containing molybdate were layered onto gradients containing molybdate ((GRAD(+)) while cytosols free of molybdate were layered onto gradients not containing sodium molybdate ((GRAD(-)). The gradients were subjected to ultracentrifugation (VTi-80 rotor, 520000xg'x120 min x 2°C), fractionated (40x5drop fractions) and the fractions counted in a scintillation counter, following the addition of 4 mℓ cocktail. Positions of the marker proteins are indicated by arrows : myoglobin (M, 2.03S), ovalbumin (Ov, 3.6S), bovine serum albumin (BSA, 4.6S) and aldolase (Ald 7.3S).

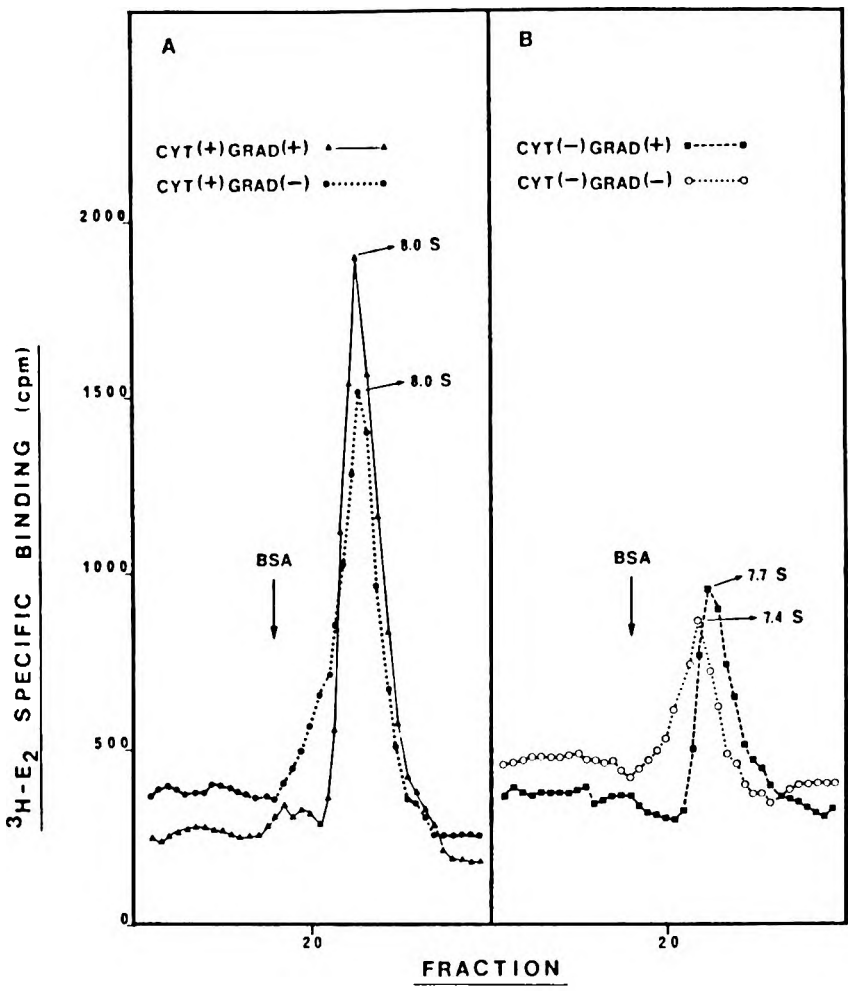


Figure 3.7 : Reversibility of the effect of sodium molybdate on the VMU ER as studied by sedimentation-dialysis experiments, monitored by SDG. Cytosols were homogenized in the presence (CYT(+)) (A) or absence (CYT(-)) (B) of sodium molybdate. The gradients were prepared containing (GRAD(+)) or not containing ((GRAD (-)) sodium molybdate. Aliquots of (200µℓ) incubation mixture were analysed on hypotonic 10-35% SDG gradients and were processed as described in section 3.2.7. Different combinations of conditions relative to the sodium molybdate status were employed: SDG profiles of the VMU ER analysed in (A)CYT(+) GRAD(+) (--), CYT(+) GRAD(-) conditions and (B) CYT(-) GRAD(+) or CYT(-) GRAD(-) conditions.

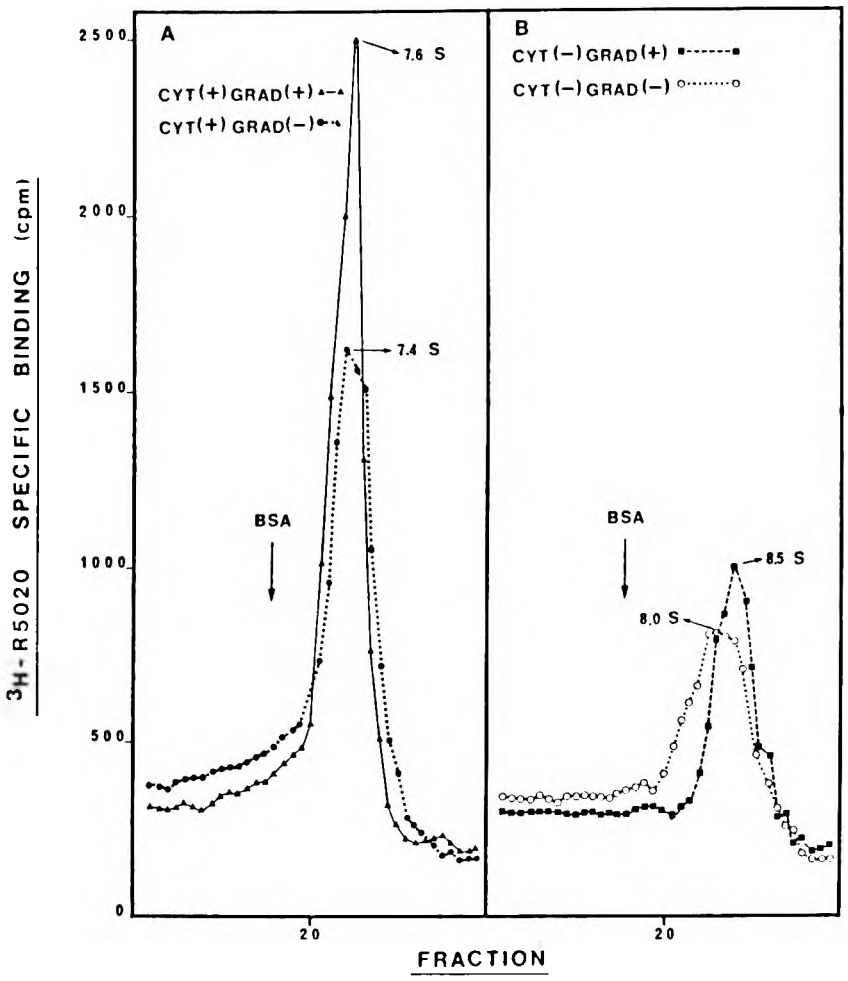


Figure 3.8 : Reversibility of the effect of sodium molybdate on the VMU PR as studied by sedimentation-dialysis experiments, monitored by SDG. Cytosols were homogenized in the presence (CYT(+)) (A) or absence (CYT(-)) (B) of sodium molybdate. The gradients were prepared containing (GRAD(+)) or not containing ((GRAD (-)) sodium molybdate. Aliquots of (200µℓ) incubation mixture were analysed on hypotonic 10-35% SDG gradients and were processed as described in section 3.2.7. Different combinations of conditions relative to the sodium molybdate status were employed: SDG profiles of the VMU ER analysed in (A)CYT(+) GRAD(+) (--), CYT(+) GRAD(-) conditions and (B) CYT(-) GRAD(+) or CYT(-) GRAD(-) conditions.

TABLE 3.4

Quantitative analysis of the effect of sodium molybdate on the stabilization of VMU ER and PR in a typical sedimentation-dialysis experiment

RECEPTOR	SPECIFIC BINDING IN ∿ 8 S (dpm/mg/protein)				% Inactivation			
	CYT(+)/GRAD(+) (1)	CYT(+)/GRAD(-) (2)	CYT(-)/GRAD(+) (3)	CYT(-)/GRAD(-) (4)	1 → 2	3 → 4	1 → 3	1 → 4
ER	6249	6465	4401	3069	-3.5	30.3	29.6	50.9
PR	17718	16429	11439	10221	7.3	10.7	27.4	35.2

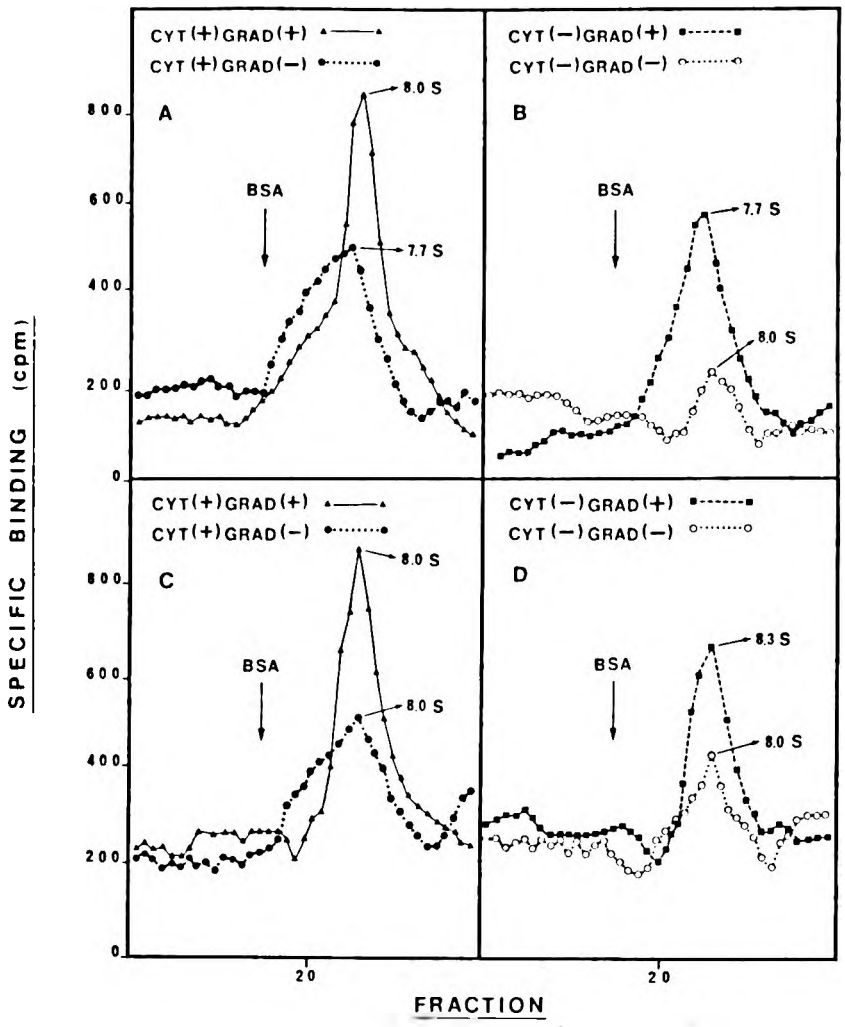


Figure 3.9 : Reversibility of the effect of sodium molybdate on the VMU ER and PR as studied by dialysis monitored in hypotonic 10-35%. Cytosols were homogenized in the presence (CYT(+)) or absence (CYT(-)) of sodium molybdate. The cytosols were dialysed in cellulose tubing (1.5 cm diameter) against a buffer with the same constitution, relative to sodium molybdate content as the homogenization buffer, or against a buffer with the opposite constitution. Following dialysis (4hx0-4°C) cytosols were labelled and processed as previously described and then finally analysed on hypotonic 10-35 % SDG, with the same molybdate status as the dialysis buffer : (A,B) hypotonic SDG profiles of the VMU ER obtained under the following conditions :(CYT(+)GRAD(+) (▲), CYT(+)GRAD(-) (●), and CYT(-)GRAD(+) (■) and CYT(-)GRAD(-)(O); (C,D) hypotonic SDG profiles of the VMU PR obtained under the following conditions : CYT(+)GRAD(+) (▲),CYT(+)GRAD(-)(●), CYT(-)GRAD(+) (■) and CYT(-)GRAD(-) (O).

TABLE 3.5

Effect of molybdate on receptor inactivation during the dialysis of cytosols (CYT(+) and CYT(-)) against buffers ($TEDAG_{10}$ and TEDAG-MO) and subsequent SDG.

Receptor	Molybdate status		Specific binding in ∿ 8 S peak (dpm/mg protein)	Inactivation (%)
	Cytosol	Buffer		
ER	+	+	3788	-
	+	-	3017	20.4
	-	-	2475	34.7
	-	+	3010	20.5
PR	+	+	10257	-
	+	-	7992	22.1
	-	-	3947	61.5
	-	+	7390	28.0

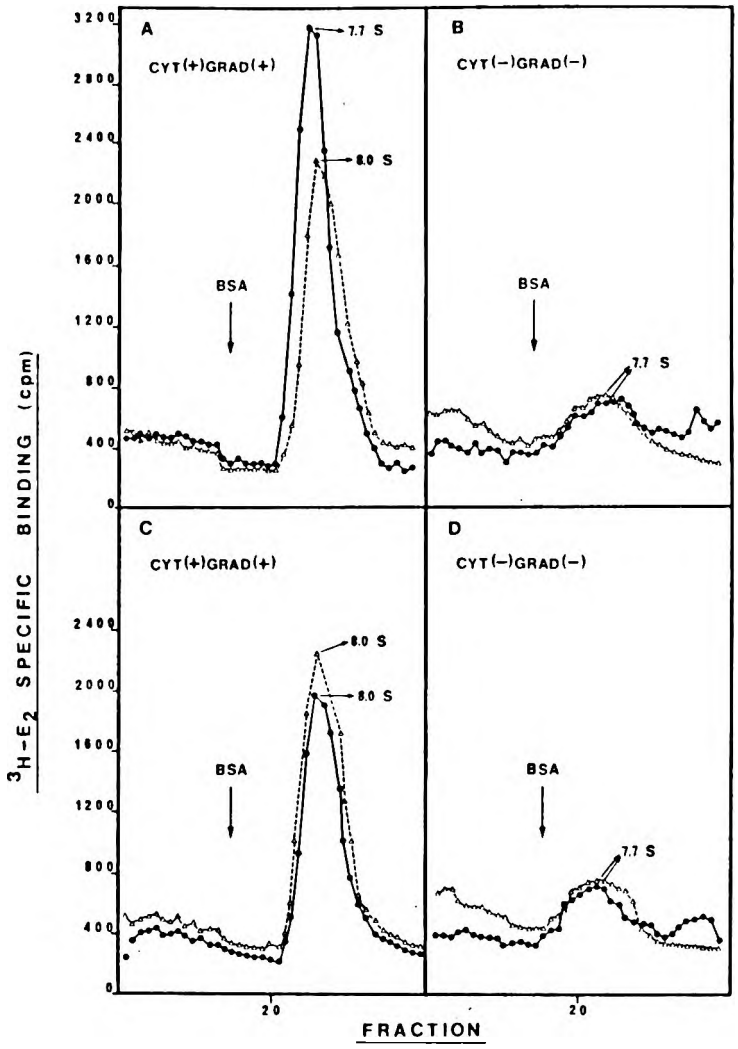


Figure 3.10: Influence of PMSF and Leu on sedimentation profiles of ER in CYT(+) and CYT(-) cytosols. Cytosols were prepared in the presence (●) or absence (Δ) of 2mM Leupeptin (A,B) and in the presence (●) or absence (Δ) of 1 mM PMSF (C,D). CYT(-) cytosols analysed on gradients not containing molybdate (GRAD(-) whereas CYT(+) cytosols were run on gradients containing molybdate GRAD(+). The position of the ^{14}C-labelled marker protein (BSA, 4.6S) is indicated by an arrow.

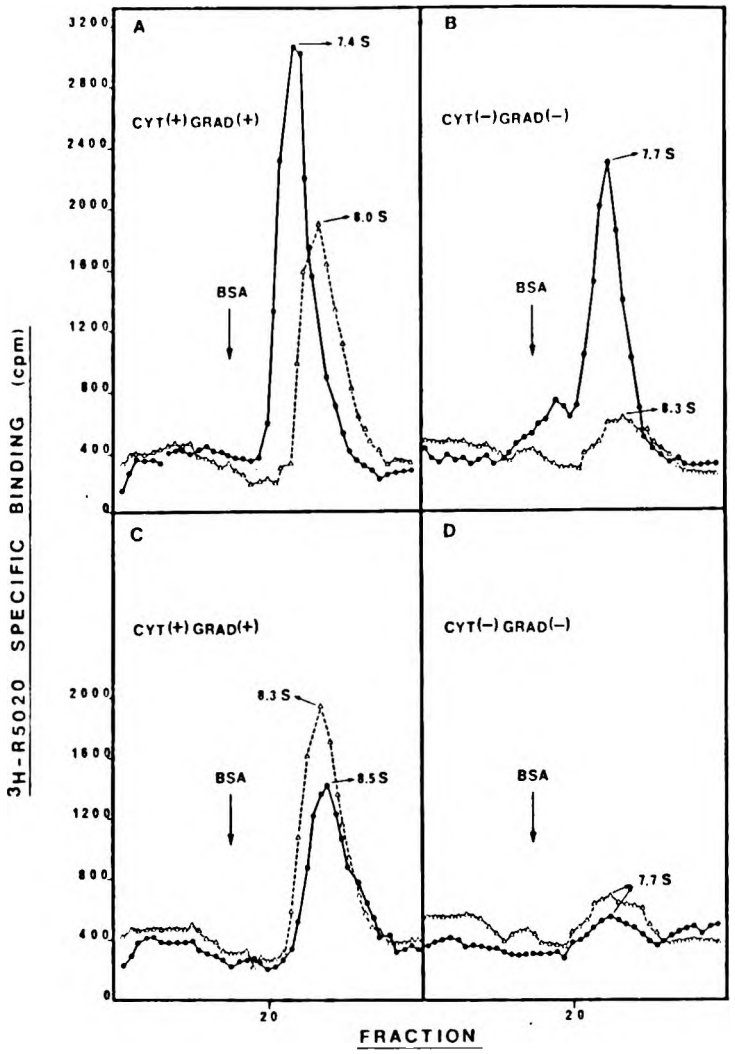


Figure 3.11 : Influence of PMSF and Leupeptin on sedimentation profiles of PR in CYT(+) and CYT(-) cytosols. Cytosols were prepared in the presence (●) or absence (Δ) of 2mM Leupeptin (A,B) and in the presence (●) or absence (Δ) of 1 mM PMSF (C,D). CYT(-) cytosols analysed on gradients not containing molybdate (GRAD(-)) whereas CYT(+) cytosols were run on gradients containing molybdate (GRAD(+)). Marker position (^{14}C-BSA; 4.65) is indicated by BSA.

TABLE 3.6

Effect of proteolytic inhibitors Leu and PMSF on the molybdate stabilization of VMU ER and PR during preparation of cytosols and SDG analysis.

Receptor	Molybdate status		Proteolytic inhibitor			
	Cytosol	Gradient	Leu (2mM)	PMSF (1mM)	Specific-binding in 8 S peak (dpm/mg prot)	Inactivation (%)
ER	+	+	-	-	16455	0
	-	-	-	-	9314	43.4
	+	+	+	-	19677	- 19.6
	-	-	+	-	9582	41.8
	+	+	-	+	13292	19.2
	-	-	-	+	8506	48.3
PR	+	+	-	-	16006	0
	-	-	-	-	9861	38.4
	+	+	+	-	19919	- 24.4
	-	-	+	-	17667	- 10.4
	+	+	-	+	12765	20.2
	-	-	-	+	7961	50.3

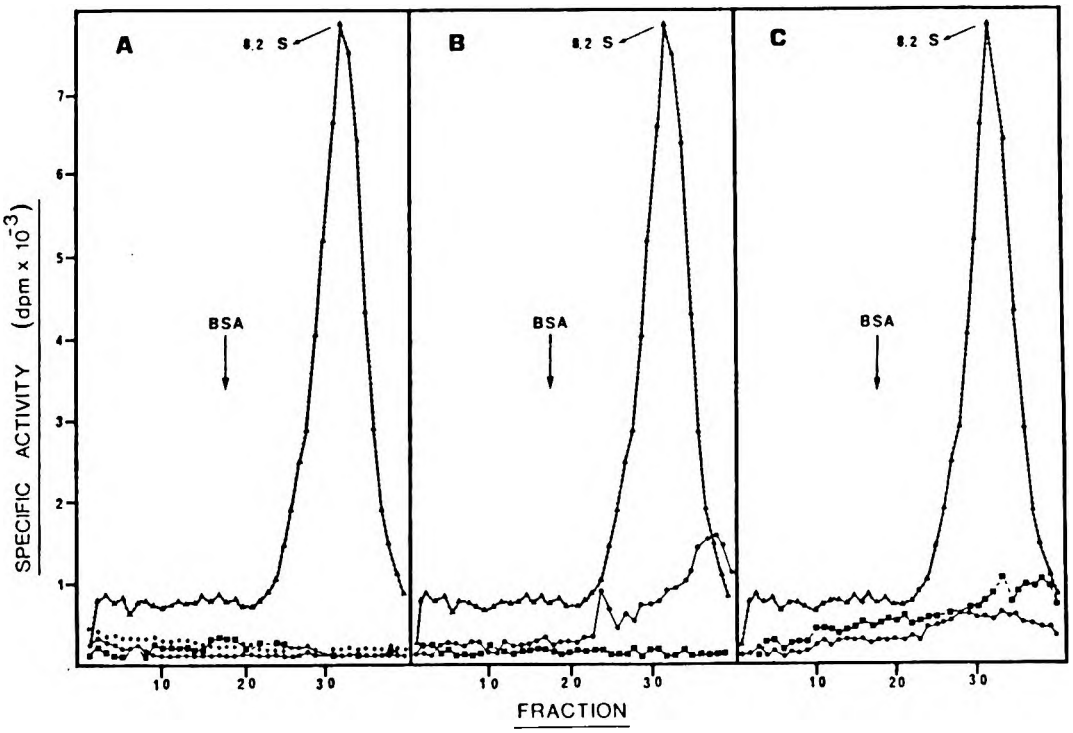


Figure 3.12 : Effect of proteolytic inhibitors and thiol reactive agents on VMU ER SDG profiles.
VMU were sliced up and thin slices were homogenized in 1.5 mℓ of $TEDAG_{10}$ buffer containing the inhibitor at the following final concentrations : panel A: DTNB (•)10mM, NEM (■) 5 mM and IAA (•) 10mM. Panel B : Leupeptin (•) 2mM and TLCK (■) 5mM. Panel C : PMSF (■) 5mM and O-Phen (•) 5mM. The cytosols were subjected to SDG analysis. Cytosols were incubated with ^3H-E_2 in the presence or absence of 250 fold excess of cold ligand. The DCC treated supernatant (2000xg'x10 min) was layered (200µℓ) on top of linear 10-35% hypotonic SDG, and centrifugation was executed at VTi-80 (520000xg' for 2 h. Five drop fractions were collected and the fractions were monitored for radioactivity.

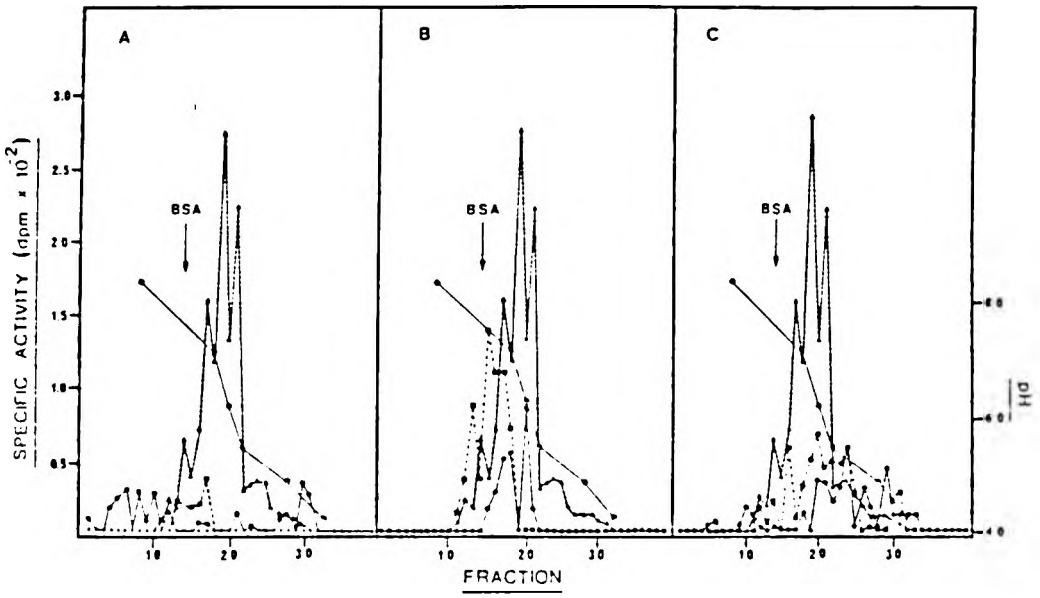


Figure 3.13 : Effect of proteolytic inhibitors and thiol reactive agents on VMU ER IEF profiles.
VMU were sliced up and thin slices were homogenized in 1.5 mℓ of $TEDAG_{10}$ buffer containing the inhibitor at the following final concentrations : panel A: DTNB (●)10mM, NEM (■) 5 mM and IAA (*) 10mM. Panel B : Leupeptin (●) 2mM and TLCK (■) 5mM. Panel C : PMSF (●) 5mM and O-Phen (■) 5mM. The cytosols were subjected to IEF analysis. Cytosols were incubated with ^3H-E_2 in the presence or absence of 250 fold excess of cold ligand. The DCC treated supernatant (2000xg'x10 min) was applied (40µℓ) to the gels on small rectangles of filter paper and ran for 2 hours in the conditions described in Section 3.2.10.
Controls are indicated by ▲.

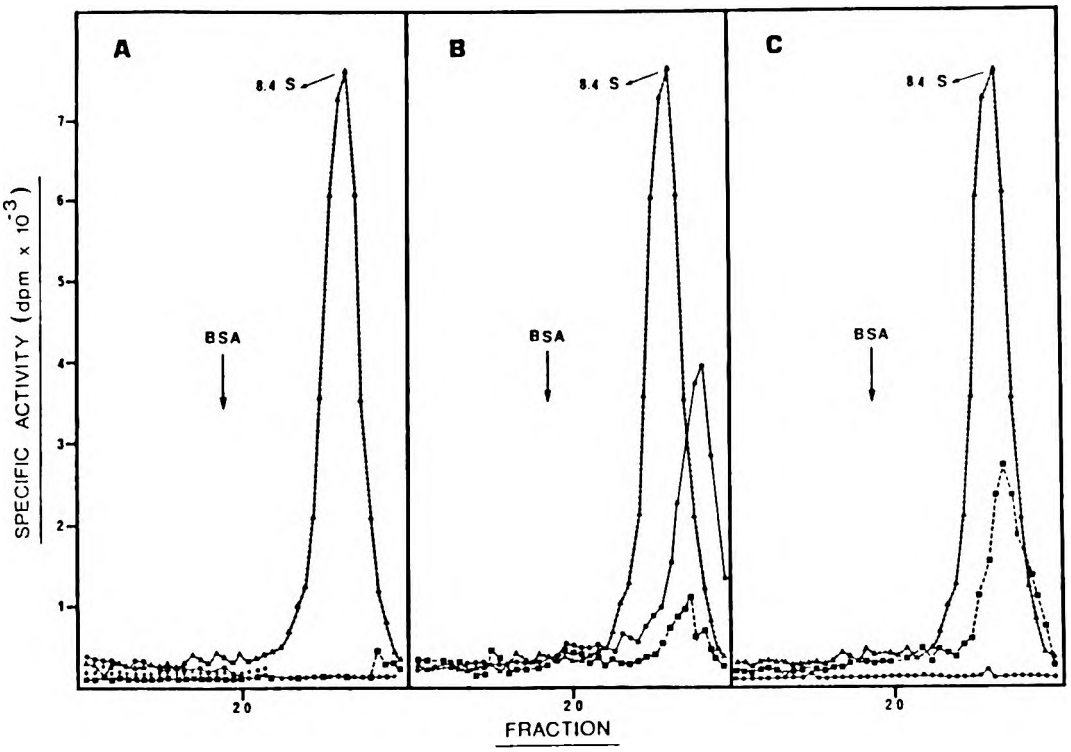


Figure 3.14 : Effect of proteolytic inhibitors and thiol reactive agents on VMU PR SDG profiles.
VMU were sliced up and thin slices were homogenized in 1.5 mℓ of $TEDAG_{10}$ buffer containing the inhibitor at the following final concentrations : panel A: DTNB (●)10mM, NEM (■) 5 mM and IAA (*) 10mM. Panel B : Leupeptin (●) 2mM and TLCK (■) 5mM. Panel C : PMSF (●) 5mM and O-Phen (■) 5mM. The cytosols were subjected to SDG analysis. Cytosols were incubated with R-5020 in the presence or absence of 250 fold excess of cold ligand. The DCC treated supernatant (2000xg'x10 min) was layered (200µℓ) on top of linear 10-35% hypotonic SDG, and centrifugation was executed at VTi-80 (520000xg' for 2 h. Five drop fractions were collected and the fractions were monitored for radioactivity.

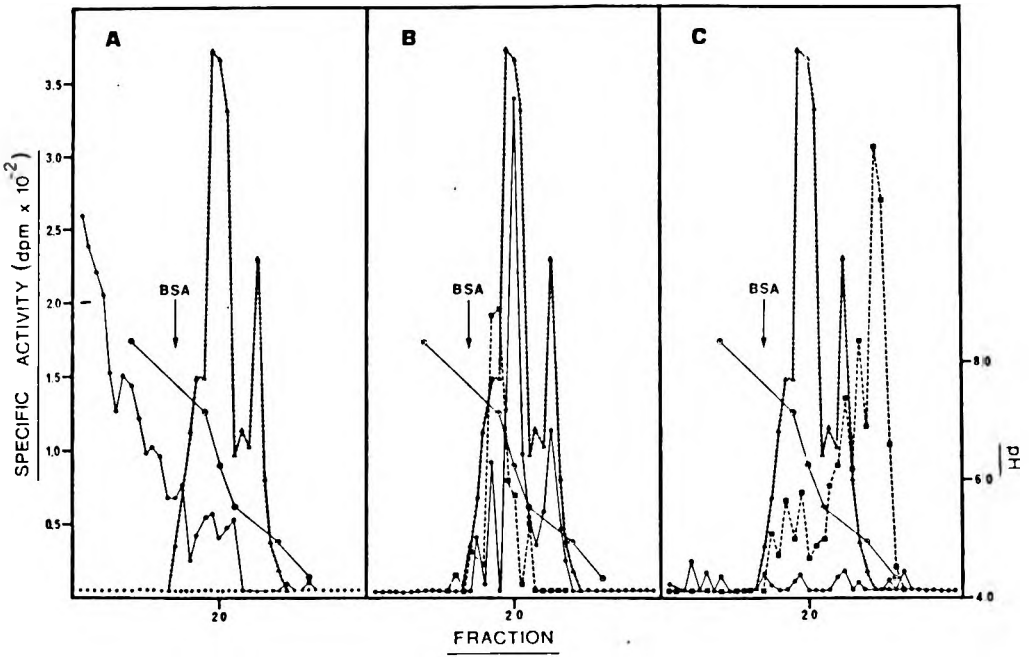


Figure 3.15 : Effect of proteolytic inhibitors and thiol reactive agents on VMU PR IEF profiles.
VMU were sliced up and thin slices were homogenized in 1.5 mℓ of TEDAG$_{10}$ buffer containing the inhibitor at the following final concentrations : panel A: DTNB (●)10mM, NEM (■) 5 mM and IAA (*) 10mM. Panel B : Leupeptin (●) 2mM and TLCK (■) 5mM. Panel C : PMSF (●) 5mM and O-Phen (▲) 5mM. The cytosols were subjected to IEF analysis. Cytosols were incubated with ^{3}H-E_3 in the presence or absence of 250 fold excess of cold ligand. The DCC treated supernatant (2000xg'x10 min) was applied (40μℓ) to the gels on small rectangles of filter paper and ran for 2 hours in the conditions described in Section 3.2.10.

TABLE 3.7

Effect of proteolytic inhibitors and thiol reactive agents on the ligand binding capacity of VMU ER and PR.

Inhibitor	Binding capacity (fmol/mg protein)		% inactivation	
	ER	PR	ER	PR
None	195	387	0	0
O-Phen	138	188	29.2	51.4
PMSF	21	54	89.2	86.1
DTNB	9	23	95.4	94.1
IAA	10	24	94.9	93.8
Leu	109	300	44.1	22.5
NEM	36	89	81.5	77.0
TLCK	35	129	82.1	66.7

TABLE 3.8

ffect of proteolytic and thiol inhibitors on the pI values and SDG profiles of VMU ER and PR

nhibitor	ER		PR	
	Isoelectric points (pI)	Sedimentation coefficients (Svedbergs)	Isoelectric points (pI)	Sedimentation coefficients (Svedbergs)
one	6.6, 5.8	8.2	6.5, 5.0	8.4
-Phen	6.5	8.4	5.2, 4.6	8.7
MSF	5.1	8.4	n.p.	8.2
TNB	4.5	n.p.	6.5	n.p.
AA	n.p.	n.p.	n.p.	n.p.
eu	6.1, 7.0	6.1, 9.9	6.1	9.5
EM	7.2	n.p.	n.p.	n.p.
LCK	7.2	n.p.	7.0.	8.9

n.p. = no peaks; peak surface area severely reduced)

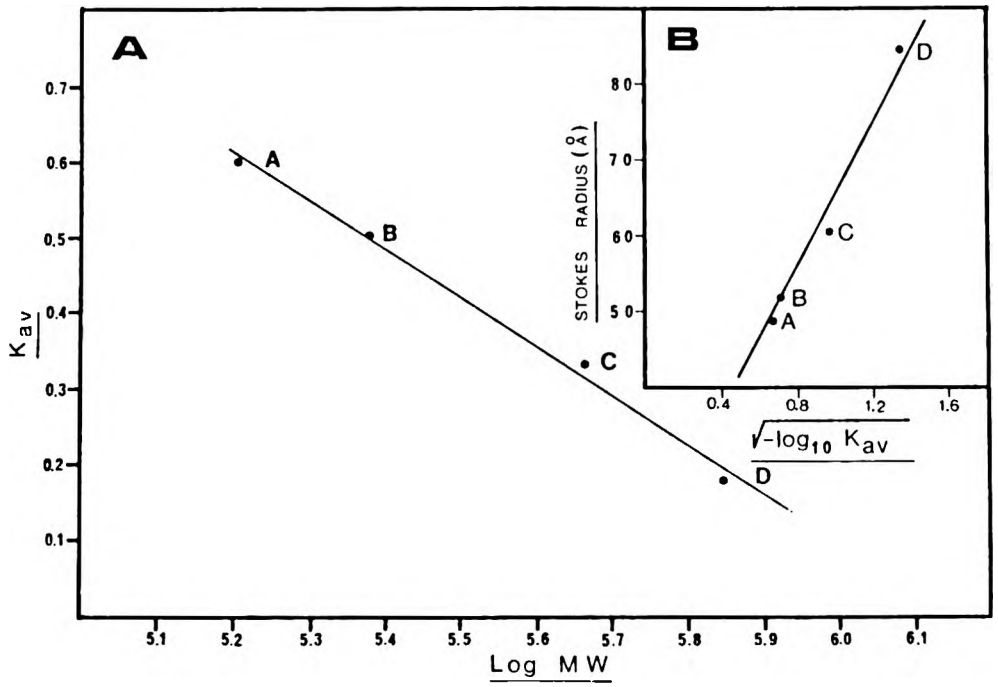


Figure 3.16 (A) A calibration curve for the molecular mass determination of the VMU ER on ultrogel AcA-22 columns. The standard proteins used were : Catalase (MW=232000 daltons) thyroglobulin (MW=669000), aldolase (MW=158000 daltons) and ferritin MW=440000 daltons).

(B) A calibration curve for the determination of the Stokes radius (r*) of the VMU ER on an Ultrogel AcA-22 column. Standard proteins employed were catalase (r*=52.2A), thyroglobulin (r*=85.0A), aldolase (r*=.1A) and ferritin (r*=61.0A).

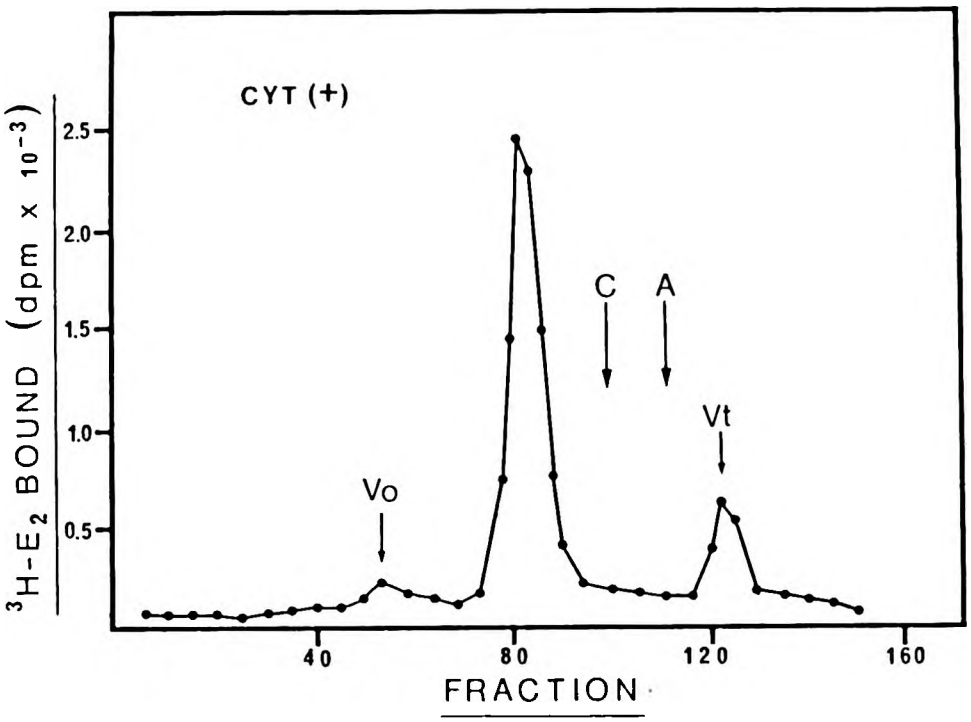


Figure 3.17 : A representative column elution profile obtained with size exclusion chromatography on an ultrogel AcA-22 column. Cytosols were incubated in the presence of 20mM sodium molybdate (CYT(+)). Incubation with ^{3}H-E$_2$ was executed at 20°Cx30min. Incubation with 250 fold excess of unlabelled ligand (for NSB) was performed simultaneously. The supernatant (500µl) of 2000xg'x10 min was layered on the column. The mobile phase was TEDAG buffer and the flow rate was 4.9 mℓ/cm. Marker proteins used were catalase (C) and aldolase (A).

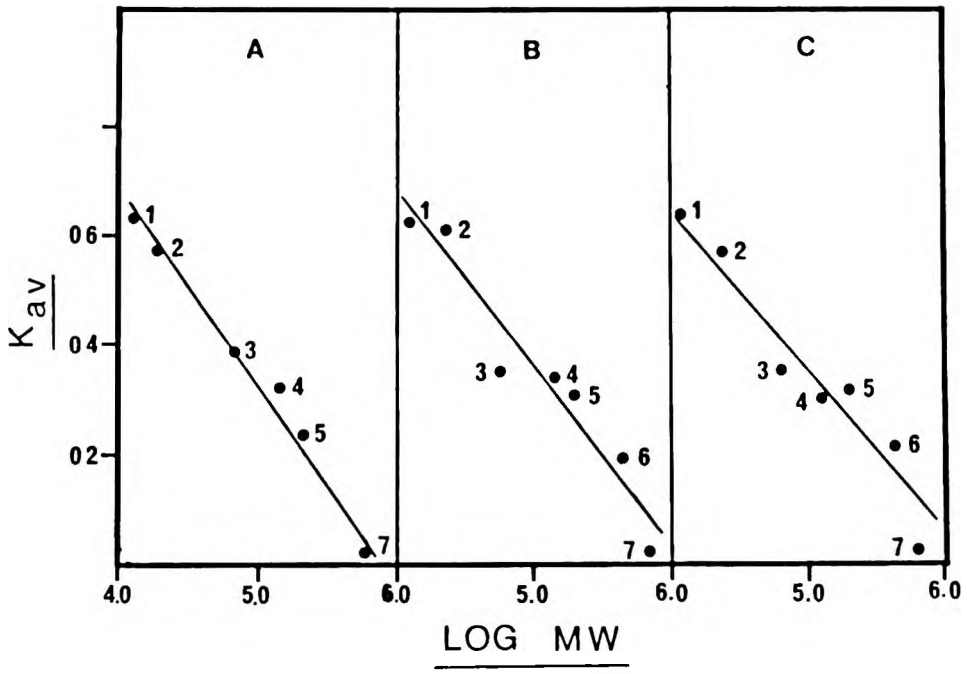


Figure 3.18 : Representative calibration curve for the Waters I-250 HPLC gel column. The different marker proteins used were : ribonuclease (1), chymotrypsin (2), albumin (3), aldolase (4), catalase (5), ferritin (6) and thyroglobulin (7). The column was eluted with different mobile phases : PEG buffer (A), $PEGK_{100}$, buffer (B) and $PEGK_{400}$ buffer (C)

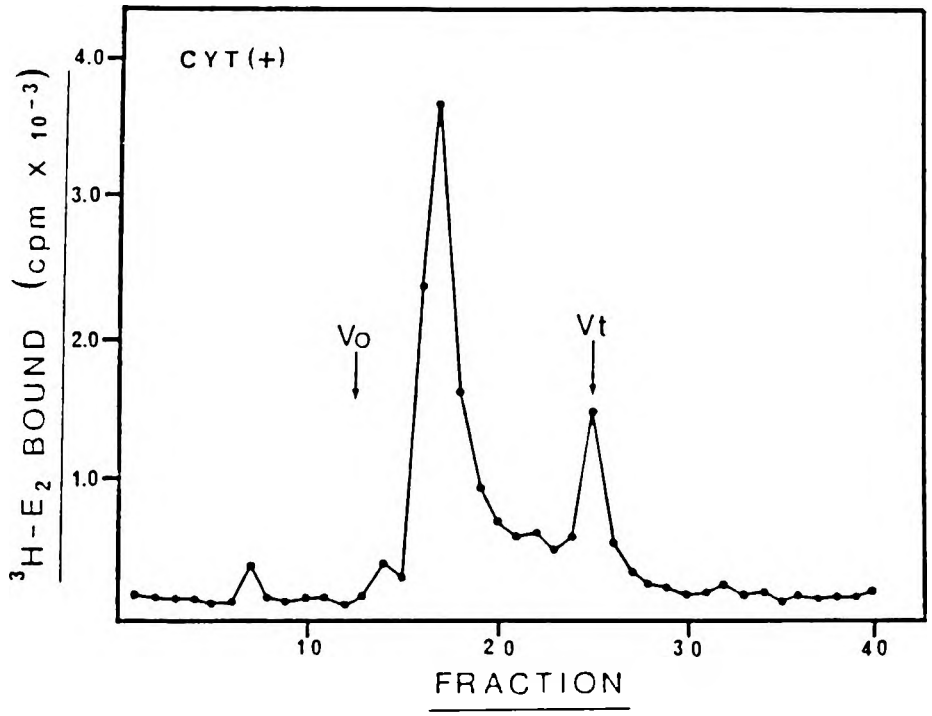


Figure 3.19 : Representative size exclusion chromatography profile of VMU ER on Waters I-250 column. Cytosols were prepared in the presence of sodium molybdate and labelled with ^{3}H-E_2 (30 minx20°C). The DCC treated supernatant was injected onto the column. A run was also executed employing a 250 fold excess unlabelled DES. Columns were calibrated with a set of unlabelled marker proteins (as indicated in Fig. 3.18). The mobile phase was PEGK buffer and the flow rate was 1ml/min.

TABLE 3.9

Molecular weights and Stokes radii of VMU ER calculated from column data:

Cytosol	Mobile phase	MW ($\times 10^{-5}$ daltons)	r* (Å)	Receptor class (Sherman et al (213))
CYT(-)	$TEDAG_{10}$+0.4 MKCl	*0.92±0.21	42.1±1.3	III
CYT(-)	$TEDAG_{10}$+0.15 MKCl	0.86±0.3	41.8±1.5	III
CYT(+)	$TEDAG_{10}$+20mM Na_2MoO_4	3.83±0.46	60.5±2.6	I
CYT(-)	$TEDAG_{10}$	4.68±0.35	63.2±2.5	I

(* r=Stokes radius; ± S.E.M. = standard error of the mean; mean of 3 assays)

TABLE 3.10

Summary of data on molecular weight of ER using different conditions on Waters I-250 HPLC gel column.

Cytosol	Mobile phase	Molecular mass ($\times 10^5$ daltons)		Calibration curve
		Peak 1	Peak 2	
CYT(+)	PEG^M	-	2.006	1
CYT(-)	PEG	4.82	2.006	1
CYT(-)	$PEGK_{100}$	-	2.113	1
CYT(-)	$PEGK_{100}$	-	1.204	2
CYT(-)	$PEGK°_{400}$	7.196	-	1
CYT(-)	$PEGK°_{400}$	8.556	-	3
CYT(+)	$PEGK°_{400}$	-	1.252	1
CYT(+)	$PEGK°_{400}$	-	1.104	3
CYT(-)	$PEGK^{30}_{400}$	7.196	-	1
CYT(-)	$PEGK^{30}_{400}$	3.556	-	3
CYT(+)	$PEGK^{30}_{400}$	-	1.526	1
CYT(+)	$PEGK^{30}_{400}$	-	1.104	3

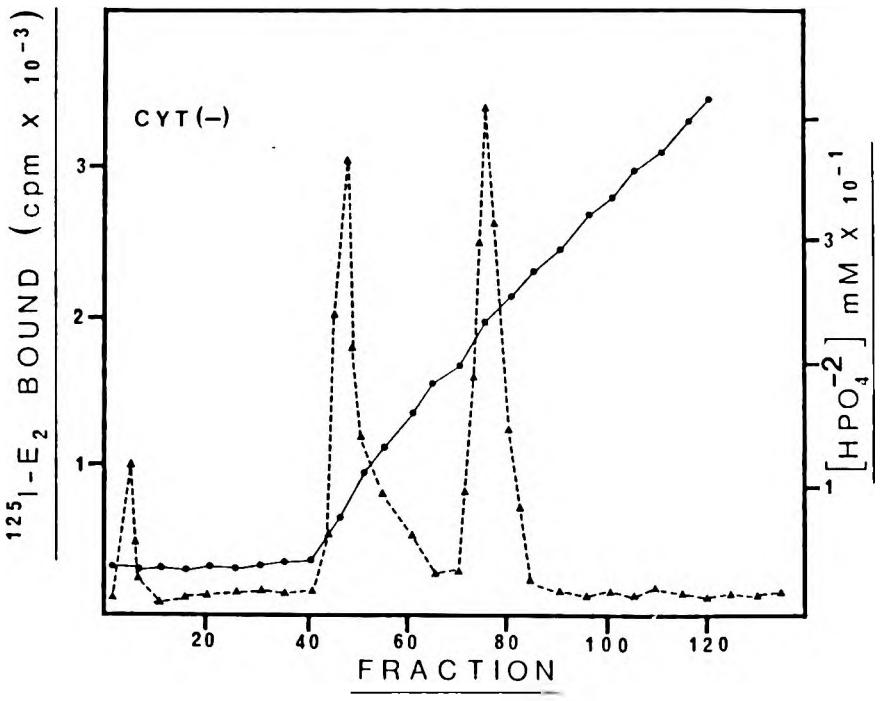


Figure 3.20 : HPLC Ion exchange chromatography of Vervet monkey uterine ER on an AX-1000 column. Cytosols free of sodium molybdate CYT(-) were charged by incubation with ^{125}I-E_2 for 18-24h at 0-4°C in the presence or absence of a 250 fold excess DES. Unbound ligand was removed by DCC and the 2000xg' supernatants applied to the columns. Columns were equilibrated with 25 mM Tris buffer (pH 8.2) and developed with a limiting buffer containing 500 mM phosphate. Flow rate was 1 mℓ/min and 1 mℓ fractions were collected.

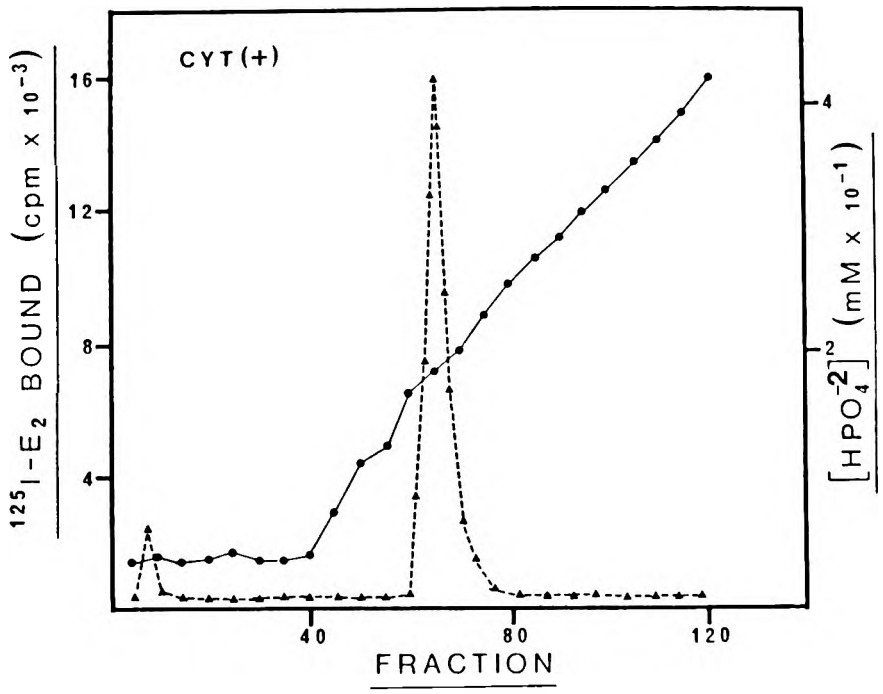


Figure 3.21 : HPLC Ion exchange chromatography of vervet monkey uterine ER on an AX-1000 column. The effect of sodium molybdate on the ionic properties is depicted in the chromatograms above. Cytosols containing sodium molybdate CYT(+) were charged by incubation with ^{125}I-E_2 for 18-24h at 0-4°C in the presence or absence of a 250 fold excess DES. Unbound ligand was removed by DCC and the 2000xg' supernatants applied to the columns. Columns were equilibrated with 25 mM Tris buffer (pH 8.2) and developed with a limiting buffer containing 500 mM phosphate. Flow rate was 1 mℓ/min and 1 mℓ fractions were collected.

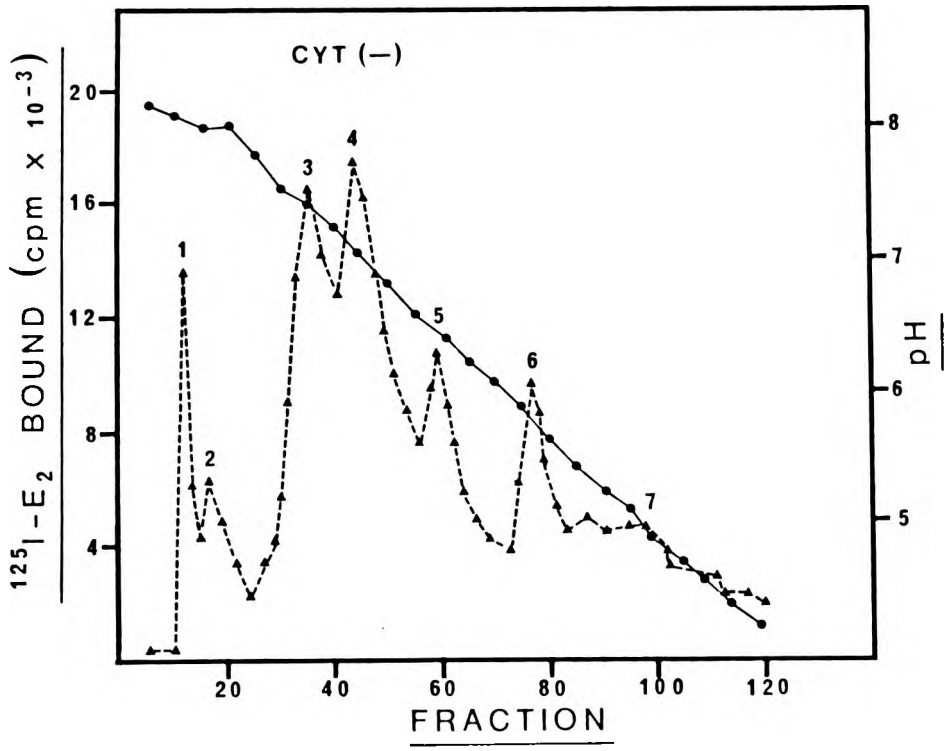


Figure 3.22 : HPLC chromatofocussing of Vervet monkey ER on an Ax-500 column. The absence of molybdate on the ionic properties of the receptor molecules is illustrated. Cytosols were prepared in the absence of sodium molybdate. Aliquots of cytosols were incubated with ^{125}I-E$_2$ with or without unlabelled DES (250 fold excess) for 18-24h at 0-4°C, treated with DCC and the 2000xg' supernatants injected onto the column. Prior to sample application the columns were equilibrated with a 25 mM Tris buffer (pH) 8.4) until the column eluate had a pH of 8.2 to 8.4.Columns were developed by eluting with a 30:70 mixture of Polybuffers (Pharmacia) 96 and 74 respectively (dilution 1:15 with 20% glycerol). The pH of the Polybuffer mixture was adjusted to 4.5. Flow rate was 1 mℓ/min and the pH of every second fraction determined.

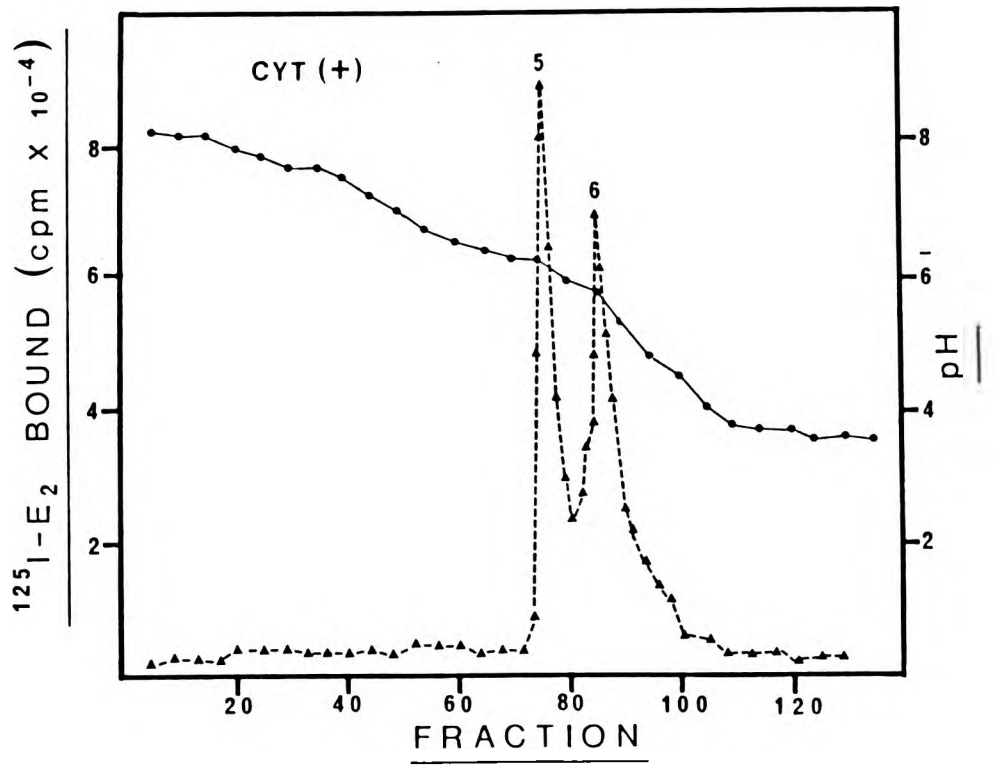


Figure 3.23 : HPLC chromatofocussing of Vervet monkey ER on an Ax-500 column. The effect of molybdate on the ionic properties of the receptor molecules is illustrated. Cytosols were prepared in the presence of sodium molybdate. Aliquots of cytosols were incubated with ^{125}I-E_2 with or without unlabelled DES (250 fold excess) for 18-24h at 0-4°C, treated with DCC and the 2000xg' supernatants injected onto the column. Prior to sample application the columns were equilibrated with a 25 mM Tris buffer (pH) 8.4) until the column eluate had a pH of 8.2 to 8.4.Columns were developed by eluting with a 30:70 mixture of Polybuffers (Pharmacia) 96 and 74 respectively (dilution 1:15 with 20% glycerol). The pH of the Polybuffer mixture was adjusted to 4.5. Flow rate was 1 mℓ/min and the pH of every second fraction determined.

TABLE 3.11

A summary of ER species obtained, following HPLC chromatofocussing of CYT(-) and CYT(+) cytosols on an AX-500 polyamine column

Peak	Isoelectric points	
	CYT(-)	CYT(+)
1	8.2	-
2	8.0	-
3	7.4	-
4	7.1	-
5	6.5	6.1
6	5.8	5.5
7	4.5-5.4	-

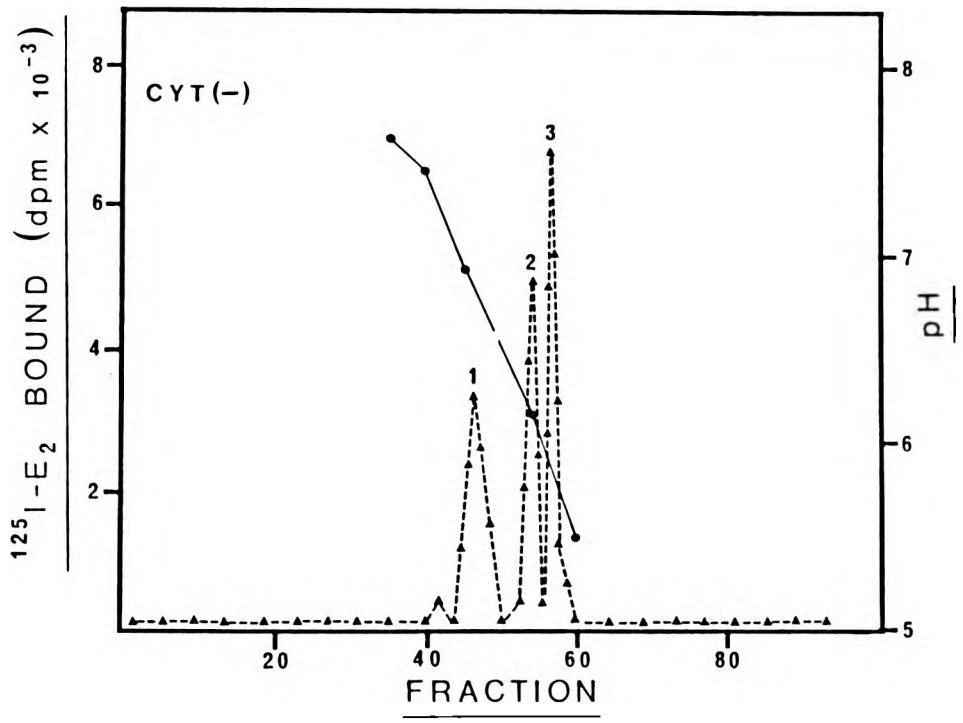


Figure 3.24 : Iso-electric focussing on Agarose gels of the Vervet monkey uterine ER. Ampholine range employed, was pH 5-8. Cytosols prepared in the absence of molybdate (CYT(-)) were incubated with ^{125}I-E_2 with or without unlabelled DES (250 fold excess) for 18-24h at 0-4°C. Reactions were stopped by treatment with DCC and the 2000xg' supernatants applied to the gels (power, 15W) the gels were cut manually into 1 mm slices and the slices placed into counting vials and the radio-activity monitored. The pH gradient of the gels was calibrated employing standard proteins : lactoglobulin (pI 5.5-5.5), myoglobin (horse, pI 7.4,7.0), carbonic anhydrase (pI 6.1), myoglobin (sperm whale, pI 7.7, 8.2).

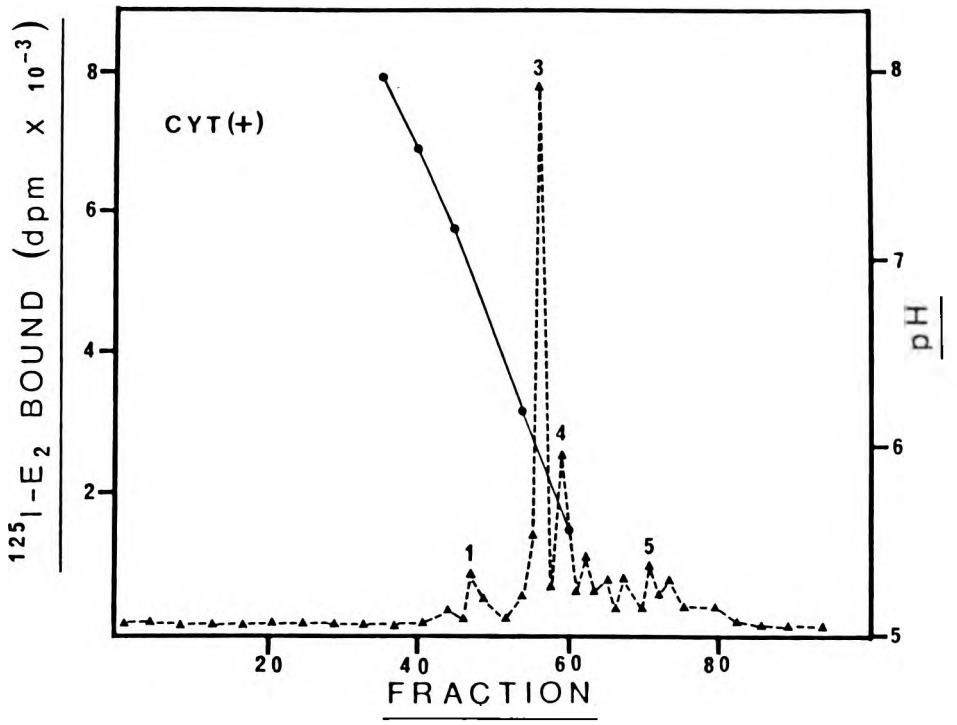


Figure 3.25 : Iso-electric focussing on Agarose gels of the Vervet monkey uterine ER. Ampholine range employed, was pH 5-8. The effect of sodium molybdate on the molecular properties of the ER is depicted in the figures above. Cytosols prepared in molybdate (CYT(+) were incubated with ^{125}I-E$_2$ with or without unlabelled DES (250 fold excess) for 18-24h at 0-4°C. Reactions were stopped by treatment with DCC and the 2000xg' supernatants applied to the gels. Following the development of the gels (power, 15W) the gels were cut manually into 1 mm slices and the slices placed into counting vials and the radio-activity monitored. The pH gradient of the gels was calibrated employing standard proteins : lactoglobulin (pI 5.5-5.5), myoglobin (horse, pI 7.4,7.0), carbonic anhydrase (pI 6.1), myoglobin (sperm whale, pI 7.7, 8.2).

TABLE 3.12

A sunmmary of ER species obtained, following iso-electric focussing on agarose gels (pH range : 5-8) in the absence and presence of sodium molybdate.

	CYT(-)		CYT(+)	
Peak	pI	*%B	pI	%B
1	6.8	4.07	6.8	16.6
2	6.2	30.3	-	-
3	5.9	28.9	5.9	39.9
4	-	-	5.6	13.0
5	-	-	5.0-5.5	30.5

(*%B = % specific binding in peak)

CHAPTER 4

EFFECT OF KCl ON SEDIMENTATION PROPERTIES OF THE VERVET MONKEY UTERINE OESTROGEN AND PROGESTERONE RECEPTORS.

4.1 INTRODUCTION:

Apart from general characteristics of the process of hormone receptor interaction as discussed in Chapters 1 and 3, there are several features that the different hormone receptor systems have in common. First, many of the receptors studied seem to possess a highly similar molecular architecture (243). Secondly, there seems to be remarkable similarity in the effects of certain chemical agents (e.g.) sodium molybdate, proteolytic inhibitors, sulfhydryl active chemicals on steroid receptor structure and function (232,235-237). Thirdly, the receptors appear to dissociate into smaller subunits in hypertonic media (25,238).

These common effects, would seem to imply that, apart from the obvious similarities in overall size and shape, there exists some common architecture in the subunit organization. The effect of 0.4 m KCl on the mechanism of action of female sex hormones was first studied in detail by Jensen & De Sombre (239). These workers demonstrated that the radioactive oestradiol taken up by the immature rat uteri, was associated with a different form of the receptor entity in the cytosol than in the nucleus. Upon ultracentrifugation in low ionic strength sucrose density gradients, the cytosolic complex sediments as a discrete band displaying a sedimentation coefficient originally believed to be 9,5 S (23), but later proved to be $\sim$ 8 S (241).

In the presence of sodium or potassium chloride, at concentrations of 0.2 M or higher, the $\sim$ 8 S complex is transformed into a more slowly sedimenting entity, with a sedimentation

coefficient close to that of bovine serum albumin (215). This "transformation" was thought to consist of a process of dissociation from the ∿ 8 S to a ∿ 4 S entity and was claimed to be fully reversible.

The effect of hypertonic media on the receptors was studied in more detail by Stanley and Korenmann (242), who demonstrated that oestrogen binding activity in the cytosolic fraction of rabbit and rat uteri underwent a reversible dissociation in 0.3 M KCl. Erdos (27) studied the state of aggregation of the receptor-oestradiol-complex as a function of the ionic strength. The labelled cytosol was incubated overnight in the presence of different concentrations of KCl and analysed on sucrose gradients containing the corresponding amounts of KCl. Up to 0.05 M the position and pattern of the radioactive peak remains unchanged, i.e. the sedimentation constant is still ∿ 8 S. Between 0.3 - 1.0 M KCl the radio-active complex displayed a sedimentation constant of 5 S. Incubation with KCl does not change the amount of ^{3}H-oestradiol bound by the receptor, and in fact the results are identical whether the extract is labelled before, simultaneously or after addition of KCl. NaCl was found to have the same effect as KCl.

Wittliff et al (13) reported that specific oestrogen binding components in cytosols of lactating rat mammary gland sedimented at 8.6S under conditions of low ionic strength and at 4.6S on sucrose gradients containing 0.4 M KCl (238). No loss in bound radioactivity under these conditions occurred, indicating that conversion of protein-associated steroid was involved. According to Jensen & De Sombre (243) both the ∿ 8 and ∿ 4 S complexes sediment at ∿ 8 S in low salt sucrose gradients, but they can be readily distinguished in the presence of 0.3 M KCl which reversibly dissociates the ∿ 8 S complexes into steroid-binding subunits. The cytosol complex sediments at 3.8 S and the nuclear complex sediments at 5.2 S. These two subunit complexes are usually referred

to as the 4 S and 5 S complexes, respectively. In their classical twostep model for steroid hormone action, presented in Fig. 4.a Jensen and De Sombre (243) gave an explanation for the different roles, played by various hormone receptor species, observed in low and high ionic strength buffers.

In this model the predominant oestradiol-receptor complex is not derived from a nuclear receptor protein, but originated from an extranuclear complex, which was translocated to the nuclear compartment, following a "temperature-dependent transformation" of the initial ~ 8 S cytosolic hormone-receptor complex.

In the light of the importance of understanding the nature of receptor-subunit organisation, it was deemed necessary to investigate the effect of KCl on the sedimentation properties of the VMU ER and PR.

4.2 MATERIALS AND METHODS:

4.2.1 Preparation of cytosols and gradients:

A pool of uteri were sliced up into small, thin sections, the pieces thoroughly mixed and divided into two equal parts (by weight). One part was homogenized in 10 mM Tris-HCl buffer (pH=7.4) containing 20mM sodium molybdate while the other part was homogenized in a similar buffer not containing sodium molybdate. Cytosols were either used immediately, or stored at -70°C in 1 mℓ aliquots, prior to the experiment.

Gradients containing 0.4 M KCl but no sodium molybdate were prepared as described in Section 2.9.2.

4.2.2 Expermimental Procedure:

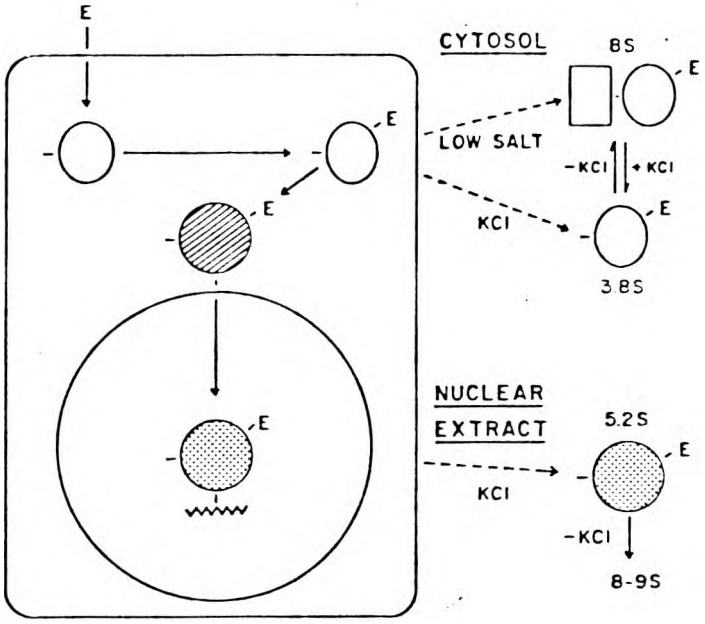


Figure 4.a: Schematic representation of interaction pathway of oestradiol in uterine cell (E). (From Jensen and DeSombre) (243).

4.2.2.1 Dissociation (in situ) of ER and PR on hypertonic (0.4 M KCl) sucrose density gradients during centrifugation : effect of sodium molybdate in cytosols on gradient profiles :

The cytosols contained (CYT(+)) or did not contain (CYT(-)) sodium molybdate (20mM). Cytosols (200µℓ, 6-8 mg/mℓ cytosolic protein) were incubated with ^{3}H-E_2 or ^{3}H-R5020 to a final ligand concentration of 5nM at 20°C for 30 min, with (non-specific binding) or without (total binding) a 250 fold excess of either DES or R5020. The reaction was terminated by transferring the incubation solution to a DCC pellet. The slurry was thoroughly vortexed and the DCC spun down at 2000xg' (15 min). An aliquot (200µl) of the resulting supernatant was layered onto sucrose density gradient containing 0.4 M KCl. Following centrifugation (VTi-80 rotor; 520000xg'x2hx2-4°C) the gradients were fractionated and processed as described in Section 2.9.2.

4.2.2.2 Dissociation of ER and PR, following reincubation in hypertonic buffer (0.4M KCl) for various times, prior to centrifugation on hypertonic gradients :

The cytosols contained (CYT(+)) or did not contain (CYT(-)) sodium molybdate (20mM). Cytosols (200µℓ; 6-8 mg/mℓ cytosolic protein) were charged by incubation with an equal volume of the proper radioligand (final concentration : 5 nM; ^{3}H-E_2 or ^{3}H-R5020 with (non-specific binding) or without an excess (250 fold) of the proper unlabelled competitor (DES or R5020) for 30 min. at 20°C. Labelled cytosols were then reincubated at 0-4°C for various periods (0, 5, 10, 20 min), following the addition of a small volume (100µℓ) of concentrated KCl (2M) in $TEDAG_{10}$ buffer to render a final concentration of 0.4 M KCl.

The second incubation was terminated by transferring the cytosolic solutions to a DCC pellet, followed by vortexing and centrifugation at 2000xg' for 15 min in the cold

(0-4°C). The clear supernatants were then layered onto precooled gradients, prepared as described before and centrifugation usually commenced ten minutes later. Following completion of centrifugation (VTi 80 rotor; 520000xg'x2h) gradients were processed as before.

4.2.2.3 Reversibility of the dissociation of ER and PR : sedimentation dialysis.

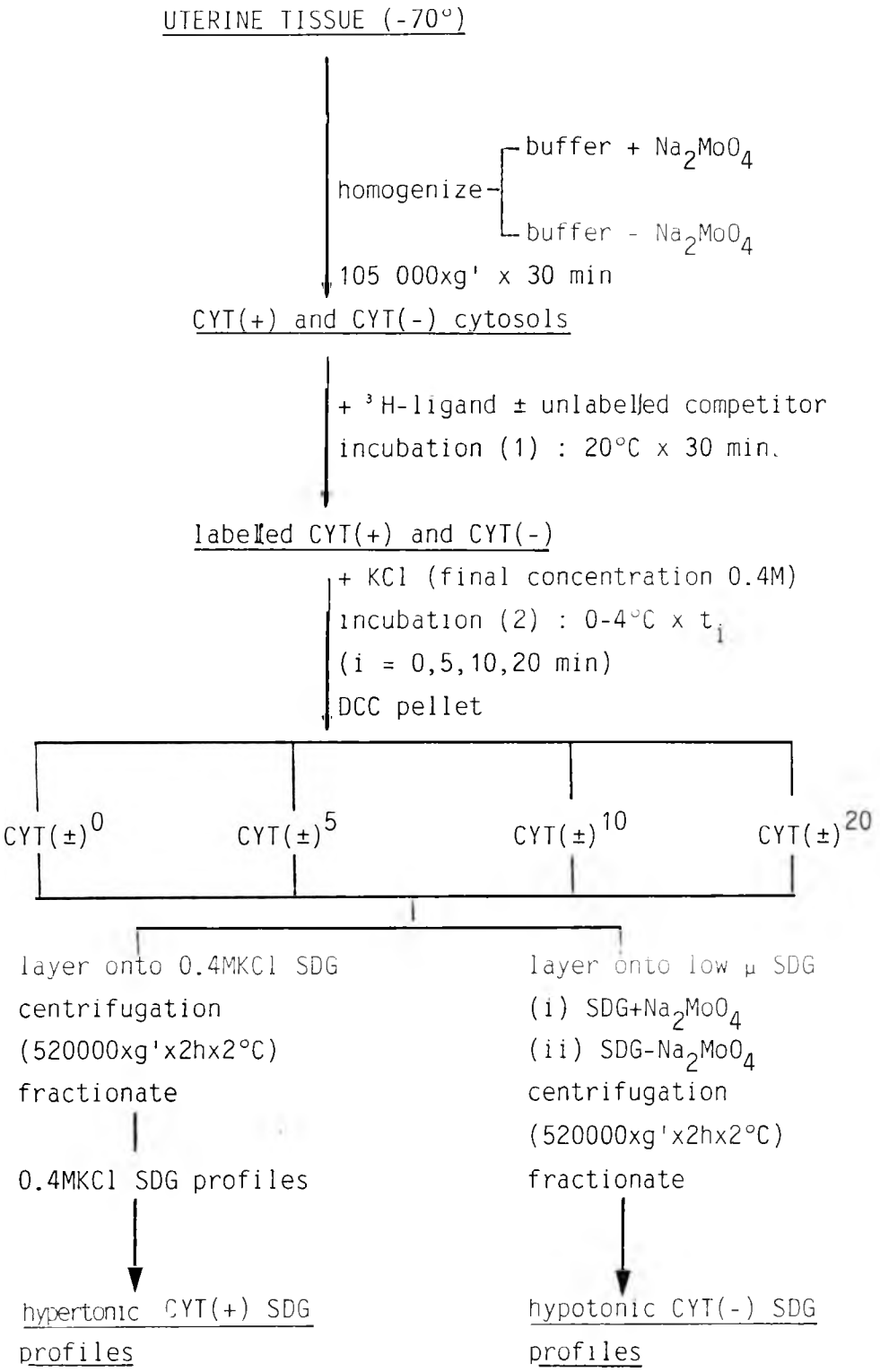
The experiment was executed as described in Section 4.2.2 above except that the second incubation was followed by centrifugation (VTi-80; 52000xg'x2hx2°C) of the resultant 0.4M KCl cytosols on low ionic strength sucrose density gradients. Gradients either contained (GRAD(+)) or did not contain (GRAD(-)) 20mM sodium molybdate.

4.3 RESULTS AND DISCUSSION :

Typical SDG profiles, obtained when VMU receptors were allowed to dissociate in situ in the high ionic strength gradient solutions (0.4 M KCl) during centrifugation, are depicted in Fig. 4.1 and Fig. 4.2. As a rule these profiles seemed to be more complex than those reported by the majority of workers in the field (221,238). Sodium molybdate only seems to effect higher yields of all the different molecular species and exerted no selective protection for a particular molecular entity after a slight shift towards lower S-values occurred. However it is doubtful whether these shifts are at all significant. A distinct difference can be observed between the ER and PR with reference to the ratio of the different peaks in the profile. For the ER the 6.9 S peak seems to be more prominent than the 5.6 S entity. The PR displayed the opposite of what is observed for the ER : here the 6.2 S species is the minor component, while the 4.6 S is the dominant molecular entity.

When cytosols were reincubated with 0.4 M KCl, prior to centrifugation on the 0.4 M KCl containing SDG (10-35% sucrose) a progressive loss of the faster sedimenting (∿ 6-7 S) entity, with a resultant increase in the slower sedimenting components (∿ 4-6 S) could be observed. Again, sodium molybdate only affected the yield, but not the relative proportions of the different molecular entities. The progesterone receptor seems to dissociate at a faster rate, compared to the ER as judged from the SDG profiles, but qualitatively it is following the same pattern. The faster sedimenting PR entity, however, seems to shift to lower S values for cytosols prepared in the presence of sodium molybdate (CYT(+)), while the shift for the ER in the presence of sodium molybdate is towards slightly higher S-values.

Reversibility of the dissociation of the ER seems to be affected by the length of time it is exposed to 0.4 M KCl during a second incubation at 0 - 4°C. The dissociation at zero time exposure (pre-centrifugation) is practically totally reversible, while a swift decrease in reversibility is observed at longer pre-centrifugation exposure to hypertonic buffers. A remarkable feature observed in this reversibility study, is that sodium molybdate tends to cause severe aggregation of the ∿ 8 S of molecules, which is aggrevated the longer the second incubation in 0.4 M KCl. Quantitatively (Table 4.1) it can be seen that in the absence of sodium molybdate (CYT(-)) the longer KCl incubation renders a linear decrease in the surface area of the ∿ 8 S peaks, while a directly proportional increase in the surface area of the 4 S peaks can be observed (Fig. 4.8). For molybdate containing cytosols (CYT(+)), the loss in the ∿ 8 S entity can be attributed to the formation of aggregates. The rate of increase in the ∿ 4 S area seems to be the same for both (CYT(+) and CYT(-)), while the rate of decrease in the ∿ 8 S peak is 82 % greater for CYT(+) than for CYT(-) cytosols.



FLOW DIAGRAM 4.1 : Methodology for investigating the dissociation of VMU ER and PR in hypertonic media.

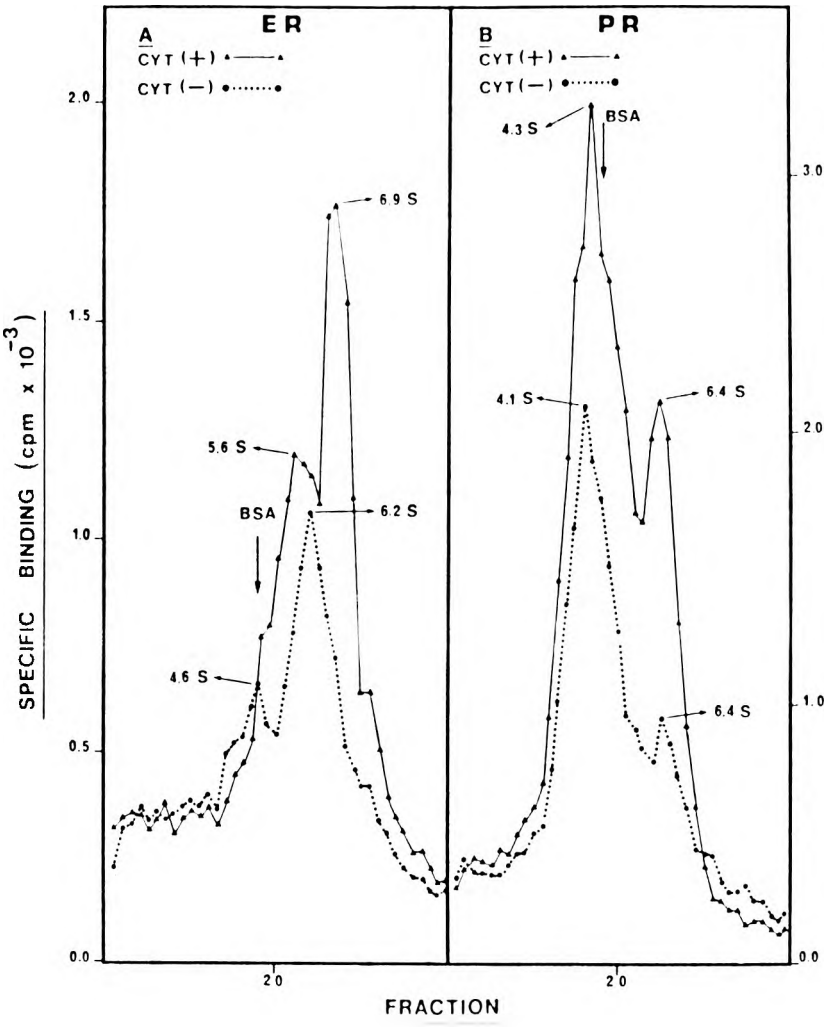


Figure 4.1: Dissociation of the VMU ER and PR on 10-35 % sucrose density gradients containing 0.4 M KCl. Comparison of ER with PR : (A) dissociation of VMU ER : (▲) CYT(+) and (●) CYT (-) cytosols; (B) dissociation of VMU PR : Cytosols were prepared in the presence (CYT(+)) or absence (CYT(-)) of 20mM sodium molybdate. Following incubation with the appropriate ligands (5nM ^{3}H-E_2 or ^{3}H-5020) for 30 min at 20°C, the charged cytosols were treated with DCC and the 2000xg' supernatants (200µl) applied to precooled hypertonic (0.4M KCl) SDG (10-35% sucrose). Centrifugation was executed at 520000xg' for 2h at 2-4°C and the gradients fractionated into 5 drop fractions.

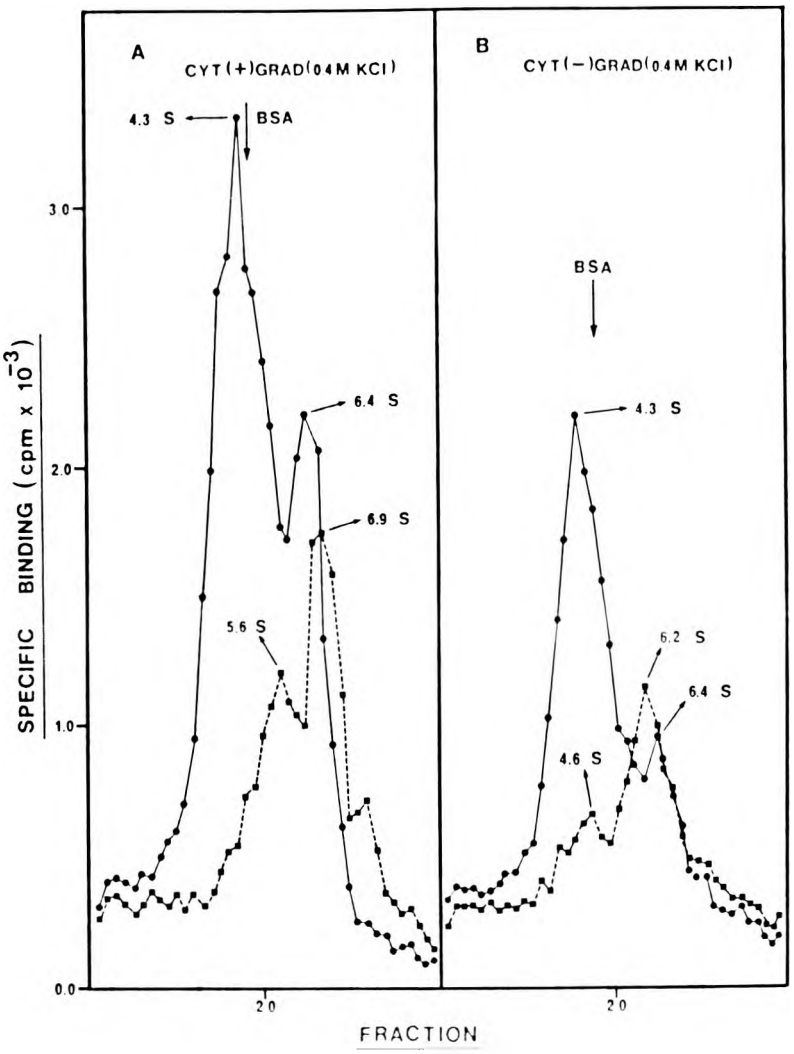


Figure 4.2: Effect of sodium molybdate on the dissociation of the VMU receptors on 10-35 % SDG containing 0.4 M KCl. Comparison of the dissocation of ER and PR receptors in CYT(+) and CYT(-) cytosols : (A) dissociation of ER and PR in CYT(+) cytosols : (■) ER, (●) PR; (B) dissociation of ER and PR in CYT(-) cytosols : (■) ER and (●) PR. Cytosols were prepared in the presence or absence of 20 mM sodium molybdate. After incubation with the appropriate ligand (^{3}H-E$_{2}$ or ^{3}H-R5020) for 30 min at 20°C in the absence (total binding) or presence (non-specific binding) of 250 fold excess of either DES or R 5020. The DCC treated supernatants (2000xg' x10 min) were layered onto hypertonic (0.4 M KCl) 10-35 % SDG,following ultra-centrifugation (VTi-80 rotor, 520000g'x120 minx2°C). The gradients were fractionated into 5 drop fractions and the fractions monitored for total and non-specific bindings.

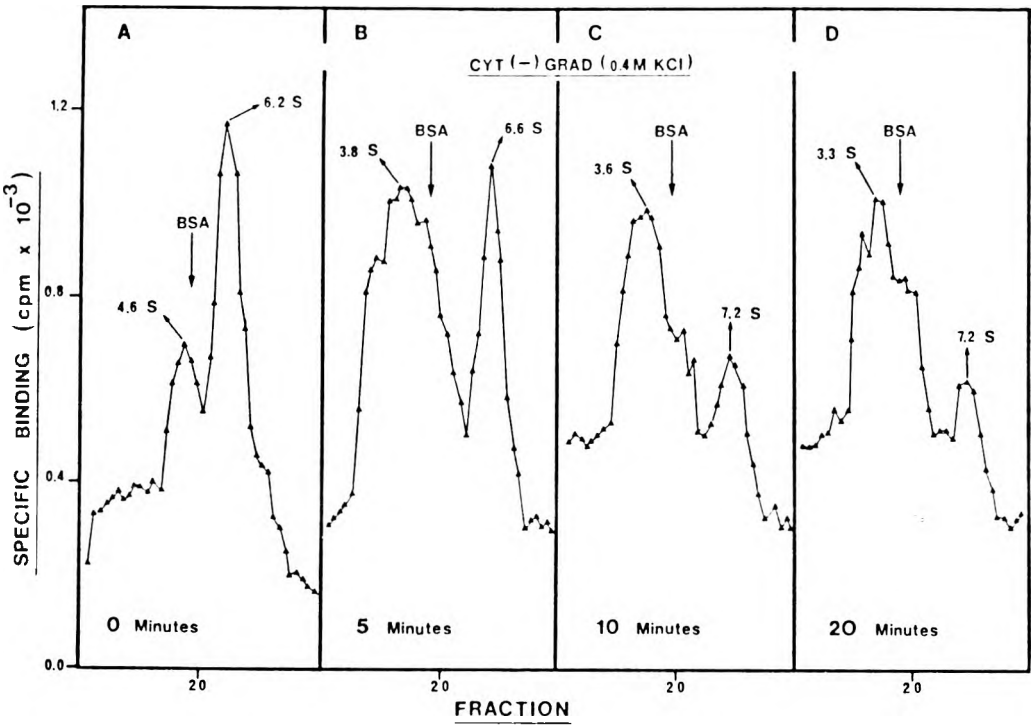


Figure 4.3: Dissociation of VMU ER, following different pre-centrifugation exposure times to 0.4 M KCl. Cytosols were prepared in the absence of sodium molybdate (CYT(-)). Cytosolic receptors were charged (20°Cx30 min) with radioligand (^{3}H-E_2; final concentration, 5 nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess of the unlabelled ligand (DES). Prior to DCC treatment, a solution of KCl (2M) was added to the reaction mixture (final concentration of KCl, 0.4 M) and the resulting solution reincubated on ice (0°C) for varying time intervals : (A) zero time incubation : following the addition of the KCl, the reaction was immediately stopped by the addition of DCC, (B) 5 minutes; (C) 10 minutes and (D) 20 minutes. The resulting 2000xg' supernatants of the DCC treated incubation mixtures were then subjected to ultra-centrifugation of 10-35 % SDG (VTi rotor; 520000 x g'x120 min) at low temperature (2°C) after which the gradients were fractionated (40x5 drop fractions) and counted for radioactivity.

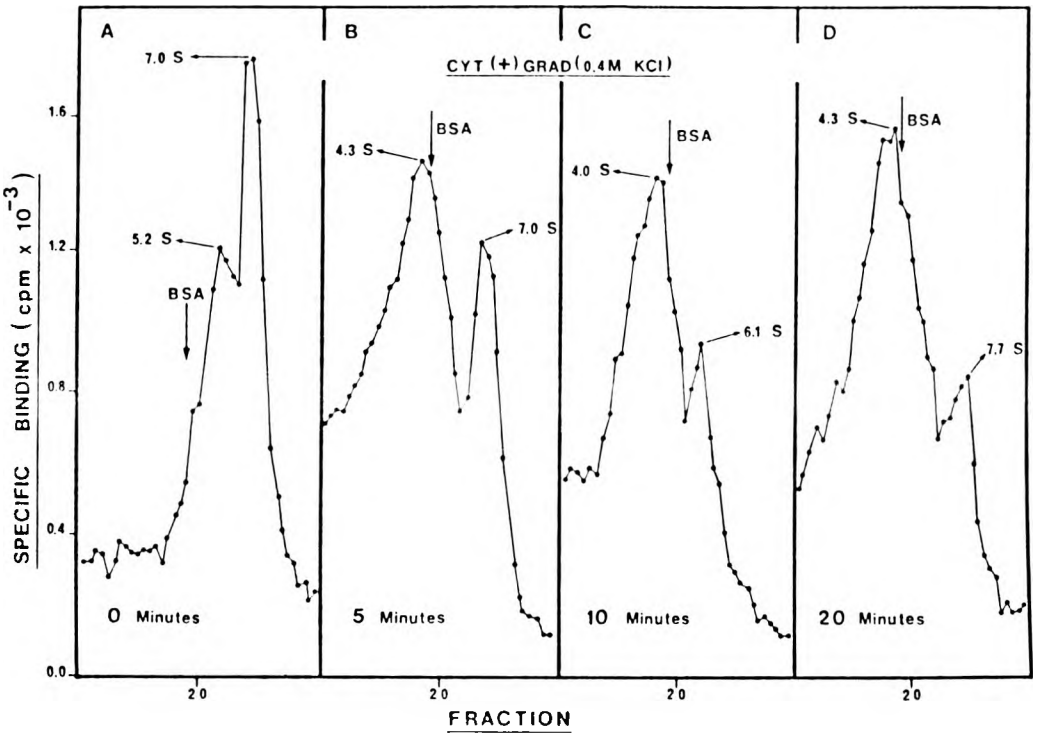


Figure 4.4: Dissociation of VMU ER, following different precentrifugation exposure times to 0.4 M KCl. Cytosols were prepared in the presence of 20 mM sodium molybdate (CYT (+)). Cytosolic receptors were charged (20°Cx30 min) with radioligand (^{3}H-E_2; final concentration, 5 nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess of the unlabelled ligand (DES). Prior to DCC treatment, a solution of KCl (2M) was added to the reaction mixture (final concentration of KCl, 0.4 M) and the resulting solution reincubated on ice (0°C) for varying time intervals : (A) zero time incubation : following the addition of the KCl, the reaction was immediately stopped by the addition of DCC, (B) 5 minutes;(C) 10 minutes and (D)20 minutes. The resulting 2000xg' supernatants of the DCC treated incubation mixtures were then subjected to ultra-centrifugation of 10-35 % SDG (VTi rotor; 520000 x g'x120 min) at low temperature (2°C).

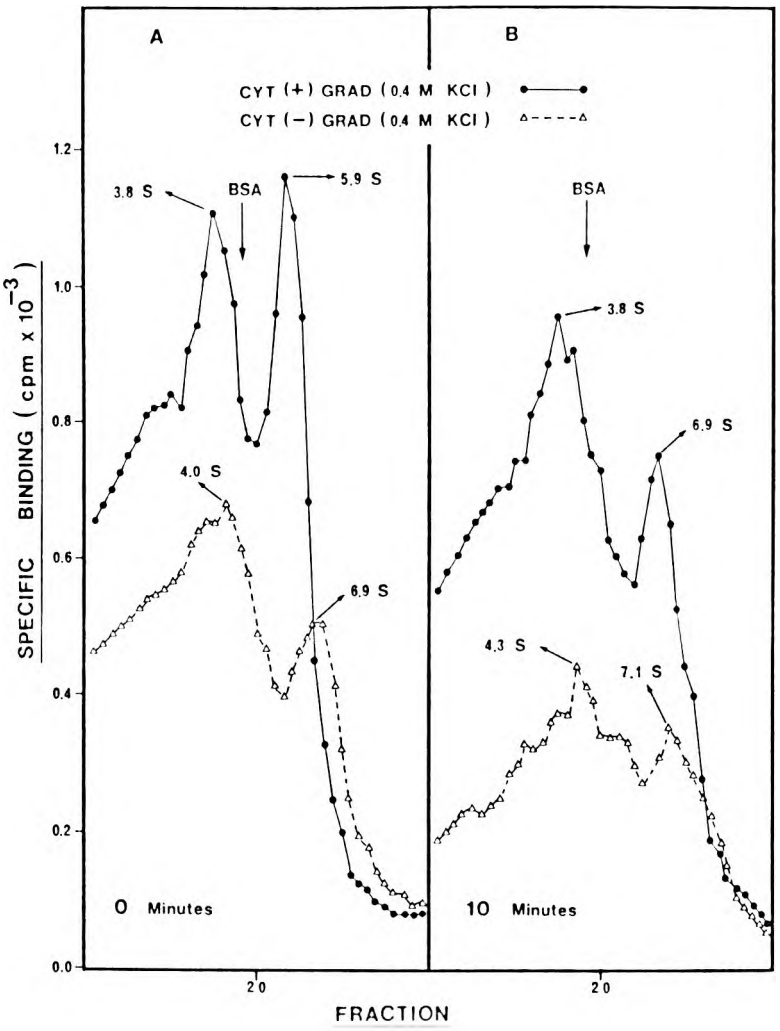


Figure 4.5: Dissociation of VMU PR following precentrifugation exposure to 0.4 M KCl. Cytosols were prepared either in the presence (CYT(+)) or absence (CYT(-)) of 20mM sodium molybdate. Following the first incubation (20°C x30 min) with ^{3}H-R 5020 (final concentration, 5nM) in the absence (total binding) for presence (non-specific binding) of a 250-fold excess if R 5020, the incubation mixture were reincubated (0°C for varying time intervals, following the addition of KCl : (A) zero time exposure (●) CYT(+), (Δ) CYT(-) (B) 10 min exposure (●) CYT (+), (Δ) CYT(+). After completion of the second incubation the incubation mixtures were treated with DCC and the 2000xg' supernatants (200µℓ) layered onto hypertonic 10-35 % SDG. The SDG profiles were obtained following ultracentrifugation (VTi-80 rotor, 520000xg'x120 minx2°C) and fractionation (40x5 drop fractions).

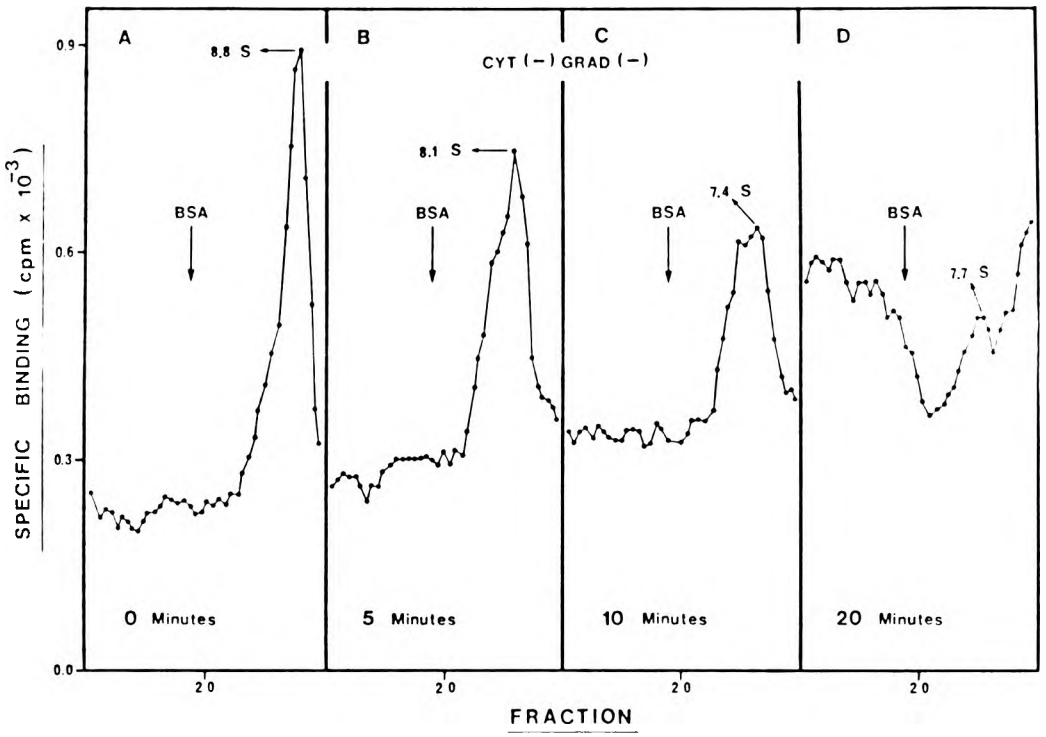


Figure 4.6: Effect of the duration of pre-centrifugation exposure of cytosolic receptors to 0.4 M KCl on the reversible dissociation of the VMU ER on low ionic strength SDG. Cytosols were prepared in the absence ((CYT(-)) of sodium molybdate. Gradients also did not contain ((Grad(-)) any sodium molybdate or 0.4 M KCl. Following the first incubation (20°Cx30 min) with ^{3}H-E_2 (final concentration, 5 nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess of DES the incubation mixture were reincubated (0°C) for varying time intervals, following the addition of KCl to a final concentration of 0.4 M KCl : (A) zero time incubation; (B) 5 min, (C) 10 min and (D) 20 min. After completion of the second incubation the incubation mixtures were treated with DCC and the 2000xg' supernatants (200µℓ) layered onto hypotonic 10-35 % SDG. The SDG profiles were obtained following ultra-centrifugation (VTi-80 rotor, 520000xg'x120 minx 2°C) and fractionation (40x5 drop fractions).

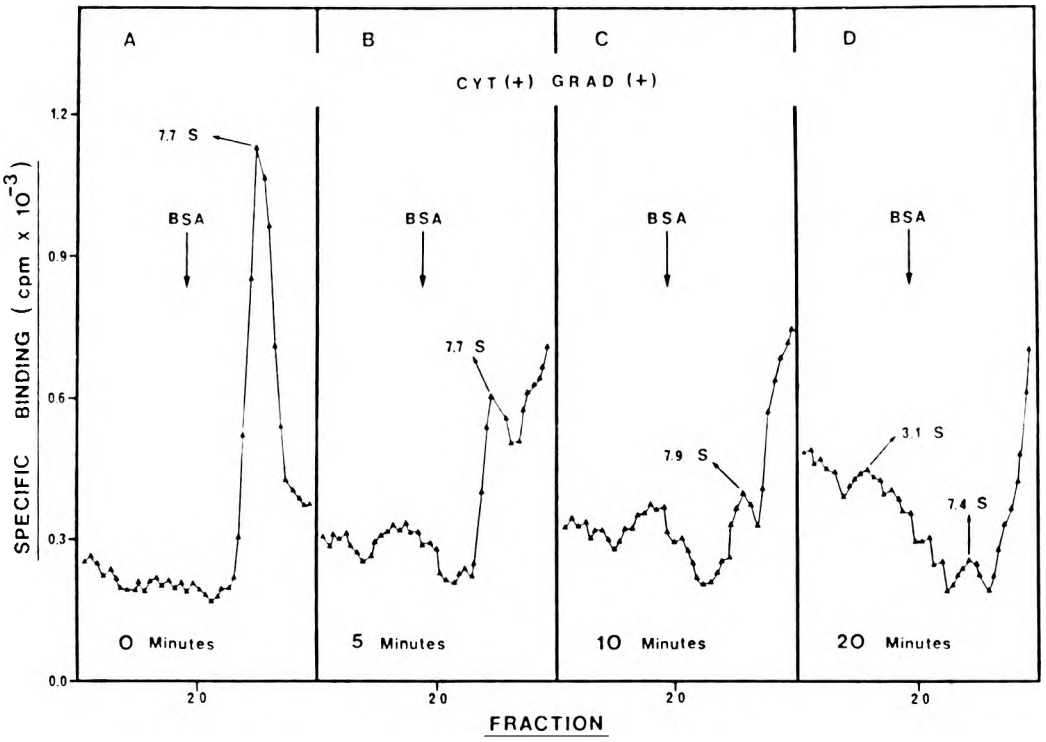


Figure 4.7: Effect of the duration of pre-centrifugation exposure of cytosolic receptors to 0.4 M KCl on the reversible dissociation of the VMU ER on low ionic strength SDG. Cytosols were prepared in the presence (CYT(+)) of 20mM sodium molybdate. Gradients also contained (GRAD(+)) molybdate but no KCl. Following the first incubation (20°Cx30 min) with ^{3}H-E_2 (final concentration, 5 nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess of DES the incubation mixture were reincubated (0°C) for varying time intervals, following the addition of KCl to a final concentration of 0.4 M KCl : (A) zero time incubation; (B) 5 min, (C) 10 min and (D) 20 min. After completion of the second incubation the incubation mixtures were treated with DCC and the 2000xg' supernatants (200µℓ) layered onto hypotonic 10-35 % SDG. The SDG profiles were obtained following ultracentrifugation (VTi-80 rotor, 520000xg'x120 minx 2°C) and fractionation (40x5 drop fractions).

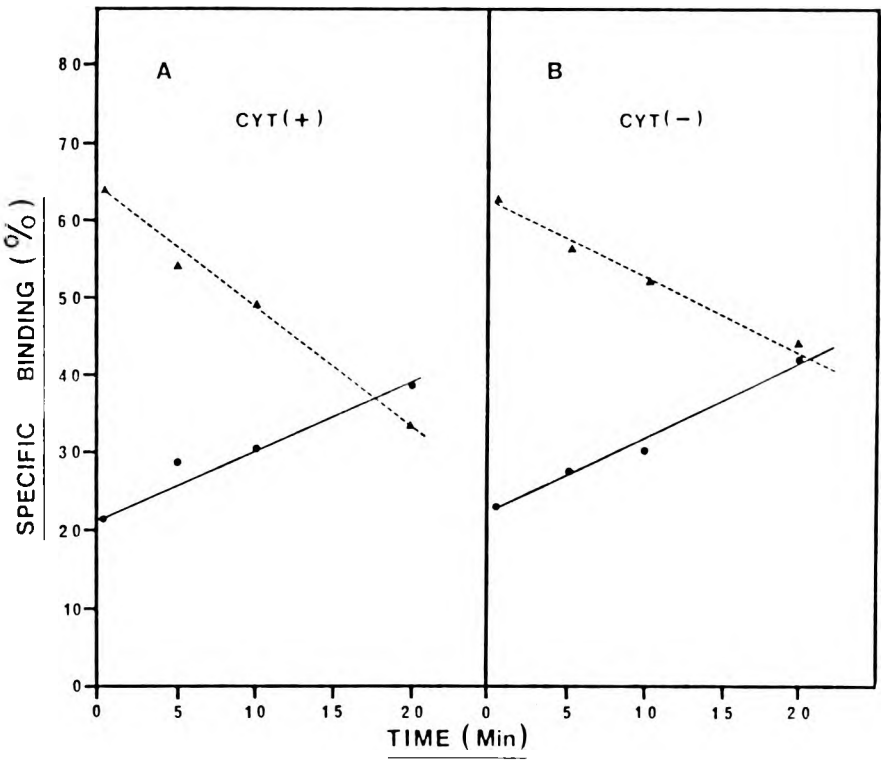


Figure 4.8: Rates of disappearance of the 8 S high affinity binding component and the simultaneous formation of smaller specific binding species in the 3 - 5 S area : (A) effect as seen with CYT(+) cytosols : (▲) disappearance of 8 S peak (●) formation of 3 5 S specific binding components; (B) effect as seen with CYT(-) cytosols : (▲) disappearance of 8 S and (+) formation of 3 - 5 S species. This figure represents an analysis of the reversibitlity experiments depicted in Fig 4.6 and 4.7

TABLE 4.1

Effect of sodium molybdate on the dissociation of VMU ER in hypertonic (0.4M KCl) gradients, when cytosols were incubated at different times in hypertonic buffers (0.4M KCl), prior to centrifugation.

Time incubated in hypertonic buffer (second incubation) (min)	% Activity							
	CYT(+)				CYT(-)			
	4 S	8 S	*A	A+8S	4 S	8 S	A	A+8S
0	22.0	46.2	17.1	63.3	23.8	62.2	-	-
5	28.7	28.0	26.0	54.0	27.7	56.1	-	-
10	31.1	20.6	29.0	49.6	26.9	52.6	-	-
20	38.4	12.8	20.1	32.9	42.0	47.8	-	-

(*A = aggregate)

CHAPTER 5

ACTIVATION, TRANSFORMATION AND TRANSLOCATION OF VMU ER AND PR.

5.1 INTRODUCTION

According to the classical, two-step model for steroid hormone action, proposed by Jensen et al (215) and Gorski et al (245), in the early 1960's, the initial cytoplasmic steroid receptor complex must be converted ("activated") into a form, exhibiting a high affinity for nuclear elements (245-246). Binding of this "activated" complex to the chromatin, initiates transcription and the subsequent synthesis of specific hormone-induced proteins (246). A lot of uncertainty, concerning the actual nature of the "activated" species still exists. Several investigators made some interesting observations on the molecular and kinetic properties of the "activated" complex in recent years (248-249). These observations seem to support a model for ligand-induced receptor dimerization or association of dissimilar subunits, converting the non-activated $\sim$ 4 S (MW $\sim 7.5 \times 10^4$ daltons) to a $\sim$ 5 S "activated" (MW $\sim 1.4 \times 10^5$ daltons).

Receptor transformation seems to be hormone and temperature-dependent, and only the "activated" $\sim$ 5 S receptor form exhibits a high affinity for target cell nuclei. An immediate effect of this binding of the activated complex to the chromatin is the increase in RNA polymerase activity (247). Other studies (248) have also concluded that the $\sim$ 5 S oestrogen binding protein consists of the $\sim$ 4 S oestrogen receptor plus an additional protein. This additional protein was found to be present (248) in target, as well as non-target, tissues. Notides (244) on the other hand, suggested that in the absence of hormone, the $\sim$ 4 S binding protein is associated with a macromolecular inhibitor from which it must dissociate before it can form the activated $\sim$ 5 S

complex.

Evidence has also been presented for a dialyzable, low molecular weight, heat stable inhibitory molecule or molecules in the cytosols of murine Leydig cell tumours (249) and rat uteri (250), which seemed to inhibit oestrogen receptor activation. A similar low molecular weight inhibitor of glucocorticoid receptor activation has also been reported (251-252). In the glucocorticoid receptor system, activation is associated with a change in isoelectric point of the receptor without any significant change in sedimentation behaviour, as is observed for the oestrogen receptor (253, 254).

In the case of the oestrogen receptor it was observed that the activation of cytosols by dialysis did not require the presence of hormone (250) an observation which tend to render the process of receptor activation even more intriguing. The progesterone receptor of chick oviduct can be transformed by heating (temperature activation), as well as by high salt treatment (salt activation), to acquire the capacity to bind to nuclei ATP-Sepharose, DNA-cellulose, or to phosphocellulose (255).

Recent studies have shown that sodium molybdate, which is a potent phosphatase inhibitor (271), stabilizes the glucocorticoid receptor (256) as well as the androgen and progesterone receptors (257). The molybdate oxyanion appears to interfere with the transformation of the progesterone receptor (257) and it is thought that receptor transformation may involve a dephosphorylation process (231-232).

It is still unsolved whether receptor transformation normally occurs *in vivo* in the cell cytoplasm or in the nucleus. In the light of the recently proposed nuclear model for steroid hormone action (46,45,258), it seems even more important to clear up uncertainties about the process of receptor activation and transformation (259). Although

temperature dependent receptor transformation can occur in the cytoplasm in vitro, Yamamoto(248) recently reported that the ∿ 4 S to ∿ 5 S conversion occurs much more rapidly in the presence of DNA. In addition, Siiteri et al (259) reported finding a ∿ 4 S receptor in target cell nuclei; a finding which seems to support the new model. Pulse-chase experiments revealed that the level of the nuclear ∿ 4 S form decreases with a concomitant increase in the level of the ∿ 5 S form, suggesting that the ∿ 4 S oestrogen binding protein is transformed within the nuclear compartment. This ∿ 4 S to ∿ 5 S conversion within the nucleus has already been reported for several target tissues e.g. hypothalamus, pituitary and uterus. The rates of receptor activation, however, were found to differ considerably among the three types of tissues (260).

It has recently been reported that the steroid binding portion of the receptor molecule is distinguishable from that portion of the receptor molecule required for nuclear binding. Mild proteolysis of cytoplasmic receptors for a variety of steroid classes generated a small (MW∿ $2\text{-}4\text{x}10^4$ daltons) fragment of the receptor ("meroreceptor") and which seem to retain the high affinity steroid binding properties, but lacks the capacity to bind to target cell nuclei(65).

More accurate information on the relation between nuclear and cytoplasmic forms of the oestrogen receptor has been derived from immunological investigations. Jensen and coworkers (208) recently succeeded in generating antibodies to highly purified, nuclear oestradiol-receptor complexes from calf uteri. These antibodies cross reacted with both the nuclear and cytoplasmic oestrogen receptors from a variety of mammalian species (calf, rat, mouse, guinea pig and human). However, they did not seem to cross react with androgen or progesterone receptor complexes, indicating that definite immunogenic differences exist among the receptor proteins for different classes of steroid hormones (208).

These monoclonal antibody preparations to the nuclear oestradiol receptor have been provided a novel tool in the study of receptor structure and function, as well as for the quantitation of the steroid hormone receptors in target tissues(261).

Studies on human breast cancer cells in culture (56, 262) indicated that approximately 75 % of the total oestrogen receptors in these cells are contained in the nucleus even in the apparent absence of hormones. Many human breast tumours have also been found to contain substantial levels of "free," or unoccupied, nuclear receptors (196, 263), a fact which might indicate that this unusual distribution is not an artifact of *in vitro* assay conditions.

It has been suggested that phosphorylation affects the binding of steroid receptors to hormones and to nuclei (266). However, so far only limited evidence has been found to prove that steroid receptors are indeed phosphorylated (269). A nuclear phosphatase, inactivating the oestradiol receptor *in vitro* (267,268) as well as *in vivo* (269) and a Ca^{2+}dependent kinase which reacts with the receptors (270), have been reported to be present in oestrogen target tissues. These enzymes were partially purified and characterized by Auricchio *et al* (271) and in so doing these researchers provided first direct evidence that the oestradiol receptor must be a phosphoprotein and that the "*in vitro*" phosphorylation-dephosphorylation process could regulate its hormone binding activity (270).

The process by which the hormone receptor complex becomes "activated" would appear to differ for different hormone classes (264). This process might involve the dissociation of certain inhibitory factors or some changes in conformation, resulting from macromolecular (homologous or heterologous) associations (265). No single model, as yet, could explain the complex relationship between receptor-ligand

interaction, transformation of the complexes, the nuclear binding of the hormone receptor complex and the resultant induction of various molecular responses. These dynamic features of steroid hormone-receptor interactions provide numerous key points at which hormone action may be regulated in order to provide the fine physiological control seen in diverse target tissues (246). It may also be an important level where differences in normal and abnormal tissues may occur.

We subsequently decided to investigate these processes as they occur in the VMU. Since we could not obtain abnormal (malignant or benign) uterine tissues for our experimental model, we decided to do some parallel studies on the dynamics of activation-transformation and translocation in normal and abnormal human uterine tissues.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

Reagents were the same as those described in Chapter 2. Calf thymus DNA was bought from Sigma. The cellulose employed in the synthesis of DNA cellulose was Munktell 410 Cellulose (Bio-Rad, Richmond, California).

The diphenylamine reagent was prepared as follows : 0.3 g diphenylamine, 0.3mℓ concentrated H_2SO_4 and 20 mℓ acetic acid: just before use, 0.1 mℓ of diluted acetaldehyde (1.6/100mℓ H_2O) was added to the diphenylamine reagent.

5.2.2 Preparation of cytosols and nuclei for binding studies

Uteri were cut into small pieces while still frozen and homogenized by one of 2 different methods :

(a) a Ultra Turrax (Janke & Kunkel) homogenizer

(b) a Dounce glass homogenizer

The homogenates were first centrifuged at 800xg' for 10 min (0-4°C), after which the 800xg' supernatants were decanted and then recentrifuged at 105000xg' for 30 min (0-4°C). The crude nuclear fraction was washed (3 times) with Tris-HCl buffer (pH=7.4) and approximately 2 mℓ of the final nuclear suspension were layered onto a 1.2 M sucrose pad (2 mℓ) in a 5 mℓ centrifuge tube. Following centrifugation at 6900xg' for 45 min (0-4°C), the washed and resuspended pellets were assayed for DNA prior to the execution of the nuclear binding experiments. Cytosols (105000xg' fraction) were processed at the same time. A comprehensive description of the procedure is shown in Flow diagram 5.1.

5.2.3 Protein and DNA determination

Protein concentration was assessed, employing the procedure of Lowry et al(273). described in Section 2.8, while the DNA content was assayed by a slightly modified method, developed by Burton(274).

To every 1 mℓ of either standard DNA solution or sample, 0.22 mℓ perchloric acid (PCA, 2.5 N) was added. After 10-30 min on ice and 5 min centrifugation at 2000 x g', the pellet was resuspended in 1 mℓ 0.3 N PCA. This suspension was subsequently hydrolysed for 30 min at 90°C, followed by rapid cooling and recentrifugation for 5 min at 2000 x g' . Two hundred and fifty µℓ of this hydrolysate was added to 500µℓ of diphenylamine reagent, the reaction mixture was allowed to stand for 16 hours at room temperature and the absorbance measured at 600 nm.

Standard curve for DNA determination: 30mg of calf thymus DNA were dissolved in 100mℓ of 5 mM NaOH. 7 different DNA dilutions were prepared ranging from 25 to 300 µg/mℓ DNA.

The standard curve is depicted in Fig 5.1.

5.2.4 Preparation of DNA-cellulose

DNA-cellulose was prepared according to Albert and Herrick (272) with only minor modifications : Fifty grams of cellulose were washed several times with boiling ethanol to remove adsorbed pyridine. The ethanol was removed by suction and the cellulose susequently washed repeatedly with 0.1 M NaOH 1 mM trisodium EDTA (Tris-EDTA) and 10mM HCl solutions at room temperature. After washing the cellulose with water to neutrality, it was lyophylized to dryness and then employed in the preparation of DNA-cellulose.

A solution of DNA (1-3 mg/mℓ in 10 mM Tris-HCl containing 1 mM Na_3 at pH 7.4) was transferred to a glass beaker. Clean, dry cellulose was added with occasional stirring, until the paste thickened (approximately 1 g cellulose per 3 mℓ). The lumpy mixture was spread out in an evaporating dish and left at room temperature and then covered with gauze until it was dry. After grinding the lumps to a powder with a glass rod, any remaining water was removed by overnight lyophilization. The thoroughly dry powder was then suspended in approximately 20 volumes of Tris-EDTA and left at 4°C for one day. After two quick washes, to remove any free DNA, the DNA-cellulose was stored as a frozen slurry in Tris-EDTA,containing 0.15 M sodium chloride.The DNA content of the DNA-cellulose was assayed as described in section 5.2.3.

5.2.5 Correlation of DNA content with number of uterine nuclei

In order to express nuclear binding as a function of DNA content and to correlate this way of expressing binding data with the total binding capacity per target cell nucleus, we endeavoured to employ the Coulter counter in the quantitation of nuclei and subsequent calculation of the binding of receptor-complexes to nuclei.

Nuclei were prepared by the method described in section

5.2.2. The washed nuclei were diluted serially and subsequently counted in Coulter counter. Results are depicted in Fig. 5.2.

5.3 ANALYSIS OF NUCLEAR RECEPTORS FOR VMU ER

5.3.1 Quantitative assay of nuclear receptors : the ligand exchange technique

5.3.3.1 Current methods:

The [^{3}H]-oestradiol exchange assay has proven useful for measuring nuclear oestrogen receptor complexes in the uterus (252) pituitary glands and hypothalami (281) lactating mammary gland (238), corpi lutei (246), chicken oviducts (288) and in Leydig cell tumours (249). Clark et al (278, 280) employed a modified method for the measurement of progesterone and glucocorticoid receptors in the nuclear and cytoplasmic compartments of rat uteri as well as in rat and chicken liver nuclei. Other workers have reported the use of the exchange method to measure androgen receptors in the nuclei of the testis cells (277). Clark also used the same method to assay oestrogen receptor complexes bound to chromatin (278).

In this section we wish to present the method currently in use in our laboratory. This method was derived and modified from most of the techniques mentioned above, but with a bit more emphasis on Clark's method (278,280).

5.3.1.2 Experimental Procedure:

Uterine tissues were homogenized in a ground-glass (Dounce) homogenizer or an Ultra Turrax PT10 homogenizer in $TEDAG_{10}$

buffer to the pellet (1mℓ/50mg) followed by thorough vortexing and subsequent recentrifugation at 800xg for 10 min. The crude washed nuclear pellet was resuspended by vortexing in $TEDAG_{10}$ buffer (same ratio as above) and 100µℓ homogeneous aliquots of the nuclear suspension dispensed into disposable culture tubes, which contained 5 different concentrations of ^{3}H-oestradiol or ^{3}H-oestradiol plus a 100 fold excess of diethylstilbestrol(DES) and 100µℓ of buffer.

Assay tubes were placed in a 37°C shaking water bath for 30 min. Exchange was terminated by the addition of 1 mℓ ice-cold $TEDAG_{10}$ buffer to each tube, followed by centrifugation at 800xg' for 10 min (0-4°C). The nuclear pellet was washed (3 times) by resuspension in 1.5 ml ice-cold $TEDAG_{10}$ buffer, vortexing and centrifugation described above. Subsequently, 1.0 ml 100 % ethanol was added to the washed nuclear pellets and assay tubes were placed in a 30°C water bath for 10-20 minutes. The tubes were thoroughly vortexed and centrifuged at 800xg' for 10 minutes. The total ethanolic extract was added to 4 ml of scintillation cocktail and samples were counted in a Beckman LS 1800 scintillation coulter to an efficiency of 25-30 %.

5.3.2 Qualitative assay of nuclear receptors: : SDG analysis :

5.3.2.1 Introduction : extraction procedures for the nuclear receptors :

In studies of specific hormone binding in the nucleus salt extraction of receptors and subsequent analysis on sucrose density gradients are probably the most common method of qualitative analysis (234). However this technique does not seem to be ideal, since only a fraction (7 %) of the nuclear receptors can be re-extracted (285). Another disadvantage of this method is the fact that the oestradiol receptor complexes normally undergo dissociation during extraction with high concentrations (0.3-0.6 M KCl) of salt (279-283).

These problems have been found to be greatly magnified in human uterine tissue(284). Salt extraction of nuclear receptors in this tissue appear to be highly inefficient. Rat et al (285) reported that mild trypsinization of calf uterine cytosol produced a stable form of the cytoplasmic receptor. Mild proteolysis not only effectively released nuclear receptors from high affinity chromatin "acceptor" sites, but also did not cause any impairment in oestradiol binding activity (286).

5.3.2.2 Experimental procedure

The mild trypsinization procedure was slightly modified for the extraction of vervet monkey uterine nuclear receptors and was compared to the salt extraction method and DNAse I treatment. VMU tissue slices were incubated ∿ 25 mg per incubation) with a final concentration of 5 nM [^{3}H]-oestradiol without (total binding) or with a 250 fold concentration DES (non-specific binding). Incubations were conducted at 27°C for 30 min in a CO_2 incubator (5% CO_2 : 92 % air). The uterine tissue slices were then thoroughly rinsed with ice cold $TEDAG_{10}$ buffer and homogenized in a Dounce glass homogenizer or with an Ultra Turrax PT10 (Janke and Kunkel) at 3x10 second bursts (50 second cooling periods in ice inbetween). The homogenate was centrifuged at 800xg' for 20 min. The 800xg' pellet was washed three times with ∿3 mℓ of $TEDAG_{10}$ buffer, by resuspension (vortexing) and recentrifugation at 800xg' for 10 min (0-4°C). The washed nuclear pellet was subsequently extracted by a number of procedures for one hour at 0°C, followed by centrifugation at 180000xg' for 15 min in a Beckman Ti50 rotor. The resulting supernatant was assayed for radioactivity following removal of unbound ligand by treatment with a DCC pellet:

The following treatment and extraction procedures were employed:

(a) Trypsinize nuclear pellet for 15 min at 0°C, fol-

lowed by extraction with 0.4 M KCl for 15 min at 0°C (trypsin-KCl method);

(b) extract nuclear pellet with 0.4M KCl for 15 min at 0°C (0.4 M KCl method);

(c) hydrolysis with deoxyribonuclease I (DNAse I) for 30 min at 0°C (DNAse method).

(a) Trypsin KCl method:

In the case of the trypsin-KCl extraction, the nuclear pellet was first treated with trypsin: a nuclear pellet obtained from 150 mg monkey uterine tissue (~350μg DNA) was suspended and in 0.5 mℓ $TEDAG_{10}$ buffer and incubated with 50μg of trypsin(Sigma) for 15 min at 0°C, followed by the addition of 0.5mℓ of 0.8 M KCl in $TEDAG_{10}$ buffer (final concentration was 0.4M KCl) and incubation continued for 15 min at 0°C. Excess trypsin inhibitor (100μg) was added to terminate proteolysis, prior to centrifugation at 80000xg' for 2 hours.

(b) 0.4 M KCl method :

For the 0.4 M KCl treatment the nuclear pellet was incubated for 15 min at 0°C with 0.4 M KCl solution in $TEDAG_{10}$.

Linear (10-35 %) sucrose density gradients were prepared as described in section 2.13. The nuclear extracts (200μℓ) were layered on 0.4 M KCl sucrose gradients and the cytosols were layered on low salt gradients.

(c) DNAse method:

Nuclear pellets were also prepared in $TEDAG_{10}$ buffer (pH=7.4) and allowed to react with DNase 1 for 30 min at 0°C. Optimal DNase 1 concentration was found to be 50μg per

350µg of DNA in 0.5 mℓ of $TEDAG_{10}$ buffer (pH7.4).

5.3.2.3 Results

Fig. 5.1 represents a standard curve for the DNA assay.

A linear correlation could be illustrated between the amount of DNA present in the solution and the absorbance at 600nm (r=0.9998) for the range of 25-300 µg DNA per mℓ.

When nuclei were diluted with buffer, a linear correlation (r =0.964) was found to exist between the number of nuclei counted with the aid of the Coulter Counter and the dilution factor (Fig. 5.2). Furthermore, the DNA concentration also proved to be directly proportional (r = 0.969) to the number of nuclei (Coulter Counter) (Fig. 5.2). Although this method appeared to be quite reliable, visual inspection of the nuclear suspension with the light microscope (400 x magnification) revealed that a fairly large proportion of the nuclei (> 80 %) were broken and consequently it was decided that it would be more accurate to perform DNA assays rather than to count number of nuclei.

Fig. 5.3 represents a typical Scatchard plot for nuclear binding while Table 5.1 represents a summary of binding data obtained for ER in approximately 53 VMU. The mean value for ER was 1483±1236 fmol/mg DNA and the mean K_d was 4.02±1.8x 10^{-9}M.

Fig (5.4) compares SDG profiles of cytosolic and nuclear oestrogen receptors isolated from tissue slices. The cytosolic receptors in tissue slices rendered one main peak at 7.6 S in hypotonic gradients, while the 0.4 M KCl extract of the nuclear pellets (methods b) yielded one main peak at 4.6 S and a minor peak at 3.8 S in hypertonic gradients. A comparison between SDG profiles obtained in hypertonic gradients following the other two methods of treatment and

extraction of the nuclear pellets (method a and c) revealed peaks at 2.5 S and 4.5 S respectively (Fig. 5.5).

These results indicated that the molecular species extracted with DNase 1 (method c) had the same sedimentation coefficients as the 0.4m KCl extracted entity (method b).

When a quantitative assessment for the different extraction procedures was made, it was observed that the best extraction could be obtained with the trypsin-KCl procedure (method b) a finding which agrees favourably with results reported by other workers(282) (see Table 5.2).

5.4 DYNAMICS OF ACTIVATION, TRANSFORMATION AND NUCLEAR BINDING OF THE ER AND PR IN THE VMU:

5.4.1 Aim of investigation:

The problems surrounding the question of activation and transformation of "cytosolic" receptors as necessary prerequisites for the creation of specific binding species displaying a high affinity for nuclear components have been discussed in Chapter 1 and in the introduction to this chapter. It seems however necessary to explain that the main aim behind the experiments described in Chapter 5 is to produce creating a model system for studying the dynamics of the processes of activation and transformation of the steroid receptor species (ER and PR) in the vervet monkey uterus.

A decision was made to employ temperature induced activation-transformation in a cell free system and to follow the time course of events on high ionic strength sucrose density gradients. The sedimentation shift ($\sim$ 4 S $\rightarrow$ $\sim$ 5 S) could then be directly related to the increased affinity of the activated ($\sim$ 4 S) species for isolated uterine nuclei and

binding to DNA-cellulose. Since sodium molybdate has been reported to inhibit transformation, it was employed as an activation inhibition control for the sedimentation shift and the nuclear and DNA-cellulose binding studies.

Since all the molecular processes involved in the activation-transformation-translocation occur in vivo in the intact cell it seemed logical to employ a tissue slice approach in parallel with a cell free system. In the next sections of this chapter the development of a model system for the study of the activation-transformation and subsequent binding of the activated (transformed) species to target cell nuclei and DNA-cellulose will be described. A need was also felt to employ the model in an investigation into the possibility that differences might exist in the processes of activation-transformation-translocation between normal, benign and malignant uterine tissues. Since abnormal monkey tissues are a rarity it was also decided to employ the model system employing normal, benign and malignant human uterine tissues.

5.4.2 Experimental procedure:

(a) Tissue slice technique:

Tissue slices were prepared from a pool of 10 vervet monkey uteri. Exactly 100 mg of tissue per assay, were incubated in a final concentration of 5 nM of [^{3}H]-oestradiol for varying periods at 3 different temperatures (0°C, 20°C and 37°C) in a CO_2 : air (5:95) atmosphere. In exactly the same fashion, a duplicate set of 100 mg tissue was incubated with a 250-fold excess of DES.

At 15-30 min intervals pairs of tubes for the different incubation conditions were processed as follows: Incubation was terminated by the addition of a relatively large volume(5mℓ) chilled $TEDAG_{10}$ buffer, followed by thorough vortexing and

centrifugation (800xg'x10 min) to remove free ligand. This process was repeated three times. The washed tissue slices were then homogenized manually in a Dounce homogenizer (0-4°C). The 800xg' nuclear pellets were washed (3x) with $TEDAG_{10}$ buffer and then extracted with 1 mℓ of absolute ethanol. Radio-activity was assayed, following the addition of 4 mℓ of scintillation cocktail and counting in a Beckman LS 1800 counter at an efficiency of 25-30 %.

(b) Cell Free System :

A batch of 10-15 VMU were cut into small pieces, which were thoroughly mixed and then divided (by weight) into 2 equal parts. One part was homogenized in $TEDAG_{10}$ buffer containing 20mM sodium molybdate (TEDAG-MO) and the other part in buffer not containing sodium molybdate($TEDAG_{10}$). The homogenates were centrifuged at 800xg'x10 min and the resulting cytosols were separated from the crude nuclear preparation. The cytosols were then centrifuged at 105000xg' for 30 min (0-4°C). The crude nuclear sediments were washed (3x) with the appropriate buffer (with or without sodium molybdate). Cytosols were then incubated for 16 hr at 0°C with a final concentration of 5nM of ^{3}H-oestradiol or ^{3}H-R5020 in the presence (non-specific binding) or absence (total binding) of a 250 fold excess of the appropriate cold ligand (DES or R5020 respectively). The first incubation was then followed up by a second incubation at 30°C (activation) for varying times (0, 5,15 and 30 min).

Incubation was terminated by the addition of the "activated" cytosol to a DCC pellet, followed by vortexing and centrifugation (2000xg'x15 min). The activated cytosol was subsequently divided into 4 different parts which were employed to assay:

(1) total and non-specific cytosolic binding,
(2) sucrose density gradient profiles,

(3) binding to uterine nuclei and
(4) binding to DNA-cellulose

5.4.3 Results

The time course of ligand distribution between the cytosolic and nuclear compartments at different incubation conditions is depicted in Fig. 5.6. As expected, the different temperatures, prevailing during incubations, affected the rate of uptake and distribution of the ligand receptor complexes between the cytosolic and nuclear compartments.

A quantitative analysis of the uptake and distribution of ligand-receptor complexes as a function of both time and temperature of incubation is summarised in Table 5.3 and Fig. 5.6. At 0°C nuclear uptake is suppressed and reaches a plateau at 30 min (29.6 %). Nuclear uptake increases with the duration of incubation and also with temperature. At 37°C a distinct shift of complexes is observed from the cytosolic (71.3 % at 30 min) to the nuclear compartment (65 % at 75 min) as a function of time. This shift also takes place at 20°C but at a reduced rate. This observation fits in with the view, that cellular uptake of the steroid hormone (17-β-oestradiol) is followed by an intracellular redistribution of the ligand, resulting in maximum nuclear accumulation.

At physiological temperatures (37°C) a rapid depletion is noted in the cytosolic complexes with a concomitant increase in nuclear uptake. Because the cytosolic maximum (71.3 % at 30 min) is reached prior to the nuclear maximum (65 % at 75 min) it could imply that some processing of the cytosolic complexes (activation-transformation) is needed to speed up nuclear uptake. Because of several difficulties in the execution of the tissue slices study, it was decided to concentrate on the cell free system. This system consisted of an isolated cytosolic fraction, which is charged with

radioligand, prior to activation (30°C x ti) and binding to nuclei and DNA-cellulose.

The temperature dependent activation of the ER and PR in CYT(+) and CYT(-) cytosols as a function of the duration of the second incubation at 30°C (assessed by SDG centrifugation) is depicted in Fig. 5.7-5.8 for ER and Fig. 5.9-5.10 for PR. The first panel in the figures always serves as a control profile against which the activation transformation process depicted by the profiles in the other panels must be judged.

As a rule at zero time activation for the ER and PR, a main component, sedimenting at 4.3 S was observed in CYT(+) and CYT(-) for both ER and PR. A gradual shift from 4.3 S to 3.98 S and 5.75 S could be demonstrated (Fig. 5.8 and 5.10). At 15 min activation-transformation, the major species is a 5.5 S component. This shift is not observed when sodium molybdate was employed in the preparation of the cytosols (CYT(+)) Fig 5.7.

Even at 30 min activation-transformation the CYT(+) ER sediments as a 4.0 S species a relatively large 8.2 S peak persists. Cytosols employed in the SDG analysis of activation-transformation were simultaneously used in binding assays to uterine nuclei and DNA-cellulose and the results are shown in Fig. 5.11 below.

A gradual increase in nuclear binding (and binding to DNA cellulose) was observed, following activation in the absence of molybdate (CYT(-)). Maximum binding (36.5 % for ER and 51.2 % for PR) occurred after 15 min of activation. Simultaneously, the maximum sedimentation shift could be seen in the hypertonic SDG profits (4.3 S to 5.52 S for ER and 3.7 S to 4.6 for PR). In the presence of molybdate

(CYT(+)),nuclear binding was inhibited (maximum binding 12.5 % for ER and 16.5 % for PR) while the SDG profiles revealed an absence of the sedimentation shift for both ER and PR.

5.5 DYNAMICS OF ACTIVATION - TRANSFORMATION AND NUCLEAR BINDING OF ER AND PR IN NORMAL HUMAN UTERINE TISSUES:

5.5.1 A comparison between the distribution and levels of ER and PR in normal Vervet monkey and human uterine tissues:

Individual Vervet monkey uteri were separated into the anatomically different parts e.g. myometrium, endometrium and cervix. Pools of the various parts were prepared (6-7 uteri) and homogenised in $TEDAG_{10}$-MO buffers as described before. Binding assays for both ER and PR were conducted in parallel with low ionic strength SDG analyses in gradients containing 20 mM sodium molybdate (GRAD(+)) for the pools of individual parts of the uteri.

Human uteri were collected, following clinical hysterectomy for non-pathological (sterilization) or pathological (benign or malignant tumours) reasons. Each uterus was treated in the following manner postoperatively: in the theatre the whole uterus was placed in ice and transported to the laboratory. Blocks of tissue were removed from the fundus, corpus and cervix while the endometrium was scraped off with a curette. A representative sample was obtained from benign or malignant tumours. These "normal" as well as "abnormal" uterine tissues were obtained for every patient. Histological data were obtained for all representative samples collected and used in assays. Binding assays as well as SDG analyses for both ER and PR were done on all the different uterine samples.

5.5.2 Activation, transformation and nuclear binding of PR in normal and abnormal human uterine tissue :

Experimental procedure:

A cell free system was employed as described in Section 5.4. Following overnight incubation of cytosols (CYT(+) and CYT(-)) at 0-4°C with ^{3}H-R5020 (5nm) the receptors were "activated" by reincubation at 30°C for various time intervals (0- 30 min).

SDG gradients were developed on high salt (0.4 M KCl) while nuclear and DNA-cellulose binding studies were performed on the same cytosols. This procedure was repeated for both normal, benign leiomyomatous uterine tissues, as well as malignant cervical carcinomas.

5.5.3 Results :

Binding and SDG data for the different parts of the Vervet monkey uterus is depicted in Table 5.4 and Fig. 5.12 and 5.13.

Similar data for normal and abnormal human uterine tissues is summarised in Table 5.8 while SDG profiles are presented in Fig. 5.14 to Fig. 5.19.

Low ionic strength sucrose density gradients (10-35 %) clearly reveal a 7.1 - 7.7 S specific binding peak for both ER and PR present in the different parts of the VMU (Fig. 5.12 and 5.13). Differences in sedimentation values seem to be insignificant, while the total activity within the main peaks differed for the different parts of the uteri. In rare cases cervix tissues exhibited a clearly defined $\sim$ 4 - 5 S entity (Fig. 5.12 panel A). Levels of PR were always found to be higher than ER levels. Quantitatively (binding assays) the endometrium contains the highest concentration of ER and PR receptors. No significant differences in dissociation constants (K_d) were found (Table 5.4).

In the case of human uterine tissues (Fig. 5.14 to 5.19) low ionic strength SDG profiles showed mainly the faster sedimenting 7.2 - 8.45 component for the different parts of the uterus and also for benign and malignant uterine tumours. This component was observed for both ER and PR. However, SDG profiles for cervix and leimyoma tissues also simultaneously display bands for slower sedimenting, specific binding molecule(s) in the 4-5 S region.

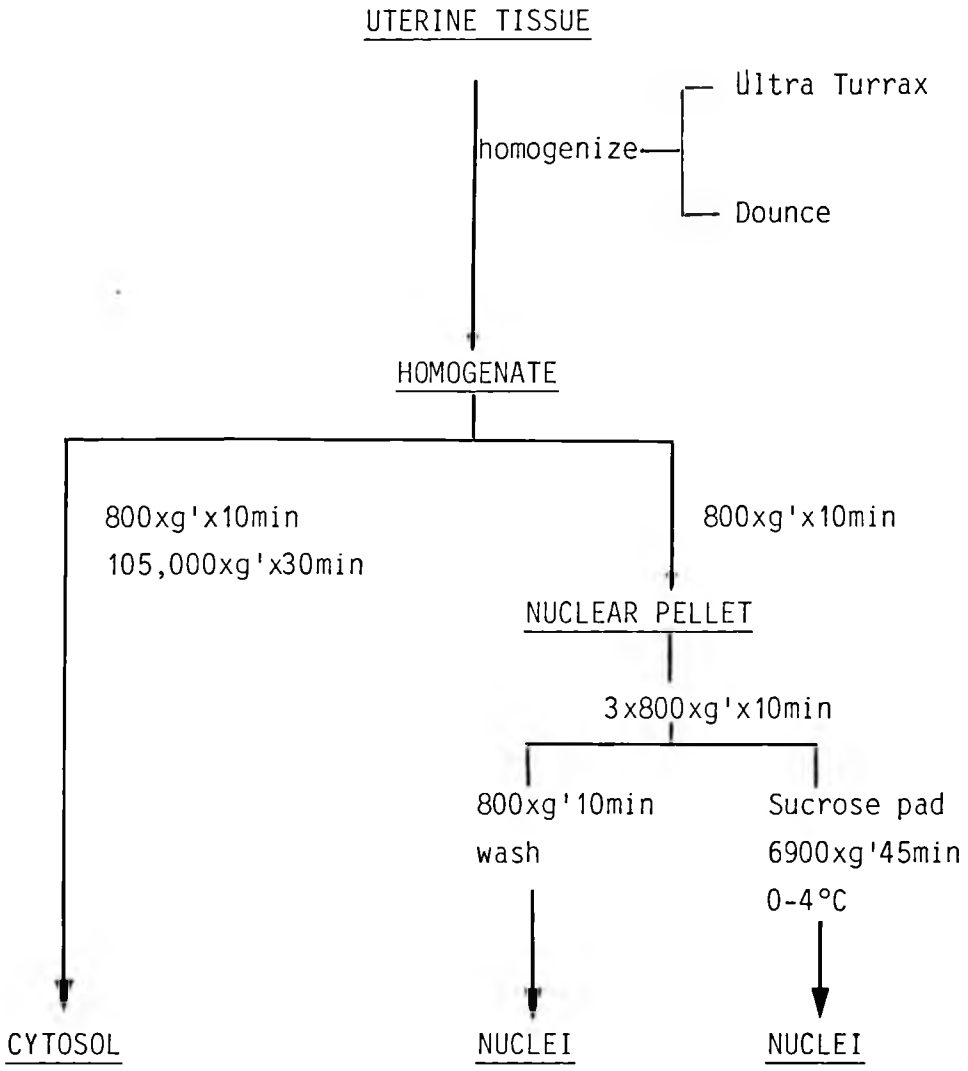
Quantitatively it would seem that receptor levels in normal human uterine tissues were comparable to that found in the VMU. Endometrial tissues generally contained the highest levels of receptors, while cervix tissue appeared to contain very low levels of ER and PR. As for the VMU practically all tissues displayed a PR/ER ratio higher than one. The wide variation observed in receptor levels can be related to the different stages in the menstrual cycle at the time of the hysterectomy. Such a variation is also obvious within different parts of the same uterus.

Binding data of activated cytosol to nuclei are shown in Fig. 5.20 while 0.4 M KCl SDG sedimentation profiles of a selected experiment are depicted in Fig. 5.21.

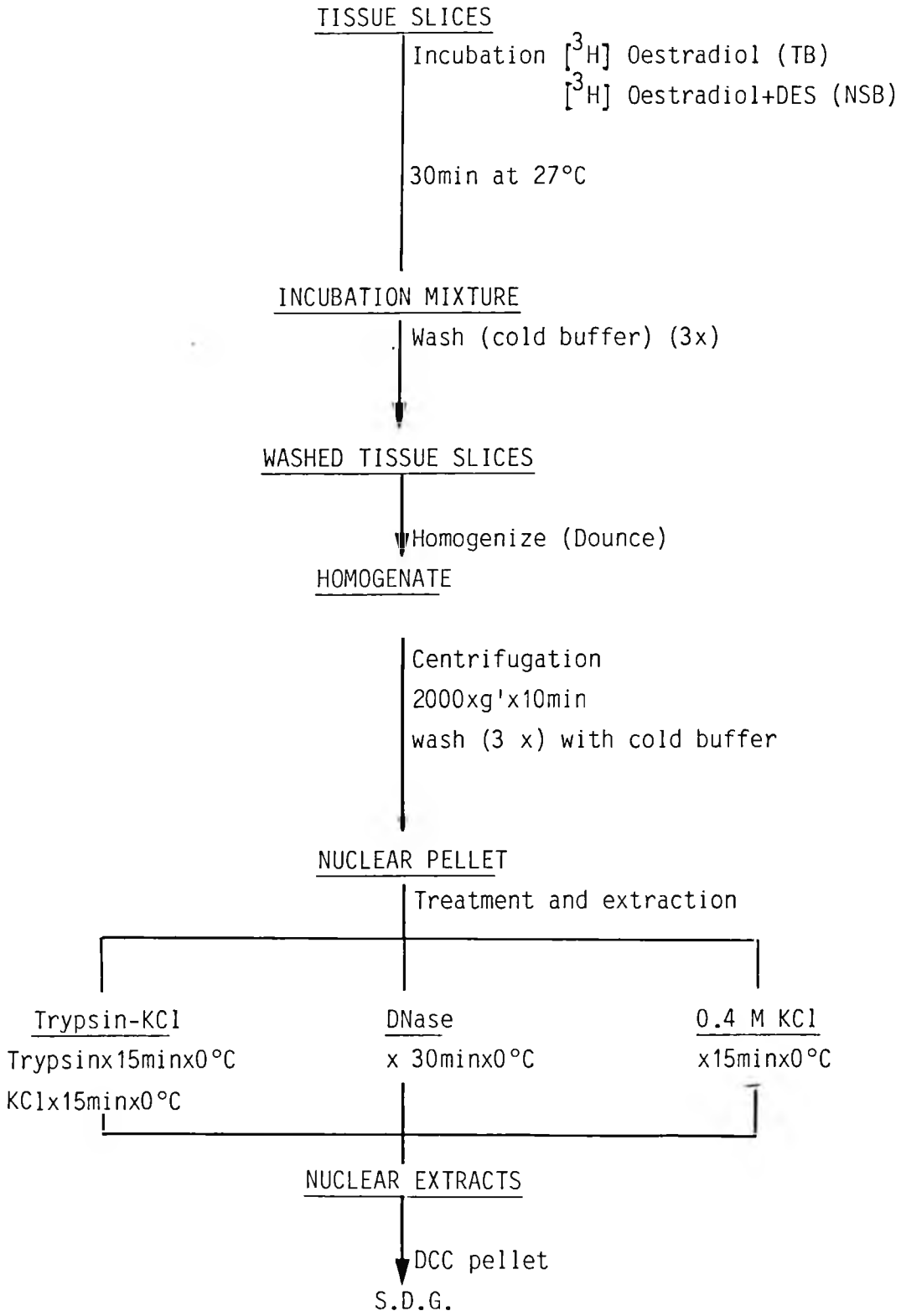
From the binding data it is again clear that maximum activation (re-incubation at 30°C) was obtained after 15 min. In the absence of sodium molybdate, a 4.6 to 5.5 S shift for ER and a 3.7 to 4.6 S shift for PR were observed. This result agreed with that demonstrated for the normal VMU. Again no shift was observed in the sodium molybdate controls a finding which agreed with the fact that nuclear binding was severely suppressed in these cytosols.

In the case of normal human myometrial tissue (Patient 38/83) nuclear binding of PR-ligand complexes was also found to be maximal (80 %) after 15 min of activation at 30°C and also showed a normal 3.7 S to 4.6 S sedimentation shift

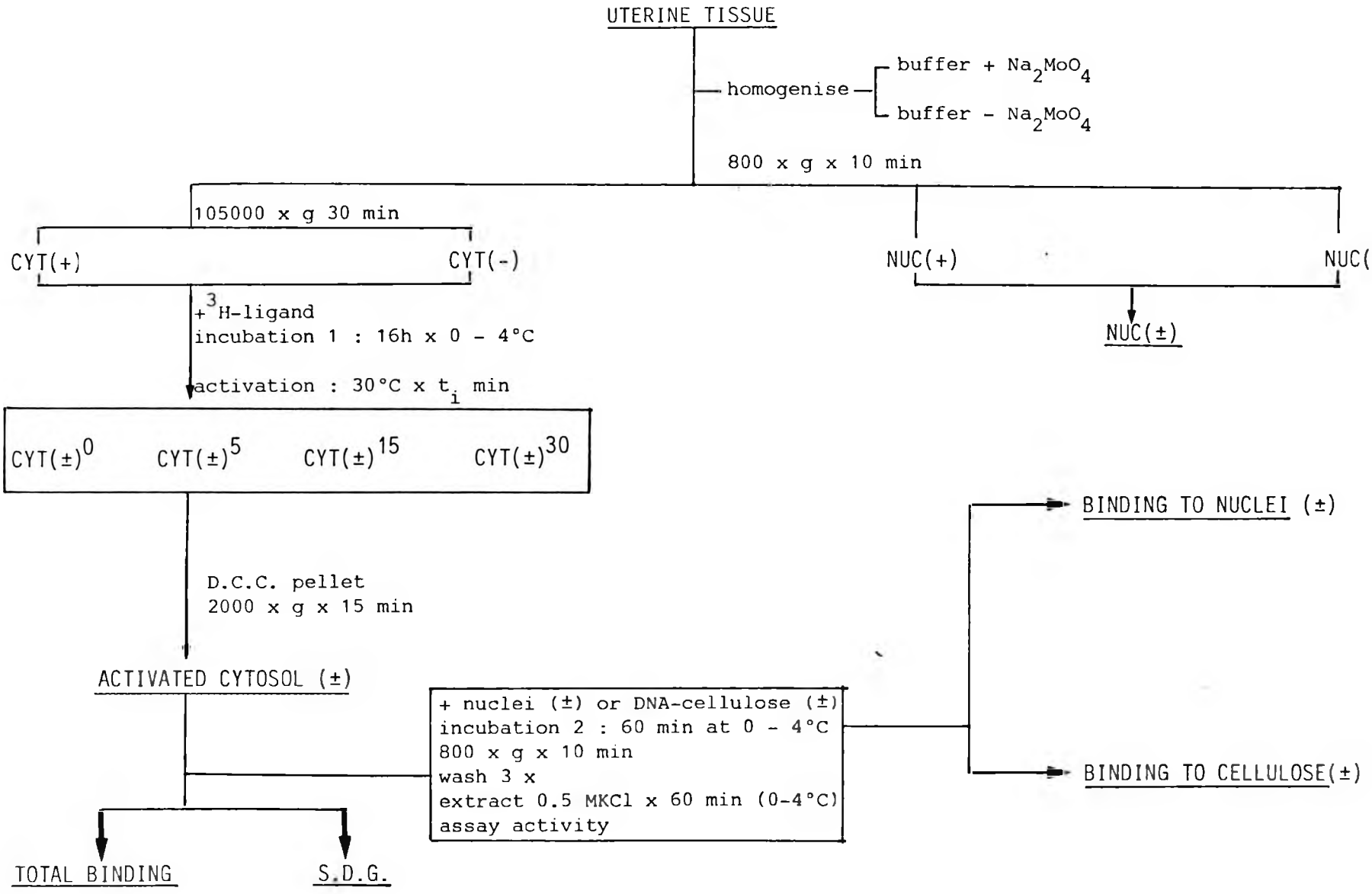
in the absence of molybdate. Abnormal myometrical tissue (leiomyoma, Patient 12/83), however, exhibited subnormal nuclear binding, while at the same time totally lacked the ability to yield a 4 S to 5 S sedimentation shift. This same tendency was observed (Fig. 5.20) in 3 samples of abnormal cervical tissue (carcinoma in situ) : subnormal nuclear binding accompanied by the lack of the sedimentation shift (Fig. 5.21). Due to difficulties in obtaining human uterine tissue, experienced at the time of this investigation only a small number (3) of abnormal gradient samples were investigated. Thus, this finding can not be generalised. However, we hope to be in a position to continue with the investigation as soon as sufficient patient material can be obtained.



FLOW DIAGRAM 5.1 : Preparation of cytosols and nuclei from vervet monkey and human uterine tissue for the investigation of the dynamics of activation-transformation and translocation of receptor-ligand complexes to the nuclear compartment.



FLOW DIAGRAM 5.2 : Comparison of various treatment and extraction procedures for the quantitative and qualitative assay of nuclear VMU ER.



FLOW DIAGRAM 5.3 : Methodology of the activation-translocation system

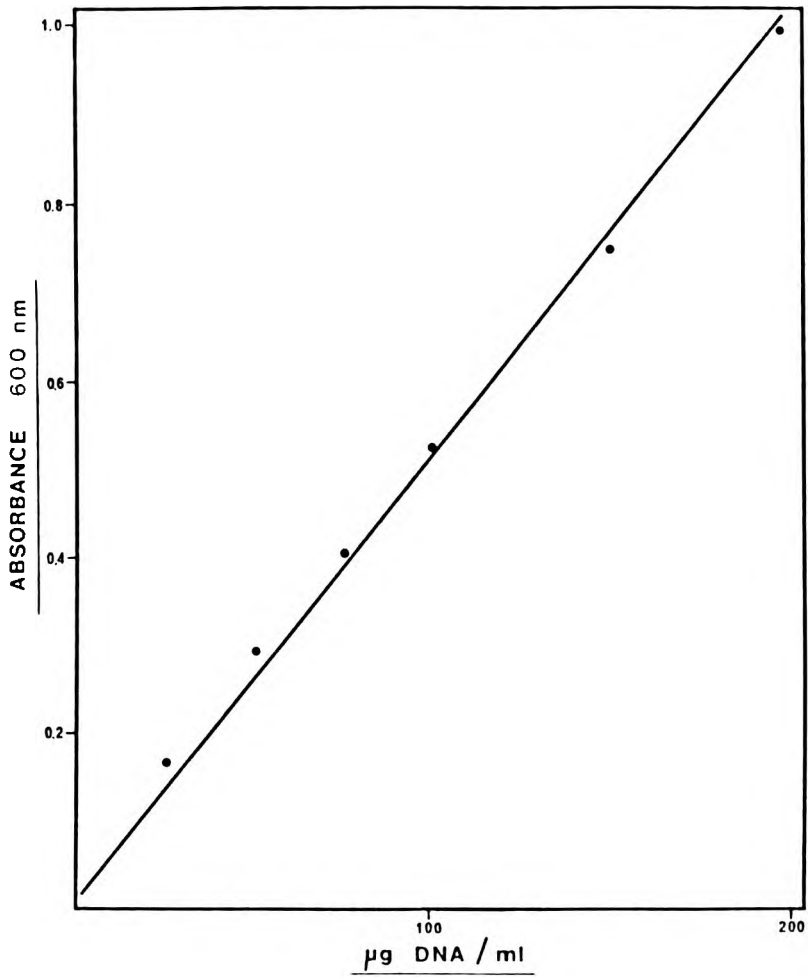


Figure 5.1 : A representative standard curve for the DNA assay. A modification of Burton's method (274) was employed (Section 5.2.3.) Different dilutions of calf thymus DNA were prepared in 5 mM NaOH, varying from 25 to 300 µg/mℓ DNA. Absorbance of the reaction mixture was measured at 600 nm and the DNA concentration was plotted against absorbance.

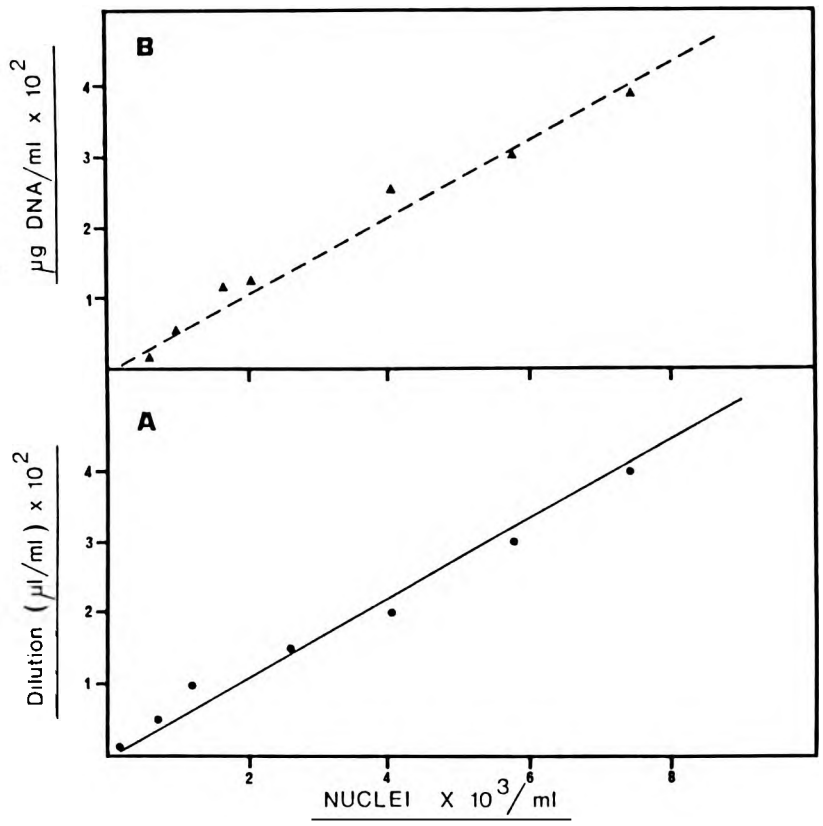


Figure 5.2 : Correlation between the total number of nuclei and the concentration of DNA. Nuclei were prepared as described in Section 5.2.2 and serial dilutions made : (A) the number of nuclei was determined for each of the dilutions by means of the Coulter counter (B) the DNA content of each dilution was also estimated, employing the method of Burton (274) described in Section 5.2.3.

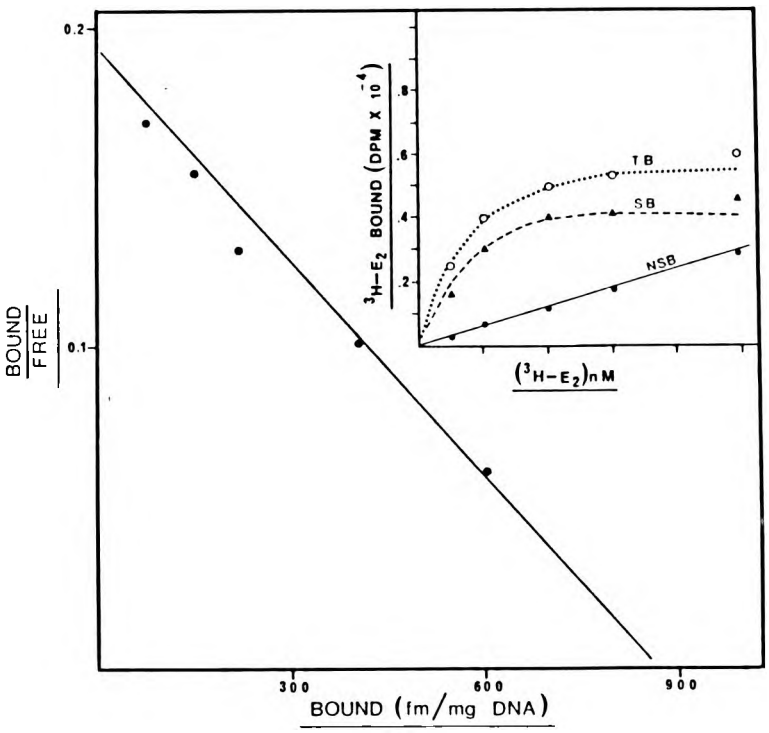


Figure 5.3 : Representative Scatchard plot for the estimation of nuclear VMU ER. Nuclei were prepared as described in Section 5.3.1.2. Aliquots (100 µℓ) of the homogenous nuclear suspension were added to tubes containing 100µℓ of 5 different ^{3}H-E$_2$ concentration (0.5-8nM) in the absence (total binding) and presence (non-specific binding) of a 100 fold excess of DES. Incubation was executed at 37°C for 30 min. The reactions were terminated by the addition of a large volume (5 mℓ) of cold TEDAG$_{10}$ buffer and nuclei spun down at 800xg' x15 min. Further washings (3x) were performed to remove all the unbound ligand and the 800xg' supernatants decanted. Ethanol (1.0 mℓ) was added to the nuclear pellet and the suspension thoroughly vortexed for 1 min after which the ethanolic extracts were monitored for total and non-specific binding.

TABLE 5.1

Summary of binding data obtained for the oestrogen receptors in VMU nuclei:

no of uteri	fmol/mg DNA	$K_d \times 10^{-9}$ M
3	3152	5.35
2	4032	4.16
1	782	5.2
∿ 15 (powder)	1818	2.15
∿ 15	1474	2.3
1	433	3.2
1	365	7.2
3	848	1.9
14	429	2.08
Tissue powder	1498	6.75
Mean	1483±1236	4.02±1.8

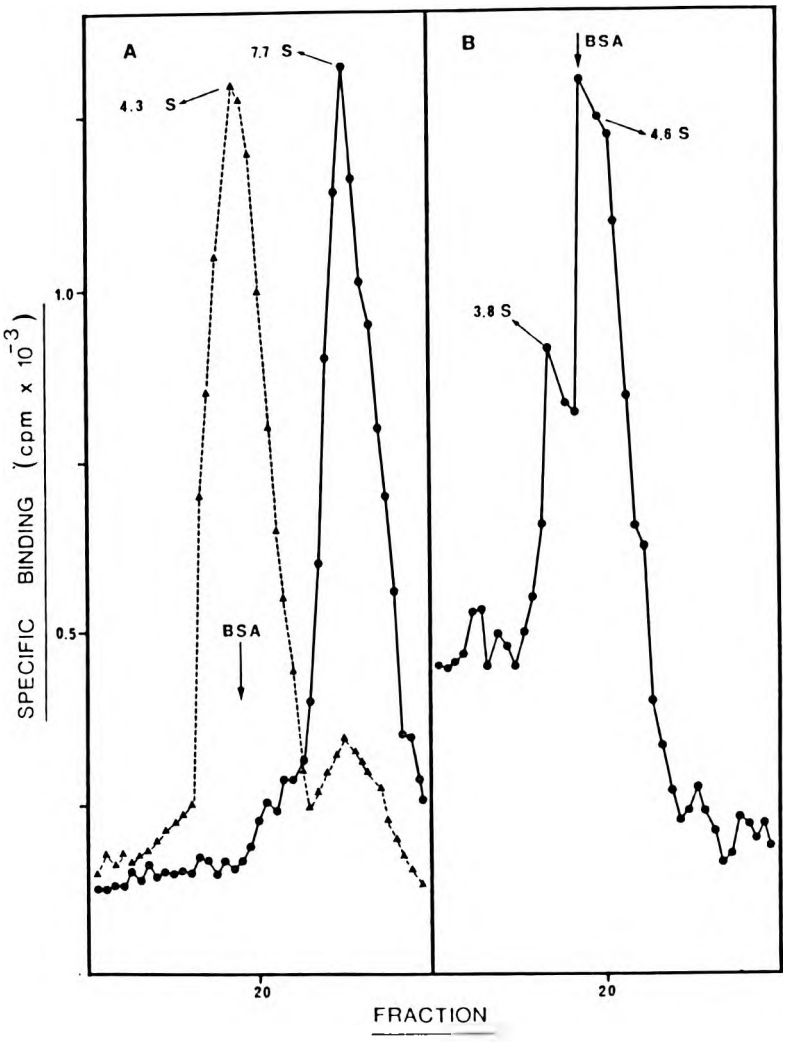


Figure 5.4 : A comparison of the cytosolic and nuclear VMU ER on sucrose density gradients. Uterine slices were incubated (20°Cx30 min) with radioligand (^{3}H-E_2 ; 5nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess DES. Upon completion of the incubation, a large volume of $TEDAG_{10}$ buffer was added to the reaction suspension. The tissue slices were spun down at 800xg'x10 min, resuspended in fresh cold $TEDAG_{10}$ buffer and the washing sequence repeated 3 times to remove unbound and non-specifically tissue adsorbed ligand molecules. The tissue slices were then manually homogenized in a Dounce ground glass homogenizer with a loose fitting pestle at 0-4°C. The homogenate was spun at 800xg' for 15 min and the nuclear myofibrillar pellet thoroughly washed, prior to extraction with 0.4 M KCl (15 min at 0-4°C). The 800xg' supernatant (crude cytosol) was recentrifuged at 105000xg' for 30 min, treated with DCC and then subjected to either hypo- or hypertonic SDG analysis : (A) 105000xg' cytosolic receptor in hypotonic SDG (●); 105000xg' cytosolic receptor in hypertonic SDG (▲); (B) 0.4 M KCl nuclear extract on hypertonic SDG.

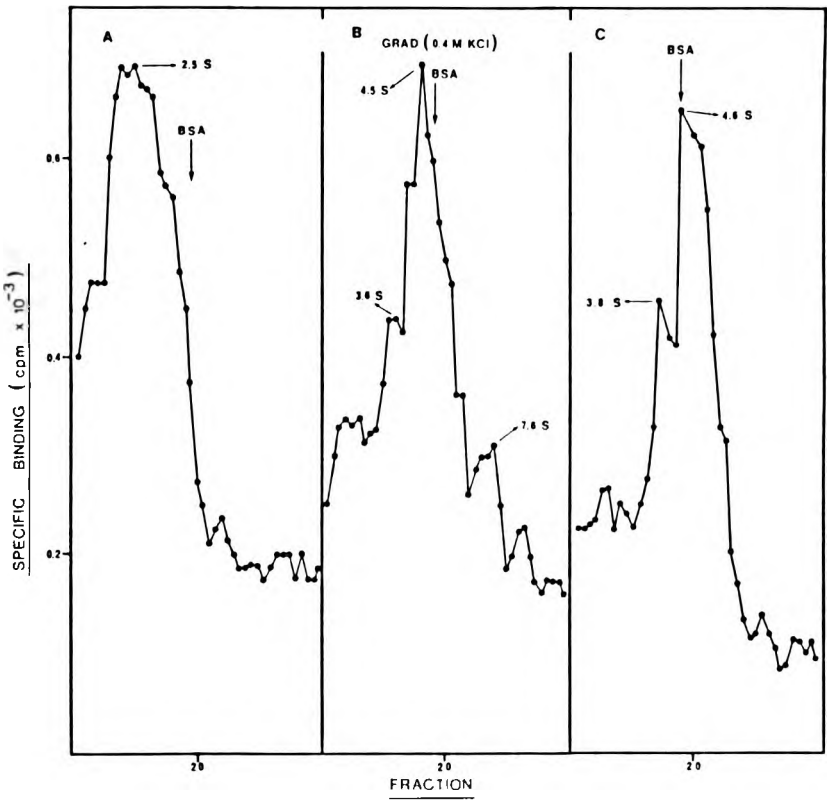


Figure 5.5 : A comparison of VMU nuclear ER species obtained with various extraction procedures : nuclear myofibrillar pellets were prepared and processed as described in the legend of Fig. 5.4 and in Section 5. Three different procedures were employed to extract the nuclear ER from the nuclear pellets : (A) buffer suspensions of the nuclear sediments were treated with trypsin (50µg) for 15 min at 0°C and then extracted with 0.4 M KCl at 0-4°C for 15 min; (B) nuclear pellets were treated with DNase I for 15 min at 0-4°C; (C) nuclear pellets were extracted with 0.4 M KCl;

TABLE 5.2

Summary of S-values for ER obtained by different treatments of nuclei

No. of assays	Method of treatment	Mean Sedimentation	Efficiency of treatment and extraction
10	0.4 M KCl (15 minx0°C)	3.8; 4.6	12.5 %
10	trypsin KCl (15 minx0°C;15 minx0°C)	2.5;	47 %
10	DNase (30 minx0°C)	3.6; 4.6	26.7 %

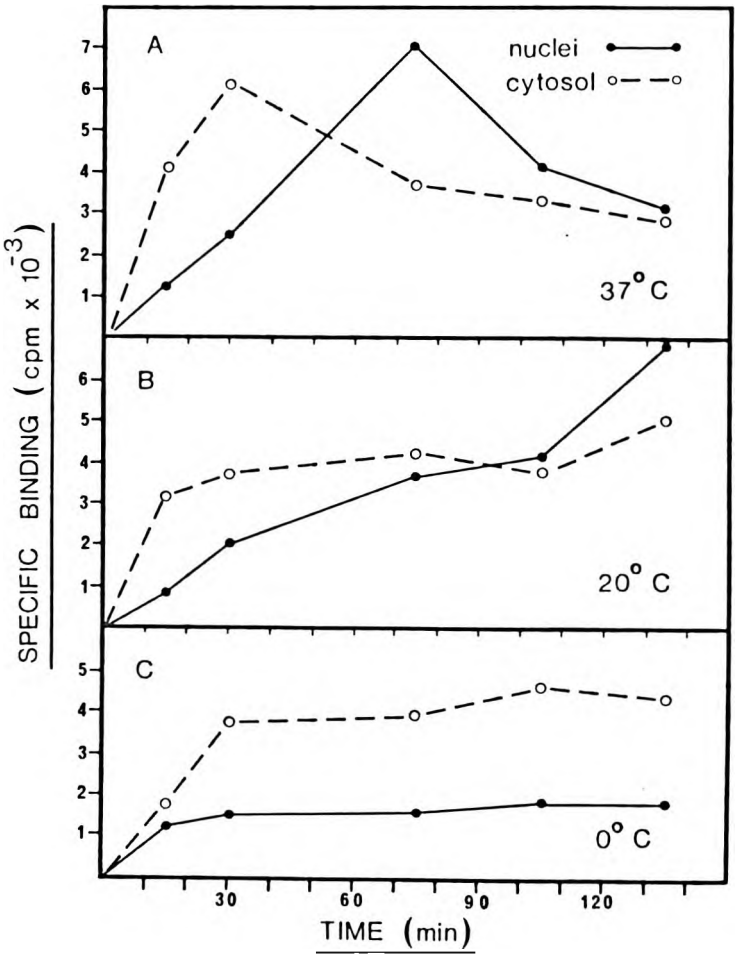


Figure 5.6 : Activation, transformation and translocation of Vervet monkey uterine ER in tissue slices as functions of time and temperature: Tissue slices were incubated with ^{3}H-oestradiol (final concentration, 5nM) without (total binding) or with (non-specific binding) a 250 fold excess of DES at the temperatures indicated above. Upon completion of incubation the reaction was terminated by the addition of a large volume (5 mℓ) of cold $TEDAG_{10}$ buffer. The tissue slices were thoroughly washed in $TEDAG_{10}$ buffer (3x with 5 mℓ buffer) to remove unbound and non-specifically tissue adsorbed ligand and then homogenized in a Dounce ground glass homogenizer. The homogenate were centrifuged at 800xg' for 10 min (0-4°C) and the crude nuclear pellets, as well as the 800xg cytosols, assayed for (A) incubation at 37°C in a CO_2 incubator (5% CO_2, 95 % air; (B) incubation at 20°C in water bath and (C) incubation on ice (0°C).

TABLE 5.3

Distribution of ligand-receptor complexes at different incubation temperatures in a time course study, employing tissue slices:

Temp of incubation (°C)	Distribution of ER between nuclear(N) and cytosolic(C) compartments					
	30 min		75 min		135 min	
	N (%)	C (%)	N (%)	C (%)	N (%)	C (%)
0	29.6	70.4	29.6	70.4	29.6	70.4
20	35.6	64.4	46.9	53.1	51.9	42.1
37	28.7	71.3	65.0	35.0	51.6	48.4

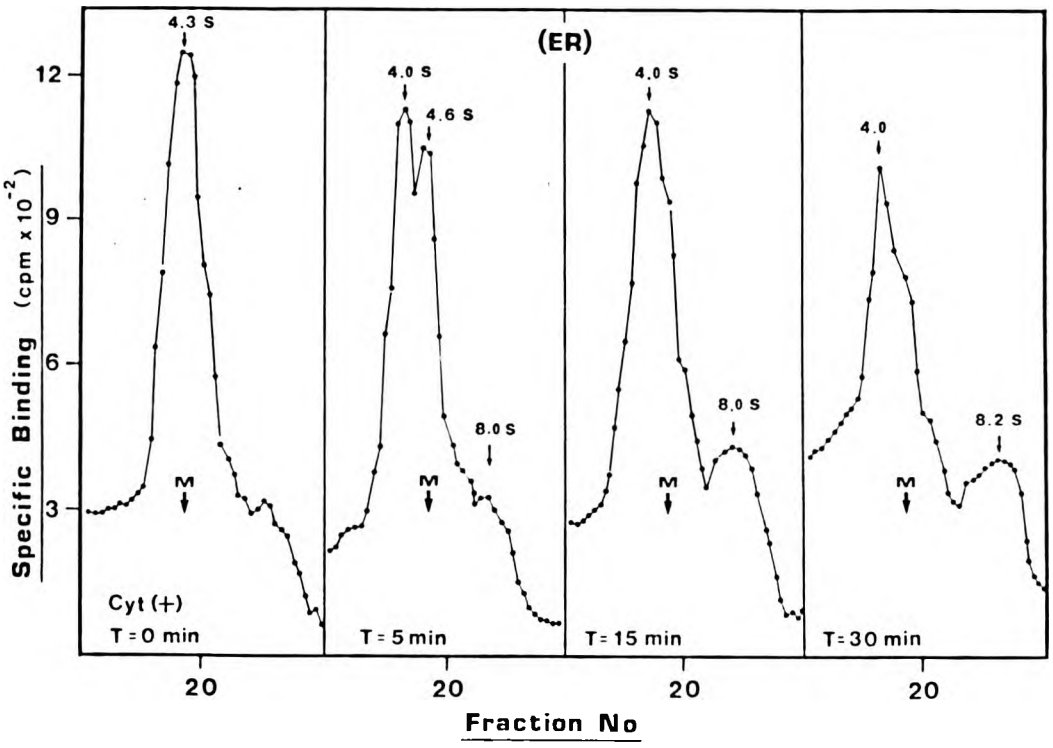


Figure 5.7 : Effect of sodium molybdate (10mM) on the time-dependent transformation of Vervet monkey uterine ER, as followed by SDG analysis. Cytosols containing sodium molybdate (CYT(+)) were incubated on ice (0°C) for 10 h with ^{3}H-E_2 (5nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess DES. At the end of the incubation the reaction tubes were transferred to a 30°C water bath for different time intervals (5 min, 10 min and 15 min). Temperature induced activation was terminated by DCC treatment on ice (0°C) and the resulting 2000 xg' supernatants (200µℓ) were subjected to ultra-centrifugation (VTi-80 rotor, 520000xg'x120 minx2°C) on 10-35% sucrose density gradients. Gradients were then fractionated (40x5-drop fractions) and the fractions monitored for radio-activity. Position of the marker protein (^{14}C-BSA; 4.6S) is indicated by M.

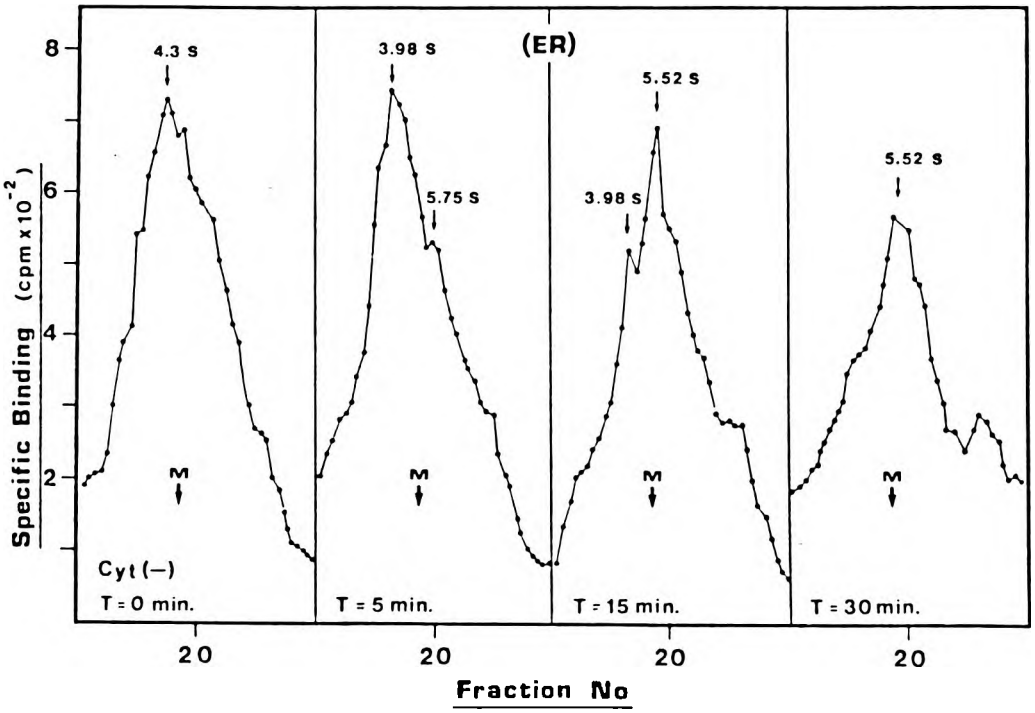


Figure 5.8 : Time-dependent transformation of Vervet monkey uterine ER, as followed by SDG analysis. Cytosols without sodium molybdate (CYT(-)) were incubated on ice (0°C) for 10 h with ^{3}H-E_2 (5nM) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess DES. At the end of the incubation the reaction tubes were transferred to a 30°C water bath for different time intervals (5 min, 10 min and 15 min). Temperature induced activation was terminated by DCC treatment on ice (0°C) and the resulting 2000 xg' supernatants (200µℓ) were subjected to ultra-centrifugation (VTi-80 rotor, 520000xg'x120 minx2°C) on 10-35% sucrose density gradients. Gradients were then fractionated (40x5-drop fractions) and the fractions monitored for radio-activity. Position of the marker protein (^{14}C-BSA; 4.6S) is indicated by M.

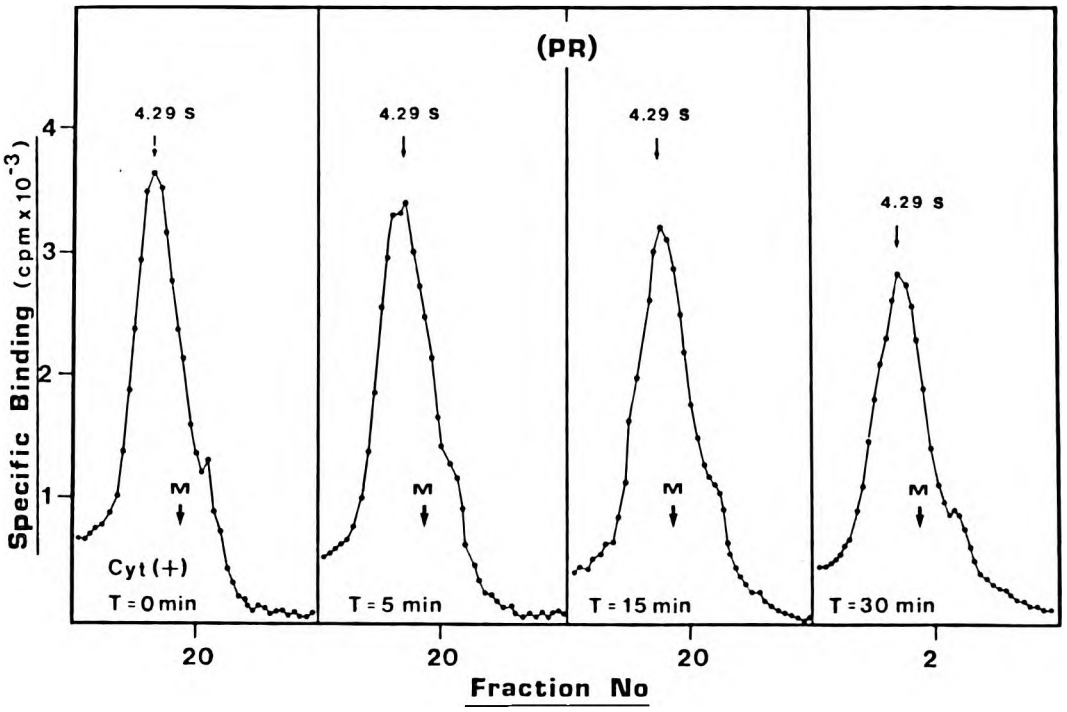


Figure 5.9 : Effect of sodium molybdate on the time-dependent transformation of Vervet monkey uterine PR as determined by hypertonic (0.4 M KCl) sucrose density gradient centrifugation. Cytosols, prepared in the presence of 20mM sodium molybdate ((CYT(+)) were incubated (0-4°Cx16 h) with ^{3}H-R 5020 (5nM final concentration) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess R 5020. Incubation was followed up by a temperature activation at 30°C for varying intervals (5 min, 15 min and 30 min), the reactions terminated by DCC treatments and the resulting 2000xg' supernatants (200µℓ) subjected to ultra-centrifugation (VTi-80 rotor 152000 xg'x120 min x 2°C) on hypertonic 10-35 % sucrose density gradients. A control set of cytosols were run simultaneously, with no activation step included, prior to SDG analysis. The marker position (^{14}C-BSA) is indicated by M.

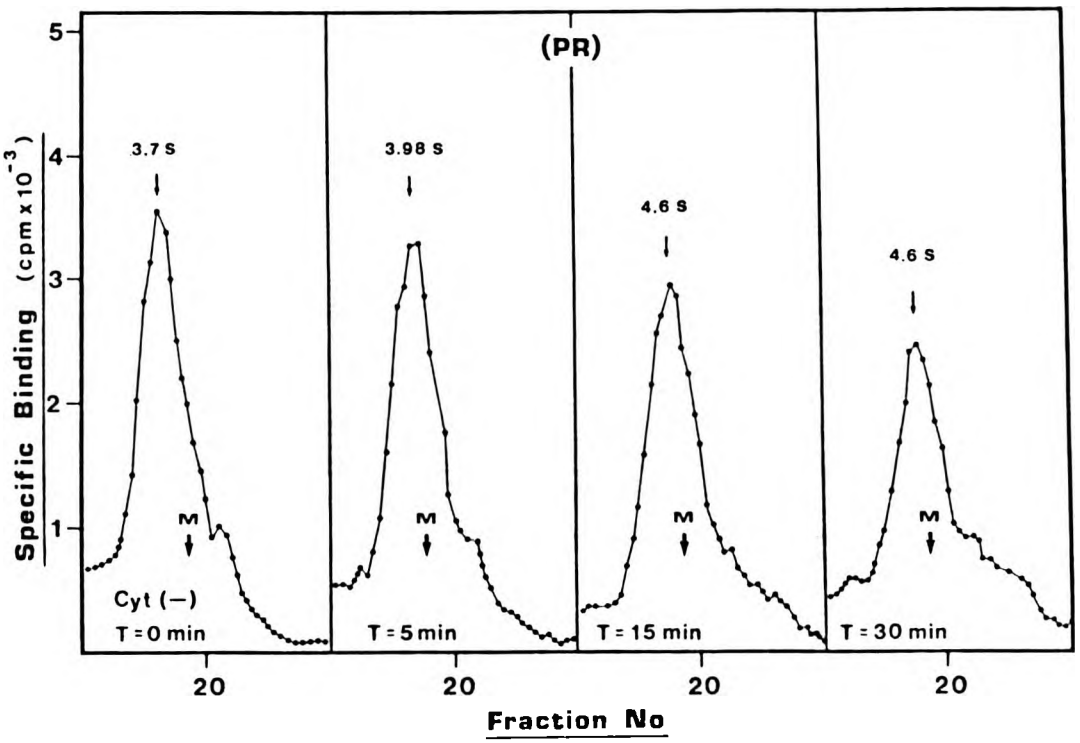


Figure 5.10: Time-dependent transformation of Vervet monkey uterine PR as determined by hypertonic (0.4 M KCl) sucrose density gradient centrifugation. Cytosols, prepared in the absence sodium molybdate ((CYT(-)) were incubated (0-4°C x16 h) with ^{3}H-R 5020 (5nM final concentration) in the absence (total binding) or presence (non-specific binding) of a 250 fold excess R 5020. Incubation was followed up by a temperature activation at 30°C for varying intervals (5 min, 15 min and 30 min), the reactions terminated by DCC treatments and the resulting 2000xg' supernatants (200µℓ) subjected to ultra-centrifugation (VTi-80 rotor 152000 xg'x120 min x 2°C) on hypertonic 10-35 % sucrose density gradients. A control set of cytosols were run simultaneously, with no activation step included, prior to SDG analysis. The marker position (^{14}C-BSA) is indicated by M.

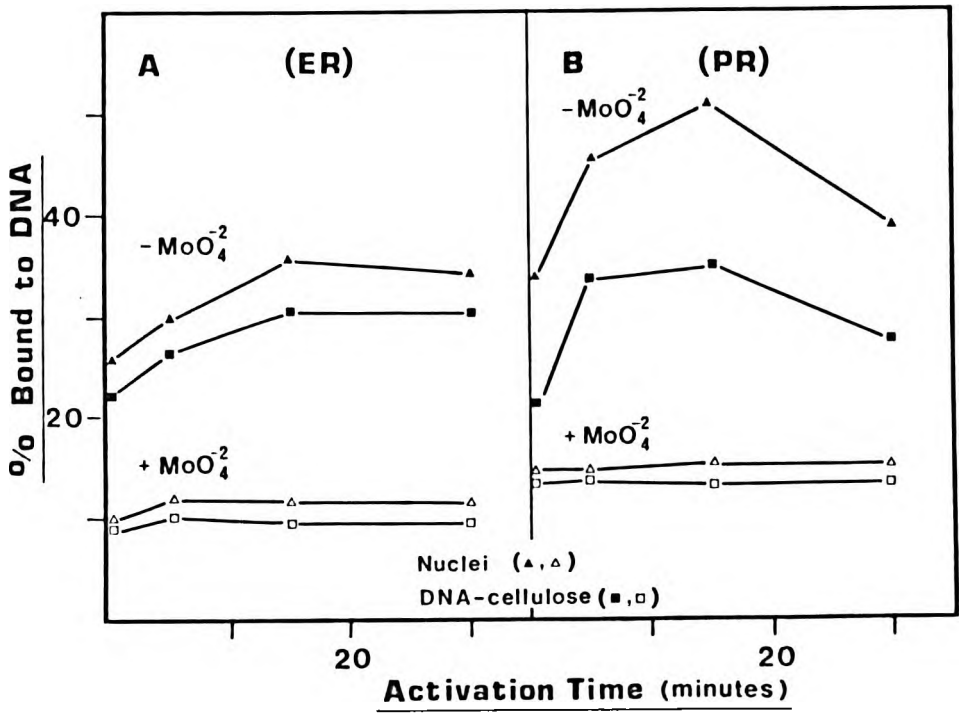


Figure 5.11: Time-dependent activation of Vervet monkey uterine ER and PR as measured by increased binding of complexes to nuclei and DNA-cellulose. Cytosols and nuclei were prepared with (□△) or without (■▲) sodium molybdate (20 mM) in homogenization buffers. Activation of cytosolic receptors was accomplished as follows: (a) first incubation : aliquots of cytosols (100µℓ) were charged with the radioligand (^{3}H-E_2 or ^{3}H-R5020); for 16 h on ice, in the absence (total binding) or presence (non-specific binding) of a 250 fold excess cold ligand (DES or R5020); (b) second incubation : all tubes were transferred to a water bath of 30°C for different periods (5 min, 15 min and 30 min). The charged and activated cytosols were treated with DCC and the 2000xg' supernatants reincubated with uterine nuclei or DNA-cellulose (see Flow diagram 5.2) for 60 min on ice. The binding assays were terminated by the addition of a large volume (5 mℓ) $TEDAG_{10}$ buffer and the 800xg' nuclear pellet washed (3x) with buffer, prior to the addition of absolute ethanol (1 mℓ). The ethanol extracts were decanted into counting vials, scintillation cocktail added (2 mℓ) and the vials counted in a scintillation counter (efficiency, 30-35%).

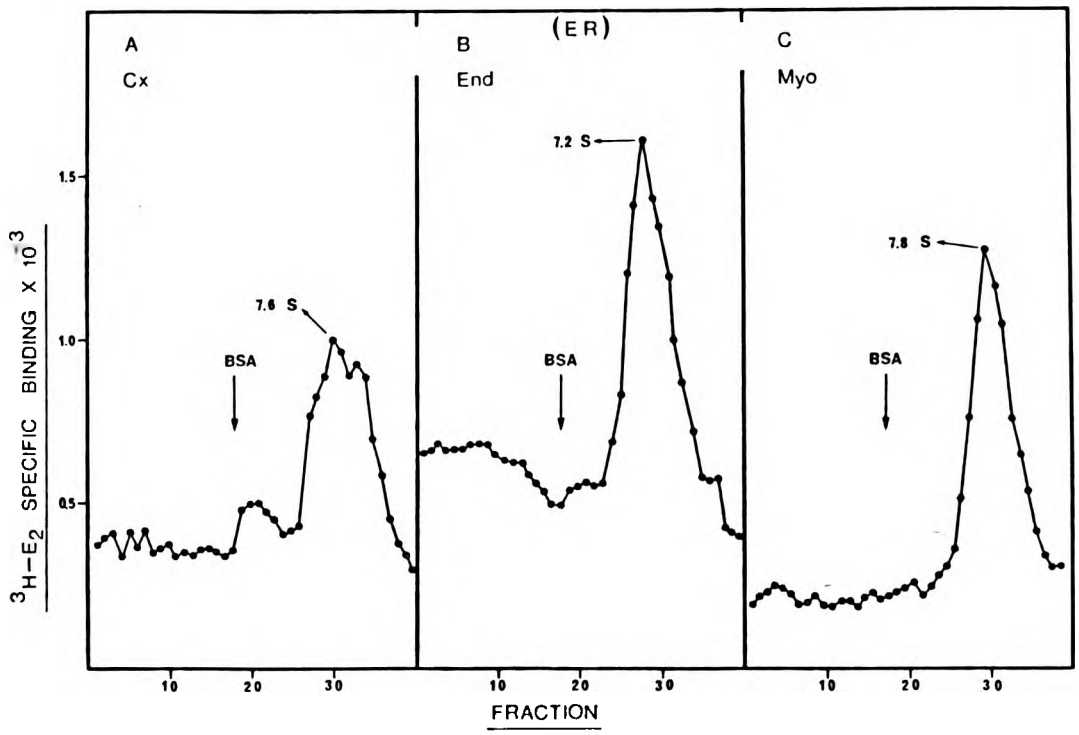


Figure 5.12 : Comparison of SDG profiles for ER in different parts of the Vervet monkey uterus : Cytosols were prepared in buffer containing 20mM sodium molybdate ((CYT(+)) and labelled with ^{3}H-E$_2$ (5nM final concentration) in the absence (total binding) or presence (non-specific binding) of an excess (250µ) of DES. The DCC treated 2000g' supernatants were layered onto hypotonic sucrose gradients subjected to ultra-centrifugation (VTi-rotor, 520000xg'x120 min x 2°C) and the gradients fractionated into 40, five drop fractions. Fractions were counted following the addition of scintillation cocktail (2 mℓ) : hypotonic, 10-35% SDG profile for the cervix cytosol (A), the endometrial cytosol (B) and the myometrial cytosol (C). Position of labelled marker protein (^{14}C-BSA) indicated by M.

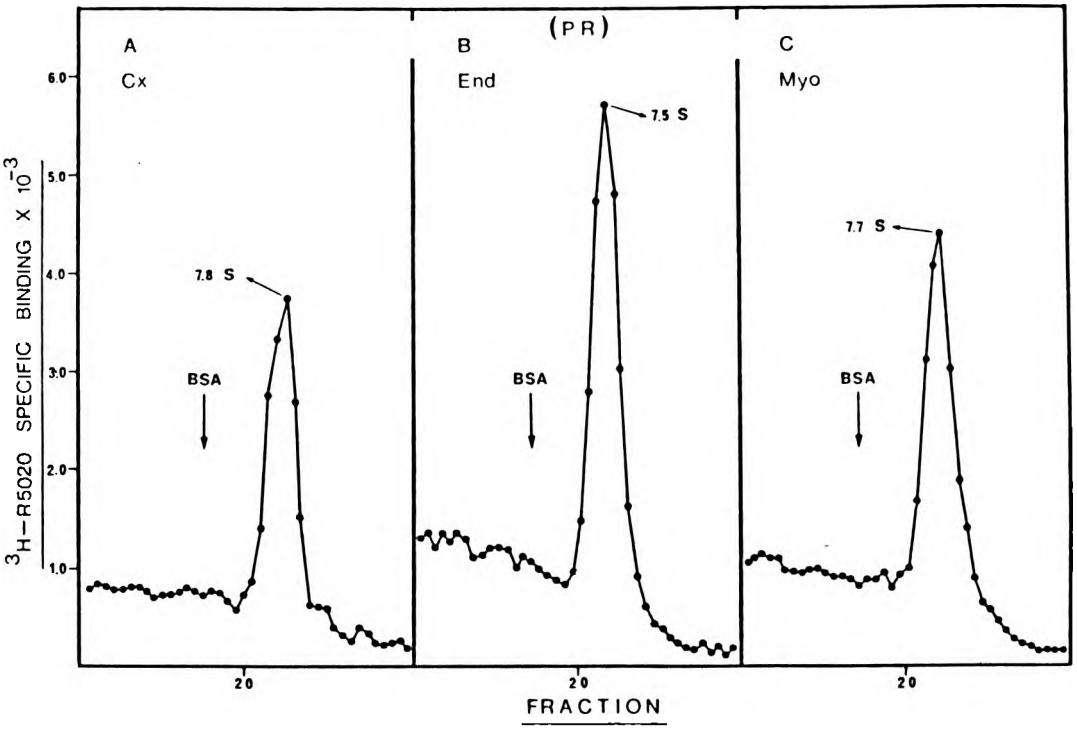


Figure 5.13 : Comparison of SDG profiles for PR in different parts of the Vervet monkey uterus : Cytosols were prepared in buffer containing 20mM sodium molybdate ((CYT(+)) and labelled with ^{3}H-R5020 (5nM final concentration) in the absence (total binding) or presence (non-specific binding) of an excess (250µ) of R5020. The DCC treated 2000g' supernatants were layered onto hypotonic sucrose gradients subjected to ultra-centrifugation (VTi-rotor, 520000xg'x120 min x 2°C) and the gradients fractionated into 40, five drop fractions. Fractions were counted following the addition of scintillation cocktail (2 mℓ) : hypotonic, 10-35% SDG profile for the cervix cytosol (A), the endometrial cytosol (B) and the myometrial cytosol (C). Position of labelled marker protein (^{14}C-BSA) indicated by M.

TABLE 5.4

Summary of binding and SDG data in different parts of the VMU

Part of uterus	Binding data				Sedimentation coefficients (S)	
	ER		PR		ER	PR
	B_{max} (fmol/mg prot)	K_d ($\times 10^{-10}$ M)	B_{max} (fmol/mg prot)	K_d ($\times 10^{-9}$ M)		
Endometrium	987	0.67	3937	1.1	7.1	7.4
Myometrium	337	0.85	3558	4.0	8.6	7.8
cervix	370	0.65	2032	4.3	7.6	7.7

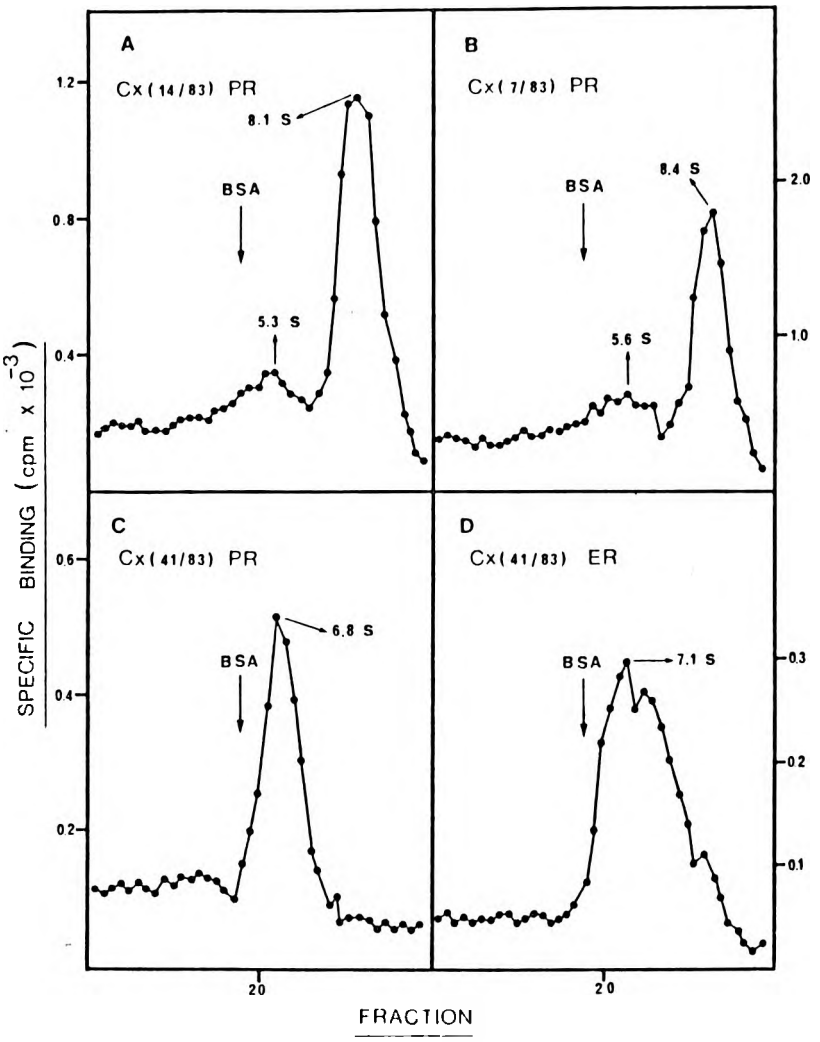


Figure 5.14: Hypotonic sucrose density gradient analysis of cytosolic ER and PR present in human uterine cervices. Cytosols were prepared in TEDAG-MO buffer (20mM sodium molybdate) and aliquots (200µℓ) of the freshly prepared cytosols incubated (30 min x 20°C) with radioligand (^{3}H-E_2 or ^{3}H-R-5020) in the presence (non-specific binding) or absence (total binding) of 250 fold excess of unlabelled ligand (DES or R5020). The DCC treated supernatants (2000xg'x10 min) were layered onto linear 10-35% hypotonic SDG. Gradients were subjected to ultra-centrifugation (VTi-80 rotor, 520000xg'x2hx2°C). Gradients were fractionated (5 dropsx40) manually, scintillation cocktail added (2 mℓ) and then monitored for radioactivity. Multipoint titration analyses were performed on all cytosols and binding data analysed according to the method of Scatchard. Marker protein (^{14}C-BSA) position is indicated by BSA: representative SDG profiles of cervix PR (A,B,C) and ER(D) of patients 14/83, 7/83 and 41/83; representative SDG profile of the cervix ER for patient 41/83.

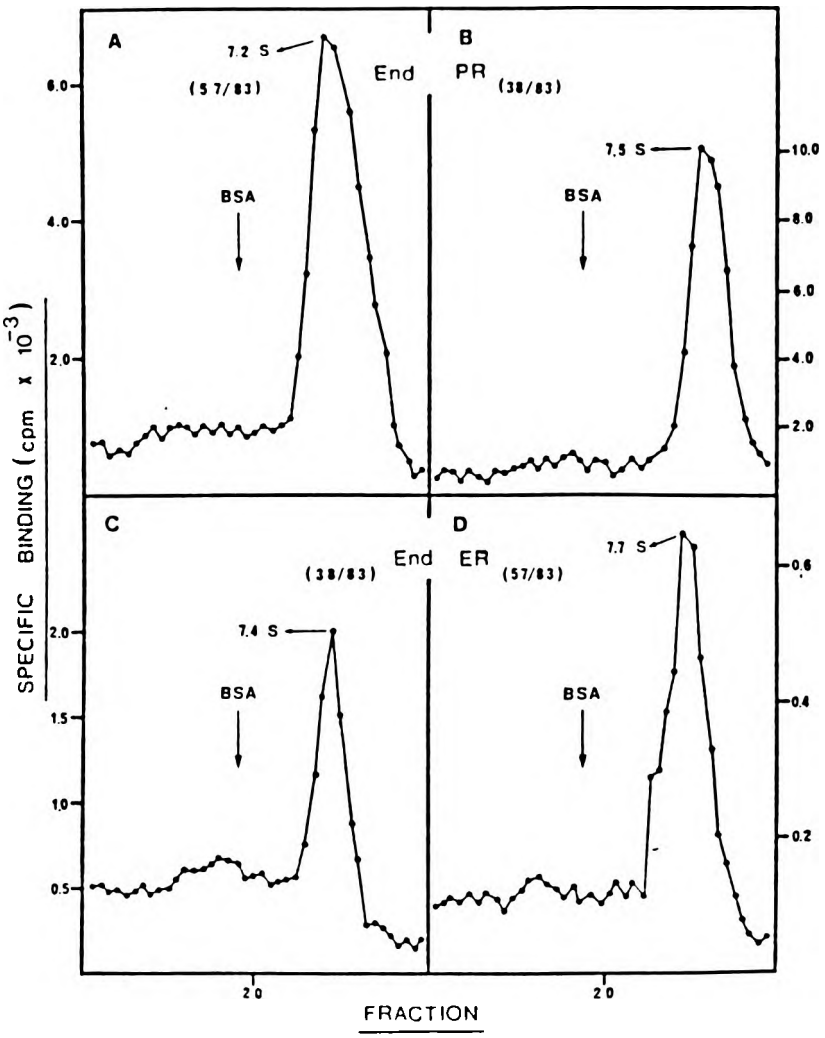


Figure 5.15: Hypotonic sucrose density gradient analysis of cytosolic ER and PR present in human uterine endometria. Cytosols were prepared in TEDAG-MO buffer (20mM sodium molybdate) and aliquots (200µℓ) of the freshly prepared cytosols incubated (30 min x 20°C) with radioligand (^{3}H-E_2 or ^{3}H-R-5020) in the presence (non-specific binding) or absence (total binding) of 250 fold excess of unlabelled ligand (DES or R5020). The DCC treated supernatants (2000xg'x10 min) were layered onto linear 10-35% hypotonic SDG. Gradients were subjected to ultra-centrifugation (VTi-80 rotor, 520000xg'x2hx2°C). Gradients were fractionated (5 dropsx40) manually, scintillation cocktail added (2 mℓ) and then monitored for radioactivity. Multipoint titration analyses were performed on all cytosols and binding data analysed according to the method of Scatchard. Marker protein (^{14}C-BSA) position is indicated by BSA: representative SDG profiles of the endometrial PR(A,B) and ER(C,D) for patients 57/83 38/83.

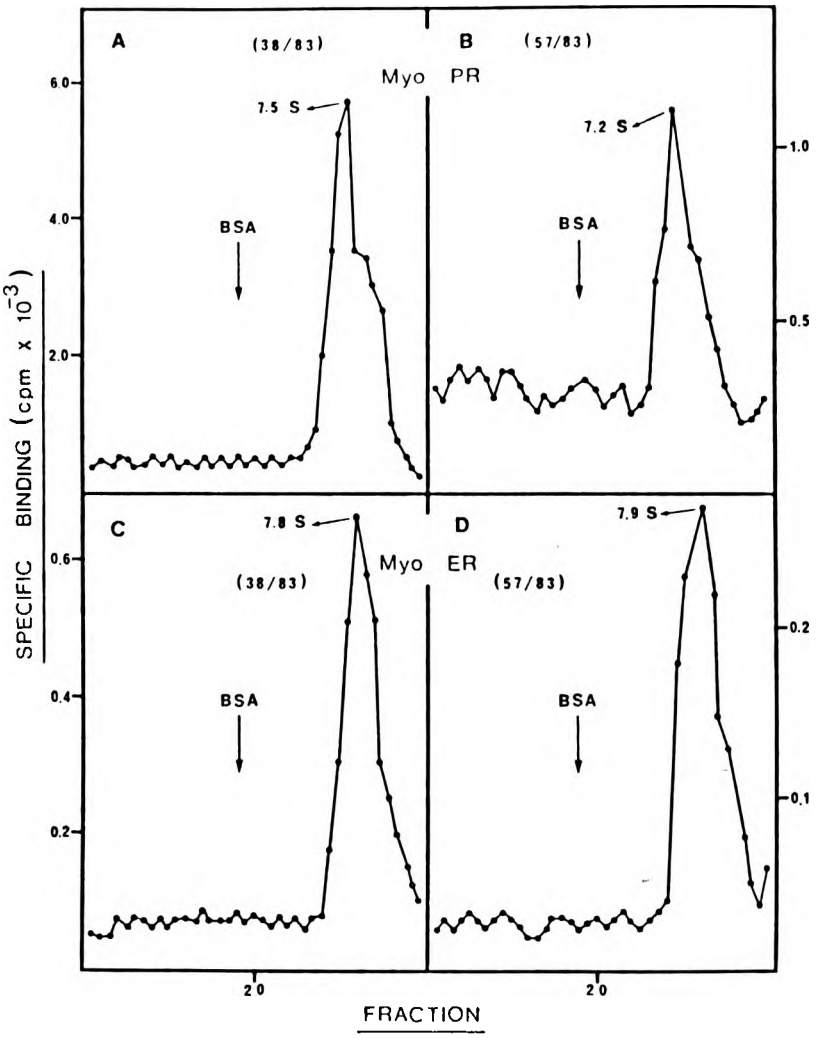


Figure 5.16: Hypotonic sucrose density gradient analysis of cytosolic ER and PR present in human uterine myometria. Cytosols were prepared in TEDAG-MO buffer (20mM sodium molybdate) and aliquots (200µℓ) of the freshly prepared cytosols incubated (30 min x 20°C) with radioligand (^{3}H-E_2 or ^{3}H-R-5020) in the presence (non-specific binding) or absence (total binding) of 250 fold excess of unlabelled ligand (DES or R5020). The DCC treated supernatants (2000xg'x10 min) were layered onto linear 10-35% hypotonic SDG. Gradients were subjected to ultra-centrifugation (VTi-80 rotor, 520000xg'x2hx2°C). Gradients were fractionated (5 dropsx40) manually, scintillation cocktail added (2 mℓ) and then monitored for radioactivity. Multipoint titration analyses were performed on all cytosols and binding data analysed according to the method of Scatchard. Marker protein (^{14}C-BSA) position is indicated by BSA: representative SDG profiles of myometrial PR (A,B,) and ER(C,D) of patients 38/83 and 57/83.

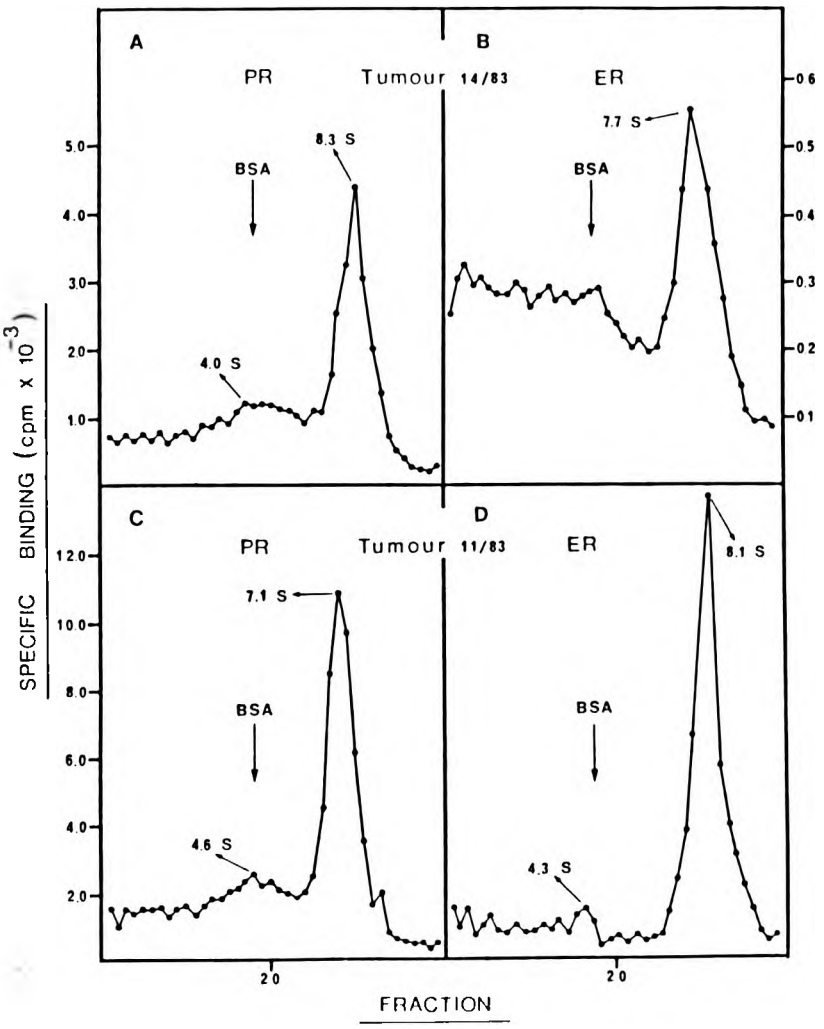


Figure 5.17: Hypotonic sucrose density gradient analysis of cytosolic ER and PR present in human uterine tumours. Cytosols were prepared in TEDAG-MO buffer (20mM sodium molybdate) and aliquots (200µℓ) of the freshly prepared cytosols incubated (30 min x 20°C) with radioligand (^{3}H-E_2 or ^{3}H-R-5020) in the presence (non-specific binding) or absence (total binding) of 250 fold excess of unlabelled ligand (DES or R5020). The DCC treated supernatants (2000xg'x10 min) were layered onto linear 10-35% hypotonic SDG. Gradients were subjected to ultra-centrifugation (VTi-80 rotor, 520000xg'x2hx2°C). Gradients were fractionated (5 dropsx40) manually, scintillation cocktail added (2 mℓ) and then monitored for radioactivity. Multipoint titration analyses were performed on all cytosols and binding data analysed according to the method of Scatchard. Marker protein (^{14}C-BSA) position is indicated by BSA: representative SDG profiles of PR, in cervix carcinoma (A) and leiomyoma (C) and of ER in carcinoma of the cervix (B) and leiomyoma (D).

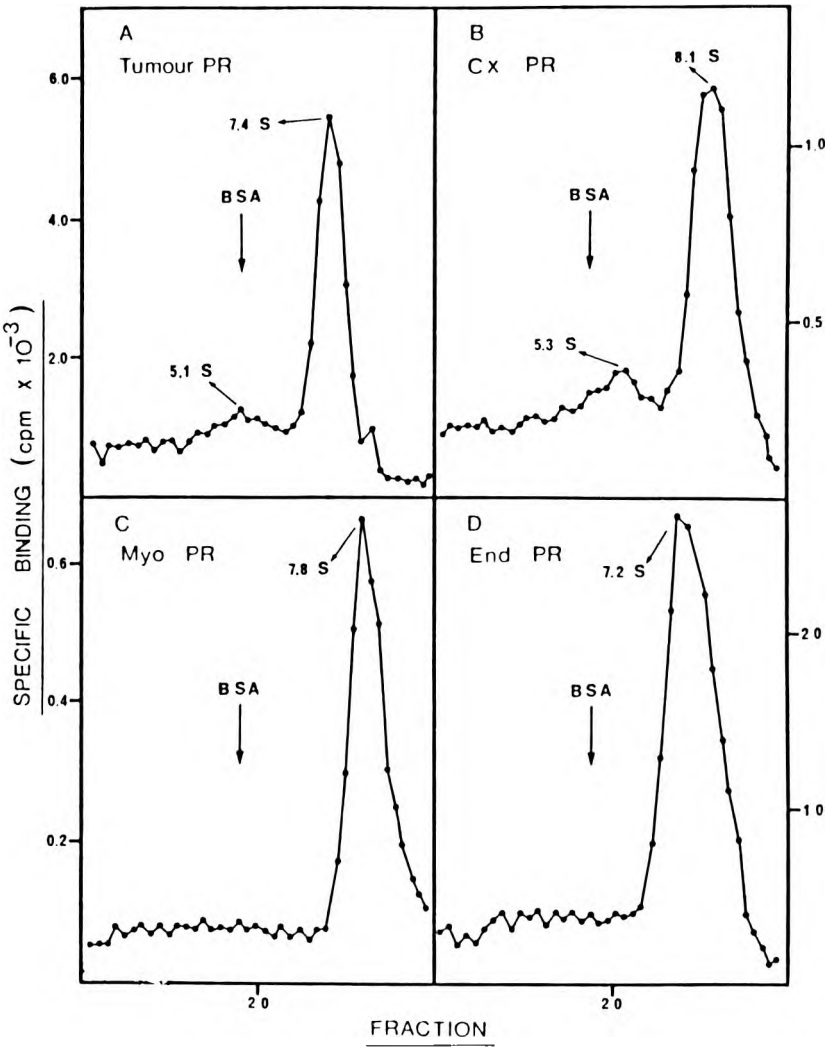


Figure 5.18: A comparative investigation of the distribution of receptor species, present in different parts of the same human uterus with the aid of SDG centrifugation in hypotonic media. Cytosols were prepared in TEDAG-MO buffer (20mM sodium molybdate) and aliquots (200µℓ) of the freshly prepared cytosols incubated (30 min x 20°C) with radioligand (^{3}H-E_2 or ^{3}H-R-5020) in the presence (non-specific binding) or absence (total binding) of 250 fold excess of unlabelled ligand (DES or R5020). The DCC treated supernatants (2000xg'x10 min) were layered onto linear 10-35% hypotonic SDG. Gradients were subjected to ultra-centrifugation (VTi-80 rotor, 520000xg'x2hx2°C). Gradients were fractionated (5 dropsx40) manually, scintillation cocktail added (2 mℓ) and then monitored for radioactivity. Multipoint titration analyses were performed on all cytosols and binding data analysed according to the method of Scatchard. Marker protein (^{14}C-BSA) position is indicated by BSA: representative SDG profiles of the PR present in human uterine tumour (microinfiltrated leiomyoma, A), cervix (B), myometrium (C) and endometrium (D); SDG profiles of the PR present in human uterine tumour (microinfiltrated leiomyoma,A), cervix (B), myometrium (C) and endometrium (D).

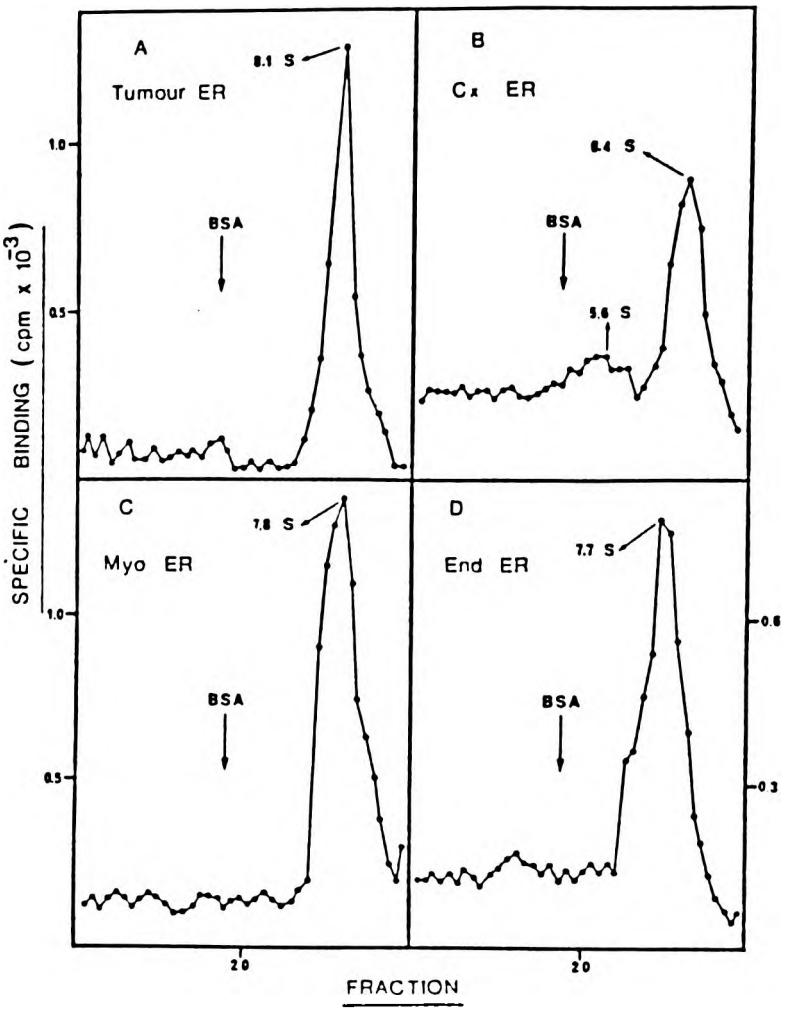


Figure 5.19: A comparative investigation of the distribution of receptor species, present in different parts of the same human uterus with the aid of SDG centrifugation in hypotonic media. Cytosols were prepared in TEDAG-MO buffer (20mM sodium molybdate) and aliquots (200µℓ) of the freshly prepared cytosols incubated (30 min x 20°C) with radioligand (^{3}H-E_2 or ^{3}H-R-5020) in the presence (non-specific binding) or absence (total binding) of 250 fold excess of unlabelled ligand (DES or R5020). The DCC treated supernatants (2000xg'x10 min) were layered onto linear 10-35% hypotonic SDG. Gradients were subjected to ultra-centrifugation (VTi-80 rotor, 520000xg'x2hx2°C). Gradients were fractionated (5 dropsx40) manually, scintillation cocktail added (2 mℓ) and then monitored for radioactivity. Multipoint titration analyses were performed on all cytosols and binding data analysed according to the method of Scatchard. Marker protein (^{14}C-BSA) position is indicated by BSA: representative SDG profiles of the ER present in human uterine tumour (microinfiltrated leiomyoma, A), cervix (B), myometrium (C) and endometrium (D).

TABLE 5.5

Summary of Patient Data

Pat. No	Tissue Origin	Diagnosis	S values Sucrose Gradients ER	S values Sucrose Gradients PR	Fmol/mg protein Scatchard ER	Fmol/mg protein Scatchard PR
41/81	Cervix	mild chronic cervicitis	4.6	8.5	174.3	279.4
	Tumour	Adenocarcinoma	7.3	4.4;7.7	192.1	485.6
37/83	Cervix	CIS c microinfiltration	6.7	6.2	6.7	229.4
	Tumour	Leiomyoma	7.6	8.4	178.0	3176.0
21/83	Cervix	microinfiltration	top	3.6;6.4	405.4	878.8
	Endo	inactive endo	3.0;6.8	3.0;6.1	71.7	865.7
	Myo	no path. changes	top	3.0;6.4	152.3	1922.4
57/83	Cervix	Squamous metaplasia	7.2	6.4	130.0	3869.0
	Endo.	proliferative	7.7	7.2	540.0	6610.0
	Myo	no path. changes	7.9	7.2	219.0	3187.0
7/83	Cervix	No path. changes	top	8.0	27.0	515.0
	Endo.	(Secretory phase)	7.1	6.9	9.0	579.0
	Myo	Leiomyoma	7.5	7.5	30.0	1266.0
11/83	Cervix	Chronic cervicitis	7.7	7.7	95.47	52.22
	Endo	Atropic	8.0	8.0	410.2	202.9
	Myo.	Leiomyoma	9.3	8.0	304.7	478.9
38/83	Cervix	Microinfiltration	6.3	6.3	160.8	976.4
	Endo.	Adenomyosis	7.8	7.5	2485.1	4431.4
	Myo.	No path. changes	7.8	7.5	1305.4	3003

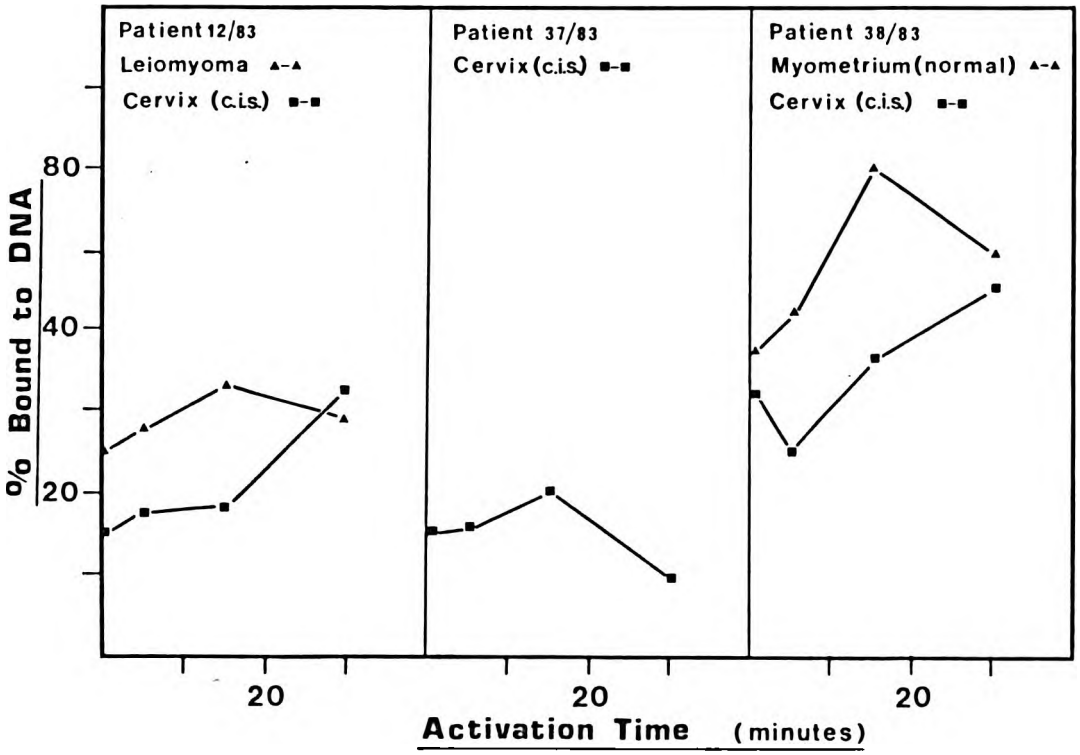


Figure 5.20: Nuclear and DNA-cellulose binding of activated PR-ligand complexes in normal and abnormal human uterine tissue cytosols as a function of the duration of activation at 30°C. Binding studies were executed as described in Section 5.4.2(b) : Patient 12/83 : leiomyoma (▲) and cervix with carcinoma in situ (■); Patient 37/83 : cervix with carcinoma in situ (■); Patient 38/83 : normal myometrium (▲) and cervix with carcinoma in situ(■).

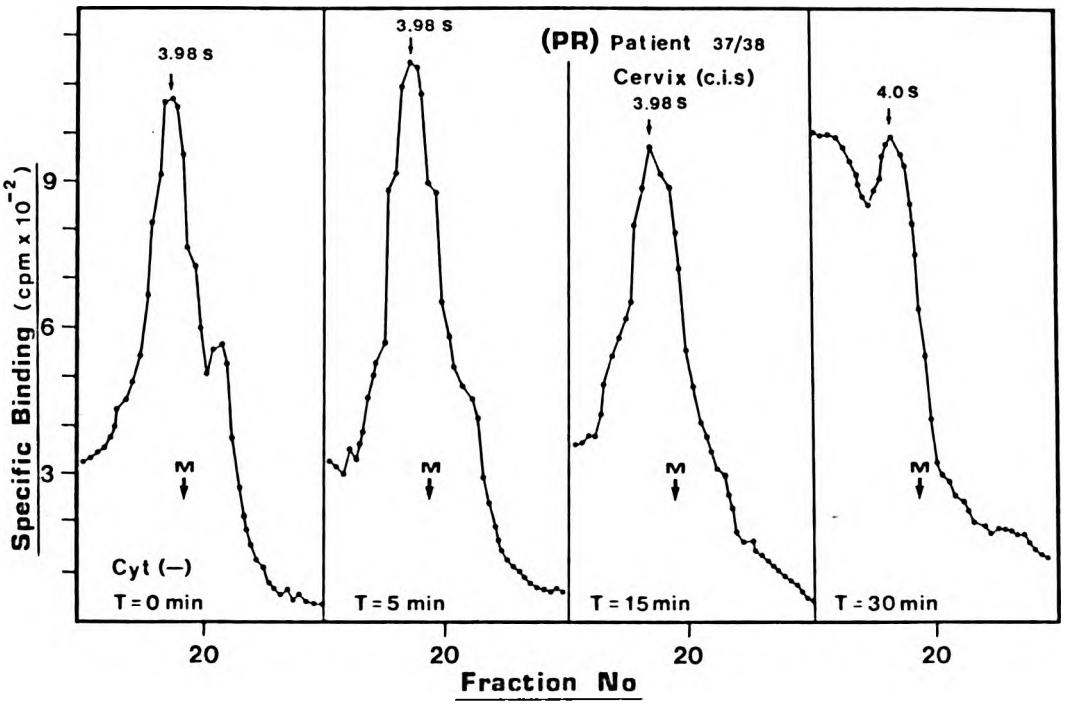


Figure 5.21 : Time dependent transformation of the PR, present in pathological human uterine cervix (carcinoma in situ) in the absence of sodium molybdate as analysed in hypertonic (0.4 MKCl) 10-35% sucrose density gradients. Cytosols were prepared, incubated and analysed on SDG for time dependent sedimentation shifts and DNS binding affinity, as previously described in Sections 5.4.2 to 5.4.3. The position of the marker protein (^{14}C.BSA) is indicated by M.

CHAPTER 6

DISCUSSION

The presence of specific, high affinity binding proteins or receptors in target cells is an important prerequisite for the onset of molecular processes triggered by steroid hormones (60,167). Following the classical observations by Jensen and Jacobson (19) and Gorski et al (25) on the accumulation of steroid hormones in target tissues of the rat, numerous reports appeared on the occurrence and distribution of steroid receptors in the target tissues of a multitude of different species, including man (143). These findings brought into perspective other, equally important observations that were made by Beatson (3) in the area of clinical studies on breast cancer patients more than 60 years ago. Beatson's work has been discussed in some detail in Chapter 1. For the purpose of this discussion, it suffices to refer only to the deductions made in the 1960's e.g. that breast tumours could be classified as hormonally responsive or hormonally unresponsive, depending on the fact that the tumours contained relatively high (hormonally responsive) or low concentrations of oestrogen receptors (35). For the past two decades large numbers of breast cancer patients were treated by hormonal regimes which were all based on this principle (262). The different types of hormonal treatment regimes currently employed, can be briefly summarised as follows:

1. Ablative procedures (removal of endocrine glands).
   - 1.1 Ovariectomy (castration)
   - 1.2 Adrenalectomy
   - 1.3 Hypophysectomy
2. Anti-hormonal therapy (anti-oestrogens)
3. Combination hormonal and chemotherapy

Large numbers of different anti-hormonal drugs were developed in the past two decades (281), but only a few are cur-

rently employed by clinicians, e.g. tamoxifen clomiphene, nafoxidine, and medroxyprogesterone acetate.
Most of the drugs studied so far however did not live up to the expectations of the clinicians (281). Apart from unpleasant side effects produced by these drugs (276) their action (tumour regression) was of short duration (222) and most of the patients developed some form of resistance against specific drugs and eventually against hormonal manipulation as a whole (35). The failure of hormonal manipulation to meet the expected results can possibly be ascribed to the following factors:

A. Incomplete understanding of the underlying hormonal mechanisms involved.
B. Inappropriate animal model (induced rat mammary tumours) employed in the laboratory evaluation of hormonal drugs.
C. The appearance of resistant cells through selection from the initial multi-centric disease or through drug reduced genetic changes.

It is believed that all these reasons apply to the problem of failures in hormonal therapy reported in recent years (79). An endeavour was made to study these mechanisms in an animal model more closely related to Homo sapiens. With this object in mind it was decided to undertake investigations in a non-human primate, e.g. Cercopithecus aethiops pygerythrus. In the light of the general finding that normal mammary tissues contain very low (to zero) levels of oestrogen and progesterone receptors it was decided initially to turn our attention on the uterus as a hormone responsive target organ.

Several reports have appeared, describing the occurrence and some properties of cytosolic and nuclear receptors in genital tissues of non-human primates (124-142). Some studies concerning oestrogen receptors in the uterus and oviduct (311) as well as in the liver (312) of green monkeys (Cercopithecus griseus) have also been reported recently.

Setchell et al (130) investigated steroid excretion during the reproductive cycle and pregnancy in the vervet monkey, Cercopithecus aethiops pygerythrus and found this Old World monkey to have menstrual patterns very similar to that of man.

The findings in this investigation of the occurrence, distribution and levels of oestrogen and progesterone receptors in Vervet monkey uteri and the results on the molecular properties of these two receptors are briefly summarised. Finally, attempts will be made to explain some of the findings relating to the in vitro activation and transformation of these receptors prior to binding to the nuclear elements.

Conditions for the assaying of ER and PR in VMU have been established. Most of the findings are in agreement with those suggested by other research groups (143-158). It is clear that the post-hysterectomy treatment of the uteri, before ER and PR are analysed, is very important in preserving the labile macromolecular components in the intact tissue. Both the receptors exhibited a significant decrease in B_{max} (16-35 % for ER and 10 - 25 % PR) when uteri were not processed at 0-4°C. Once the cytosols were prepared it seemed advisable, not to freeze and thaw again, as repeated freezing and thawing nearly always caused a significant decrease in the binding capacity of the cytosol for both ER and PR. Deterioration of oestradiol binding profiles on sucrose density gradient centrifugation could be illustrated. It would seem that relative large volumes of cytosols are best stored in small aliquots (1-2 mℓ), at -70°C, in quantities, sufficient for individual investigations, in order to avoid from repeated freezing and thawing of the labile cytosols.

In uteri of C. aethiops pygerythrus levels of ER ranged from 151 to 822 femtomoles per mg protein (mean for group assayed

is 327±165 femtomoles per mg protein). PR assays were performed on the same cytosols and the levels ranged from 444 to 2267 femtomoles per mg protein (mean of 1285±511 femtomoles per mg protein). Mean K_d values for the ER and PR-ligand complexes were found to be $3.15\pm1.4\times10^{-10}$M and $2.38\pm2\times10^{-9}$M respectively, within the group analysed (n>20). The ratio of PR and ER varied between 1.1 and 13.1 with a mean of 4.5±2.4. Only a weakly positive correlation (r = 0.25) was found to exist between levels of ER and PR in the same uteri.

The values obtained for receptor levels and dissociation constants in the course of this investigation were consistently higher than those reported for the Rhesus monkey (124) and for humans (79)(see Table 5.1). More recently, however, another group working on the Rhesus monkey reported values which compare favourably with ours, provided their published values (expressed in femtomoles per mg DNA) are recalculated to femtomoles per mg cytosolic protein (313). The same group obtained ER/PR ratios between 2.5 and 3.2 throughout the menstrual cycle, which seem to be contradictory to previous reports on the same species (124). Contradictory findings on the PR levels relative to ER levels for human tissues have also been published (99).

Association of receptors with their respective ligands seemed to be very rapid, even at 0°C and the complexes were extremely stable at 0°C and 20°C $t_{½}$ at 0°C = 7 min; $t_{½}$ at 20°C = 3 min). At 37°C inactivation was speeded up $t_{½}$ at 37°C = 0.5 min) probably due to increased action of proteolytic enzymes or perhaps some other unknwon catalytic factors. However, these data seemed to agree favourable with that published by other reserchers (284). It was for this reason that it was decided to incubate at 20°C for a duration of 30 min when performing the ER assay. Alternative incubation conditions used were at 0°C for a period of 16-24 h.

Ligand specificity investigations indicated a much narrower specificity for the ER as compared to the PR. For the ER the ligand preference was of the order : DES > EE_2 > E_3 > E_1 while no affinity was observed for any of the other steroid hormone categories. The result also seemed largely in agreement with literature reports on breast cancers(143). As for the PR, again in agreement with other investigators, it was found that this receptor had a lower specificity (224). The most remarkable characteristic was the high affinity of the PR for EE_2 (91.2 %) and the relative high affinity for other oestrogens : E_2 > E_1 > E_3 (see Table 3.2). It would seem that the VMU progesterone receptor displays a unique property by comparison with other progesterone binding proteins. Published values for the relative binding affinities of oestrogens for the PR are much lower than the values found in this work (224,314).

Scatchard plots for ER, conducted in conjunction with multiple non-specific binding assays, employing in 100 fold excess either DES, EE_2 or E_2 as unlabelled competitors, gave very similar B_{max} and K_d values when comparing identical concentrations(Fig 2.11-2.13). It could be inferred, therefore, that in spite of the high affinity of the progesterone receptor for EE_2 (RBA=91.2 %) of E_2 (RBA= 16.3 %), the use of these unlabelled competitors in the multipoint titration assay (Scatchard plots) did not significantly affected the calculated binding parameters of the ER but K_d was slightly affected by the different concentration of ligand. Data obtained on a tissue powder, freshly prepared from a pool of five VMU, indicated no significant variation (+1.9 %) in the B_{max} for the ER, when assayed with a 100 fold excess of unlabelled EE_2 or E_2 instead of DES in the determination of the non-specific binding (see Fig 2.11-2.13).

Using low ionic strength or hypotonic conditions ($TEDAG_{10}$ buffer) only an 8 S component was observed for both the ER and the PR (Fig 3.6) (S : svedbergs (1 S = 10^{-13} seconds). Mean sedimentation coefficients (in svedbergs) were found

to be 7.6±0.35 S (7.2-7.9 S) for the ER and 7.20 ±0.7 S (range 6.64-7.7 S) for the PR in the absence of 20mM sodium molybdate. Sedimentation coefficients exhibited no significant change in the presence of molybdate and were essentially the same for both receptors. A marked difference was observed between the SDG profiles of molybdate containing (CYT(+)) and non-molybdate containing (CYT(-)) cytosols. It would seem that the molybdate prevented the formation of a wider range of specific binding macromolecules, differing in their sedimentation rates within the 10 - 35 % sucrose density gradients. It is also clear from the SDG profiles that a higher yield of receptors had been obtained when cytosols were prepared in buffers containing sodium molybdate (CYT(+)). Scatchard assays corroborated these observations : the oestrogen (^{3}H-E_2)·binding capacity for CYT(+) was 27.5 % higher than for CYT(-), while the progesterone (^{3}H-R020) binding capacity for CYT(+) was 21.3 % higher. No effect was observed on the binding affinity (K_d) of the receptors.

A short exposure, of the labelled receptor-ligand complexes to 0.4 M KCl caused partial dissociation of both receptors (Fig. 4.1). Cytosols prepared in buffers containing sodium molybdate appeared to be somewhat more resistant towards dissociation, when exposed to 0.4 M KCl, followed by centrifugation on gradients containing 0.4 M KCl but no molybdate (Fig 4.2). This finding is largely in agreement with that reported very recently by Eastman-Reks et al (315). Sedimentation coefficients for the components observed in higher ionic strength (hypertonic) gradients were 7.6±0.3, 4.6±0.15 and 3.8±0.15 for the ER and 6.4±0.3 and 3.8±0.1 for the PR, respectively.

When cytosols were reincubated with 0.4 M KCl, prior to centrifugation on the 0.4 M KCℓ containing SDG (10-35 % sucrose) a progressive loss of the faster sedimenting (∿ 6-7 S) entity, with a resultant increase in the slower sedimen-

ting components (∿ 4 - 6 S) could be observed (Fig 4.3-4.4). Again, sodium molybdate only affected the yield, but not the relative proportions of the different molecular entities. The progesterone receptor-ligand complex seemed to dissociate at a faster rate, compared to the ER as judged from the SDG proflies, but qualitatively it is following the same pattern. The faster sedimenting PR entity, however, seems to shift to lower S-values for cytosols prepared in the presence of sodium molybdate (CYT(+)), while the shift for the ER in the presence of sodium molybdate is towards slightly higher s-values Fig. 4.5).

Reversibility of the dissociation of the ER seems to be affected by the length of time it is exposed to 0.4 M KCl during a second incubation at 0 - 4°C. The dissociation at zero time exposure (pre-centrifugation) is practically totally reversible, while a swift decrease in reversibility is observed, at longer precentrifugation exposure times to 0.4 M KCl. A remarkable feature observed in this reversibility study is that sodium molybdate tends to cause severe aggregation of the ∿ 8 S type of molecules following exposure to 0.4 M KCl and aggregation increased following longer periods of exposure to 0.4 M KCl (Fig 4.6 - 4.7). Quantitatively (Table 4.1) it can be seen that in the absence of sodium molybdate (CYT(-)) longer incubation in 0.4 MKCl rendered a linear decrease in the ∿ 8 S peaks with time, while simultaneously a directly proportional increase in the 4 S area was observed (Fig 4.8). In the case of the cytosols with or without molybdate (CYT(+) and CYT(-)), the rate of decrease in the ∿ 8 S peak is 82 % greater for CYT(+) than for the CYT(-) cytosols (Fig 4.8).

The presence of a 3-5S species for the VMU ER or the PR was never observed in low ionic strength hypotonic buffers. The only time a 3-5 S species was observed on sucrose gradients was after exposure of labelled cytosols to 0.4 M KCl. Short exposures to high ionic strength conditions produced partial

"dissociated" 4S species. A clear difference in the behaviour of the ER and the PR could be observed in this respect. In the case of the ER it is clear that besides the ∿ 8 S entity other species were generated while the 8 S PR seemed to "shift" to 5 S.

The results obtained for the sucrose density gradients agree to a great extent with published data on uterine receptors in other primates (142), man (107) and a number of different species (82). The effect of molybdate on SDG profiles had also been noted before (237). Molybdate is known to affect the receptor yield, a finding corroborated by our own results.

The majority of studies of receptor structure, have used radioactive steroids of high specific activity to detect the steroid-receptor complexes in crude extracts of fresh or frozen tissues. Such extracts also contain nucleic acids, ribonucleoproteins, proteases, nucleases, phosphatases and other enzymes, which may interact with or degrade the receptors during prolonged fractionation procedures. These effects may have contributed to the discrepancies found in the molecular parameters reported in the literature. Despite much work and significant progress on the stabilization, purification and structural analysis of steroid receptors extracted from mammalian tissues, the native state still remains largely unknown (298). Receptors for a single class of steroids from the same tissue have been detected in various molecular forms for at least three different functional states (298). These states are known as the "untransformed" and "inactive" receptors and can be distinguished by differences in their affinity of the receptor for steroids and in the affinity of the steroid-receptor complex for nuclei, DNA and ionic resins(244,245).

Isoelectric focussing patterns of VMU ER (agarose gels, ampholine range pH 3-10) present in cytosols prepared in the

absence (CYT(-)) of "stabilizing" sodium molybdate (20mM) exhibited 3 main peaks at pI 5.9(28.9%), 6.2(30.3 %9 and 6.8 (40.7 %) (Fig. 3.24). IEF patterns for CYT(+) cytosols, on the other hand appeared more simple, yielding one major peak at pI 5.9 (39.9 %) with two minor peaks at pI 5.6 (13.0 %) and 6.8 (16.6 %), while a broad specific binding area could be illustrated in the pI 5.0-5.5 (30.5 %) range. The apparent heterogeneous character of the VMU ER was corroborated by several other observations in the course of this investigation (SDG, anion exchange chromatography HPLC chromatofocussing).

HPLC chromatofocussing data revealed an even more complex picture (Fig. 3.22, Fig 3.23 and Table 3.11), with more high affinity binding ER species appearing upon chromatofocussing of VMU cytosols, deficient in sodium molybdate (CYT(-)) on an AX- 1000 (polybuffer : pH 4-8) polyamine column. Under similar experimental conditions, CYT(+) cytosols yielded a much simpler picture with only two major peaks at pI 6.1 and 5.5. It appeared as if the molybdate affected the ionic properties of the ER. causing it to elute at more acidic pI-values. This result could provide support for the suggestions forwarded in some research papers (226, 315) recently namely that the molybdate oxyanion physically interacts with the receptor protein, possibly with charged groups, e.g. phosphate groups or even with thiol groups.

This view was strongly underlined by the data obtained with anion exchange chromatography of the VMU ER on an AX-1000 poly-anion column. When CYT(-) cytosols were chromatographed two distinctly different ligand-binding species or subunits, appearing in a ratio of 51:49, could be eluted with a phosphate (HPO_4^{-3}) gradient (Fig. 3.20). With CYT(+) cytosols the same column yielded only one species, eluting at a salt concentration (189mM) intermediary between the species obtained with the CYT(-) cytosols (90 mM and 250mM) (Fig 3.21). This result indeed proved that the effect of the

molybdate oxyanion and the receptor molecule must be direct.

Most, if not all of the above mentioned findings are in some agreement with results reported on a number of steroid hormone receptors in recent times (298). Whiele et al (316) found similar effects of the molybdate anion of chromatofocussing patterns of lactating mammary gland and human uterine oestrogen receptors.

The effect of molybdate on size and other physicochemical parameters have been discussed previously (223-234) and would seem to apply to glucocorticoid, oestrogen and progesterone receptors. According to literature the molecular mass of the molybdate stabilized complex is approximately four times that of the product of its dissociation in hypertonic molybdate-free buffer (226). It has been shown that molybdate prevents the shift of the oestrogen bound receptors to a less negatively charged state which occurs during DEAE-cellulose chromatography. The shift of the steroid receptor complex to a less negatively charged state is characteristic of the steroid-receptor complex transformed either under cell-free conditions (230-231) or in intact cells (220). In addition molybdate is known to affect the size of the steroid-binding complex. According to Pratt et al (226) in the presence of 10mM sodium molybdate, steroid receptors form very large complexes with sedimentation values of 8-10 S.

Since the first report by Nielsen et al (228-229) an extensive investigation has been launched by numerous workers (223-234) on the subject of molybdate as a stabilizing factor for steroid hormone receptors. Consensus seems to have been reached as far as the universatility of this effect when steroid hormone receptors is concerned (220, 228, 231). Some of the most important effects of the molybdate anion, reported in the literature and confirmed here of higher yields of receptors obtained in cytosols prepared in the

presence of sodium molybdate (232) and the inhibition of both the receptor activation (231) and the subsequent binding of receptor ligand complexes to nuclei and DNA-cellulose (248,255). Recently, molybdate has also been implicated in affecting the integrity of steroid hormone receptor molecules, either by causing direct or indirect inhibition of proteolytic enzymes (300) and/or phosphatase enzymes (270). Whiele (316) reported an effect of sodium molybdate on the structural integrity of the oestrogen binding proteins in rat mammary glands and human uterine tissue, when analysed by high performance liquid chromatography chromatofocussing (HPLC-CF).

Although a host of evidence would ascribe the effect of sodium molybdate to its possible inhibitive action on either proteases (151) or phosphatases (267-268), the real physicochemical role of this compound remains unsolved. Some researchers, however claim the effect of molybdate to be completely reversible (231), while others reported results to the contrary (317). It was subsequently decided to investigate the effect of molybdate on the molecular properties of the oestrogen and progesterone receptors of the VMU.

The molecular parameters of the VMU ER and PR which were studied, in addition to the sedimentation properties were : molecular size using different columns and ionic properties using IEF and HPLC-CF. Gel permeation chromatography results of VMU ER on an AcA-22 column are in agreement with the results reported in literature (213). Classification of the data was according to Sherman's classes, numbering the forms of the ER from I to V in order of decreasing molecular size. The Stokes radii for class II and IV were found to be 8-10 % higher than those reported by Sherman and as those for class III appeared to be identical. Comparison of the Stokes radii results with those obtained by sucrose density gradient centrifugation : class I and II identify with the $\sim$ 8 S form whereas classes III and IV represent the $\sim$ 4 S form and

class V is the ~ 2 S form. The M W of the receptor in low ionic strength cytosol was 377,258 daltons with a Stokes radius of $r^* = 60$ Å while in high ionic strength the M W of the ER was 112,000 daltons and $r^* = 41$ Å.

Gel permeation or gel exclusion chromatography using classical columns such as Ultrogel AcA-22, AcA-32 (both polyacrylamide based) and Sephacryl S-300, produced different molecular species, depending on the ionic strength of the mobile phase employed in chromatography. Low ionic strength conditions tended to cause aggregation and large complexes with a molecular mass(MW) ranging from 3.77×10^5 to 8.5×10^5 daltons were frequently obtained. Stokes radii(r) for these species were of the order of 60-80Å. Physiological buffers such as $TEDAG_{10}$ (with 0.154 M NaCl) caused dissociation of the complexes and one of the most frequent species obtained had a MW of 1.12×10^5 daltons ($r^* = 41$Å). At very high ionic strength conditions (0.4 M KCl in $TEDAG_{10}$) some deterioration of the specific binding species was often observed. Sodium molybdate did not significantly change the molecular size of the complexes but exhibited a tendency to "protect" complexes from losing their ligand binding abilities. Gel permeation chromatography using HPLC produced an even higher tendency to aggregation at low ionic strength conditions. On the Waters I-250 gel columns, the most frequent complex observed was a species of a MW of 2.11×10^5 daltons, r = 61Å while a rather small entity (MW 4.34×10^4 daltons; $r^* < 34$Å was also often observed.

It was observed that molybdate inhibited the inactivation (loss of ligand binding capacity) of VMU ER and PR when cytosols were prepared in buffers containing sodium molybdate, without affecting the binding affinity. This observation has been reported by several groups in recent years (232). Furthermore molybdate did not affect the rate constants of association for receptor-ligand complexes signi-

ficantly. Dissociation of both VMU ER and PR was biphasic, indicating the existence of slow (SDC) as well as fast (FDC) dissociating complexes. Rate constants of dissociation of the complexes significantly affected by the presence of sodium molybdate (Table 3.3). However the debating points concerning the reversibility and the mechanism of the inactivation have not yet been clarified. Contradictory results have been published on the reversibility of the molybdate effect (231,317).

It is clear from the result of the sucrose density gradient profiles obtained with CYT(+) and CYT(-) cytosols when developed on either GRAD(+) or GRAD(-) gradients, that the process of inactivation in the absence of molybdate is not totally reversible (Fig 3.7 & 3.8). During the centrifugation process, receptor molecules were displaced from a compartment either containing or not containing molybdate to a compartment which either contained or did not contain molybdate (GRAD(+) or GRAD(-)). Displacement of receptor molecules from the CYT(+) cytosols to a GRAD(-) compartment exhibited very little, if any, inactivation, if any (Table 3,4). On the other hand receptor molecules from a CYT(-) cytosol showed an inactivation of 50.9 % for the ER and 35.2 % for the PR when developed on a GRAD(-) gradient. A 29.6 % and 27.4 % loss of high affinity binding were observed respectively for ER and PR when CYT(-) cytosols were developed on GRAD(+) gradients. The resulting inactivation could only be partially reversed (21,3 % and 7.8 % reactivation for ER and PR respectively) when GRAD(+) gradients were employed. In all of these experiments the affinity of the receptors for their ligands was unaffected, while inactivation, produced in the absence of molybdate, led to lower binding capacities. When cytosols (CYT(+) or CYT(-)) were first dialyzed against buffers containing (TEDAG-MO) or not containing molybdate ($TEDAG_{10}$) and subsequently centrifuged on sucrose density gradients with the same constitution as the dialysis buffers, the general tendency appeared, to be the

same as that found in the sedimentation dialysis experiment. However re-activation of the PR appeared to be much higher (33.5%) than that of the ER (14.1%). The slightly higher reactivation obtained in this experiment for the PR could possibly be attributed to removal of a dialyzable endogenous inhibitor during dialysis, a finding which might have a link, with the endogenous inhibitor referred to in a, recent paper by Leach et al (231).

Numerous reports have appeared on the effect of proteolytic enzymes on the properties of steroid hormone receptors (65,170). The effect of various proteolytic inhibitors on the ligand-binding characteristics have also been discussed by several workers (297, 300, 316).In this study synergistic effect on the specific receptor-ligand binding was observed in cytosols containing Leu (2mM). This result indicates that some receptor inactivation normally occurs during the preparation and processing of the cytosols, even in the presence of molybdate. In this particular experiment (Fig 3.10-3.11) the receptor destruction (inactivation) was most probably caused by proteolytic action which was blocked in the presence of 2mM Leu. When PMSF (1mM) was employed as proteolytic inhibitor, no synergistic effect on the binding capacity was noted for either of the two receptors investigated and again the protective effect of molybdate could be clearly seen. Some reports have appeared recently, describing methodologies for the assay of receptors in the presence of 1 mM PMSF as a precautionary step in protecting receptor integrity (318) a finding not confirmed here. In spite of the protective action of the Leu both ER and PR still seem to be inactivated in a molybdate deficient environment, indicating that the mode of action of the molybdate can not be linked to the inhibition (direct or indirect) of proteolytic enzymes.

In addition to inhibiting inactivation of steroid receptors, molybdate reversibly blocks transformation of the bound

steroid-receptor complex to the DNA-binding state. Inhibition of temperature-dependent transformation was first reported for the avian progesterone receptor by Toft & Nishigori (257). Later studies have shown that molybdate also reversibly inhibits temperature-mediated transformation of glucocorticoid (254), oestrogen (248), and androgen (255) receptors. Vanadate and Tungstate also inhibit receptor transformation (226). Transformation of the glucocorticoid-receptor complex caused by salt or by precipitation with ammonium sulphate is also inhibited by molybdate (256). as is salt-mediated transformation of progesterone receptor and oestrogen receptor (257).Toft observed that sodium molybdate only stabilizes the non-activated receptor and has no apparent effect on the stability of receptor that has been activated. Therefore, the stabilizing and inhibiting actions of sodium molybdate would appear to be related.The molecular mechanism by which molybdate stabilizes steroid receptors is not known. Although molybdate and some of the other group VI-A transition-metal oxyanions inhibit a variety of phosphatases (256) and some phosphohydrolases (e.g. Na^+, K^+ATPases (256), this action does not account for their ability to stabilize steroid receptors and inhibit receptor transformation. Rather, it seems clear that molybdate is directly interacting with the receptor. Nishigori & Toft(257) have examined the effect of a number of compounds on both progesterone-receptor transformation and endogenous phosphatase activity in chick oviduct cytosol. Although molybdate, vanadate and tungstate inhibited both phosphatase activity and receptor transformation, the results obtained with the other inhibitors suggested that these two activities might not be related. Leach et al (231) found that molybdate inhibited inactivation and transformation of glucocorticoid receptors caused by salt or ammonium sulphate precipitation at 0°C - 4°C. As these are physical methods of receptor activation and transformation that are unlikely to involve phosphatase action. It does not seem likely that phosphatase inhibition plays any role in the molybdate

effects on steroid receptors.

Other observations (224) suggest that molybdate interacts directly with the receptor and possibly exclusively with the untransformed form. The type of functional group(s) required for the proposed molybdate receptor interaction is not known. It is known that sulphydryl groups are required for receptor transformation as well as for steroid binding (298), and thus molybdate-sulphur interactions might account for all of the molybdate effects observed on steroid receptors.

A modification of Clark's method (^{3}H-E_2 exchange assay) (310) proved to be a valuable technique for the analysis of specific nuclear oestrogen-binding sites in target tissues, such as the uterus (310) as well as in human and experimental mammary tumours (263).

The subcellular distribution of the oestradiol receptor has been described before (276-278) and the results found in this investigation (Section 5.4.3) are in good agreement with the reported data. It was found that a depletion of receptors in the cytosol was always accompanied by an accumulation of receptors in the nuclear compartment (Fig. 5.6). The uptake and distribution of ligand-receptor complexes was found to be a function of both time and temperature (Table 5.3). The results were largely affected by the procedure employed in the extraction of nuclear receptors from the nuclear myofibrilar pellet. Different treatments were compared and the results found during this investigation agreed favourably with those employed by others (282). The best extraction of nuclear receptors was obtained with the trypsin-KCl procedure (method b in Section 5.3.2.2; Fig 5.4 and 5.5). Levels of nuclear ER found in the course of our investigation (1483±1236 femtomoles/mg DNA; Table 5.1) were in good agreement with results reported by other workers employing primates in their investigations (313).

The main aim behind the experiments described in Chapter 5 was to create a model system for studying the dynamics of the process of activation and transformation of the ER and PR in the VMU and to study the effect of molybdate on the system. Since all the molecular events involved in these processes occur in vivo in the intact cell tissue slices were first employed in the investigation. However the results were not quantitatively reproducible. Thus an activation "model" involving a cell-free system was developed to study the activation and transformation of ER and PR in the VMU. It was also necessary to investigate the process of activation in abnormal tissues and since abnormal monkey tissue was not available for this purpose the "activation system" employed in this case was normal and abnormal human uterine tissues. Both activation and transformation were studied in this latter system.

It is generally accepted that, according to the classical two-step model (45) the receptor-ligand complex, formed in the cytosol must be "activated" by some unknown process to form a "transformed" complex which exhibits a higher affinity for chromatin than the "untransformed" state (45). It is also accepted that this transformation process implies that the ∿ 4 S (0.4 M KCl gradients) specific binding species (untransformed, inactive) undergoes a "conformational" change to a ∿ 5 S entity. Thus, there is a link between receptor transformation and activation and the ∿ 4 S → ∿ 5 S "shift" (0.4 M KCl gradients) on the one hand and also with the ability of the "activated", "transformed" state to "translocate" to the nuclear compartment where it binds to chromatin with a high affinity on the other hand. Recently, a number of papers have appeared addressing the problem of receptor activation. A number of workers have employed inhibitors of the activation aand/or transformation process, in order to gain more insight into the problem of receptor activation (315).

It could be illustrated that temperature-induced activation of receptor-ligand complexes reaches a maximum at 15 min. of incubation at 28°C, as measured by increased binding of the complexes to uterine nuclei or DNA-cellulose. Simultaneously, a clear ∿ 4 S → ∿ 5 S shift was observed. Both these processes were inhibited by the presence of molybdate. Similarly it was shown that dissociation of receptor-ligand complexes was biphasic exhibiting the existence of fast dissociating (FDC) and slow dissociating complexes (SDC).

Temperature-induced activation clearly "transformed" the FDC to a SDC while molybdate inhibited the formation of the SDC. In the presence of 10mM molybdate, dissociation is still biphasic, implying that both FDC and SDC still exist. However, it could be illustrated that the molybdate enhances the dissociation of the FDC, while it slows down the dissociation of the SDC.

Similar observations were made on ER & PR from normal human uterine tissue suggesting that these effects are universal for steroid hormone receptors. However, in the case of abnormal human uterine tissues, especially with carcinoma of the cervix and in benign tumours like leiomyomata no ∿ 4 S → ∿ 5 S "shift" was obtained after 15 min or longer periods of temperature-induced activation. Nuclear binding too of the temperature activated complexes was abnormal in the absence of sodium molybdate. This finding would suggest an abnormality at the molecular level possibly leading to abnormal hormonal mechanisms.

There have been no previous report on any abnormality in the activation and binding events in pathological target tissues when compared to normal tissues. Wittliff et al suggested that defective ∿ 8 S + ∿ 4 S receptor profiles obtained on sucrose gradient centrifugation may be related to an abnormality in the response of certain ER positive tumours to hormonal therapy. It is as yet not known, however, whether

his observations are universally acceptable. For the time being, however it is obvious that much remains to be done before these findings can be regarded as the accepted steroid receptor mechanism in abnormal tissues. A larger sample of tumours should be investigated to obtain statistically acceptable results on this issue.

Finally it is not clear how the work described in this thesis relates to the current belief that steroid receptors do not exist within the cytosol of cells but are confined to the nucleus (45-48). Clearly the issues are still very controversial and will require a great deal of time and effort before they are finally resolved.

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