POLYCLONAL ANTIBODIES TO OESTROGEN RECEPTORS OF HUMAN MYOMETRIUM

by

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ABBREVIATIONS

The standard abbreviations, as recommended in the Biochemical Journal 'Policy of the Journal and Instruction to Author's [Biochem. J. (1981) 193, 1-27], are used throughout this thesis, with the following additions:

Alb	albumin
Anti-ER	anti-oestrogen receptor
BSA	bovine serum albumin
°C	degrees centigrade
cpm	counts per minute
DABA	diaminobenzoic acid
DHT	dihydrotestosterone
DCC	dextran coated charcoal
DES	diethylstilboestrol
DFP	diisopropyl fluorophosphate
DMBA	dimethylbenz (α) anthracene
DTT	dithiothreitol
E_2	oestradiol-17β
ER+	oestrogen receptor positive
ER-	oestrogen receptor negative
ERc	cytoplasmic or soluble oestrogen receptor
ERn	nuclear oestrogen receptor
FNA	Fine needle aspirate
$[^3H]E_2$	tritiated oestradiol-17β
Hepes	N-2-hydroxy-piperazine-N'-2-ethane sulphonic acid
H & E	Haematoxylin & eosin
HIDCCFCS	Heat-Inactivated Carcoal-Stripped Foetal Calf
MEM	Eagles minimal essential medium
h	hours(s)
HED	Hepes-EDTA-DTT
IF	immunofluorescence
IF(+)	immunofluorescence positive staining
IF(-)	immunofluorescence negative staining
PAGE	polyacrylamide gel electrophoresis

PAP	peroxidase-anti-peroxidase
PBS-A	phosphate buffered saline without Ca^{2+} and Mg^{2+}
PEP	polyoestradiol phosphate
POPOP	1,4-di-{2-(5-phenyloxazolyl)}-benzene
Pg	progesterone
PPO	2,5-diphenyloxazole
PgR	progesterone receptor
S	Svedberg units (10^{-13}S)
SD	Standard deviation from the mean
SDG	sucrose density gradient
SDGA	sucrose density gradient analysis
SDS	sodium deodocyl sulphate
SDS-PAGE	sodium deodocyl sulphate-polyacrylamide gel electrophoresis
Succ	hemisuccinate
SHBG	sex hormone binding globulin
TED	Tris-EDTA-DTT
$TEDK_{0.01}$	Tris buffer with DTT and 0.01M KCl
$TEDK_{0.2}$	Tris buffer with DTT and 0.2M KCl
$TEDK_{0.4}$	Tris buffer with DTT and 0.4M KCl
$TEDK_{1.0}$	Tris buffer with DTT and 1.0M KCl
v/v	volume for volume
w/w	weight/weight
w/v	weight for volume

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SUMMARY

Rabbit antibodies to oestrogen receptor of human origin were utilized for investigating oestrogen receptor distribution, quantification and translocation in oestradiol target cells. The purification of oestrogen receptor to homogeneity has been achieved. A single protein band was obtained at 65,000 molecular weight under denaturing conditions. The purified oestrogen receptor also had a sedimentation value in the region of 4S. The antibodies to the affinity purified oestrogen receptor had been produced in rabbit and a significant titre was found 3 months after primary immunization. The antibodies obtained had been shown to contain specific antibodies to the receptor protein by four criteria:

1. precipitation of the radioactive steroid after the addition of goat anti-rabbit antibody to a mixture of the [^{3}H] oestradiol-receptor complex and the immunoglobulin. Using the double antibody precipitation technique, the titre of the antibodies was also determined,
2. adsorption of the oestradiol-receptor complex by immunoglobulin linked to Sepharose,
3. the ability of the immunoglobulin to increase the sedimentation coefficient of the oestradiol-receptor complex. Using [^{3}H] oestradiol as the marker for oestradiol-receptor complex, it is possible to observe shifts in the sedimentation coefficients of oestrogen receptor in the absence and the presence of the antibody under both low and high salt conditions,
4. the demonstration of translocation of receptor in ZR-75-1 cells.

The immunofluorescent method, using anti-oestrogen receptor antibodies as primary and goat anti-rabbit fluorescein isothiocyanate conjugated as secondary antibody, was employed for immunohistochemical localization of oestrogen receptor. The frozen sections from rat and human uterine tissues, oestrogen receptor positive human breast cancer and the ZR-75-1 cell line all showed cross-reaction. No immunoreaction was observed in tissues from rat

spleen and oestrogen negative human breast tissues. In vitro translocation sudies were also carried out on ZR-75-1 cells and human myometrial tissues when they were incubated with diethylstiboestrol at 37°C. The in vitro translocation was then quantitated using an MPV compact linked to a fluorescence microscope. These results revealed the suitability of anti-receptor antibodies for investigating the intra-cellular dynamics of oestrogen receptor in target cells responding to oestrogen. This technique was then applied to 50 fine needle aspirates (FNAs). The quantitation of 100-150 cells was compared with the standard biochemical assay of cytosol and nuclear oestrogen receptor. FNA antibody staining correctly predicted the presence of oestrogen receptor in 20 of 21 ERc+/ERn+ tumours. There were 2 false positive results in 26 ERc-/ERn- tumours though both these tumours were heterogenous by antibody staining, in keeping with the tendency for such tumours to be hormone independent in long-term response to endocrine therapy.

The immunochemical similarity of mammalian oestrogen receptor has been demonstrated by the ability of the antibodies to cross-react with the oestrogen responsive cells and tissues. Antibodies were shown to cross-react with receptor from rat uterus as well as human uterus but did not react with either dihydrotestosterone-receptor complexes from rat prostate or with progesterone-receptor complex. These findings indicate an immunochemical similarity among oestrogen receptor from different sources, but not among receptor proteins for different steroid hormones.

1.1 STEROID HORMONES AND RECEPTORS

Hormones are chemical signals released from the endocrine glands directly into the blood stream. There are two classes of hormones: peptide hormones and steroid hormones. Once in the blood stream the steroid hormones will bind to sex hormone binding globulin (SHBG) and albumin (Clark and Peck, 1979). Recently, Strel'chyonok et al. (1984) proposed that in plasma membranes of the oestradiol-17β (E_2) target cells, there is a recognition system for the SHBG-E_2 complex which may allow these cells to take up not only free E_2 but also E_2 complexed with binding protein from the blood. The entry of the steroid into the cell initiates responses which include changes in enzyme activities, transport activities and modulation of gene expression, growth and cell division.

Target cells are characterised by the presence of specific, high affinity binding proteins. These proteins are called receptors. The structure of each receptor allows it to be recognised only by the particular hormone. Receptors for the peptide hormones are located in the plasma membrane while those for the steroids are internal though their precise location is still under much debate (see section 1.1.2).

The six classes of steroid hormones are the oestrogens, progestins, androgens, mineralocorticoids, glucocorticoids and vitamin D_3 metabolites (cholecalciferol) (Pike, 1982). Three of these hormones: oestrogen, progestins and androgens act principally on the reproductive tissues.

1.1.1 Structure and Biosynthesis of Steroid Hormones

The steroid hormones are derived from a parent structure - cholesterol. The fundamental structure of all steroids is phenanthrene to which is fused a five-membered ring structure. This

complete structure is called the cyclopentanoperhydrophenantrene nucleus. In cholesterol, a side-chain eight carbon atoms long is attached at the position of C-17. The numbering sequence is shown in Figure 1.

The Oestrogens

The female sex steroids are oestrogens and the progestins. Together they control the oestrous or menstrual cycles through feedback regulation of the hypothalamus - pituitary axis.

Oestrogens are responsible for the development of the breast and reproductive tissues. During the proliferative phase of the menstrual cycle, oestrogens promote growth and development of the uterine endometrium (see section 1.3.2). In pre-menopausal woman, they oppose the osteoporotic (calcium resorption from bone) effects of corticosteroids. The principal form of oestrogen in these women is oestradiol-17β which is synthesized from cholesterol in the ovary as a result of stimulation by gonadotrophins.

Biosynthesis of Oestrogens

Oestrogen are synthesised in the theca cells of the follicular tissue and corpora lutea of the ovaries. The parent compound, cholesterol, is converted to pregnenolone and this gives rise to 4-androstenedione and testosterone. Through various enzymes, conversion of these androgens to oestrogen takes place after spontaneous arrangement of the aromatic ring A, the final product being oestradiol-17β from testosterone and oestrone from 4-androstenedione. All enzymes involved in oestrogen biosynthesis require NADPH and oxygen for activity and are cytochrome P-450-dependent.

Other organs that produce oestrogens are the adrenals, testes and peripheral tissue. In post-menopausal women, most of the oestrogen produced comes from peripheral aromatization of adrenal 4-androstenedione. The pathways involved in oestradiol and oestrone synthesis are shown in Figure 2.

Figure 1. Numbering and lettering of steroids

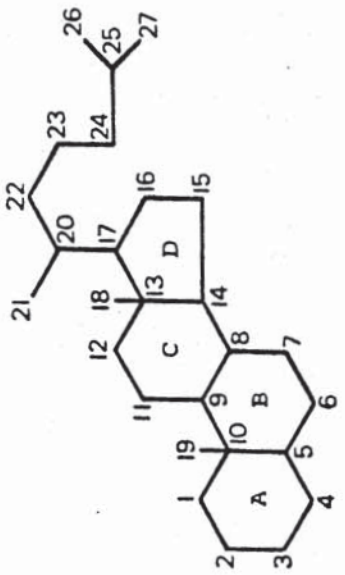
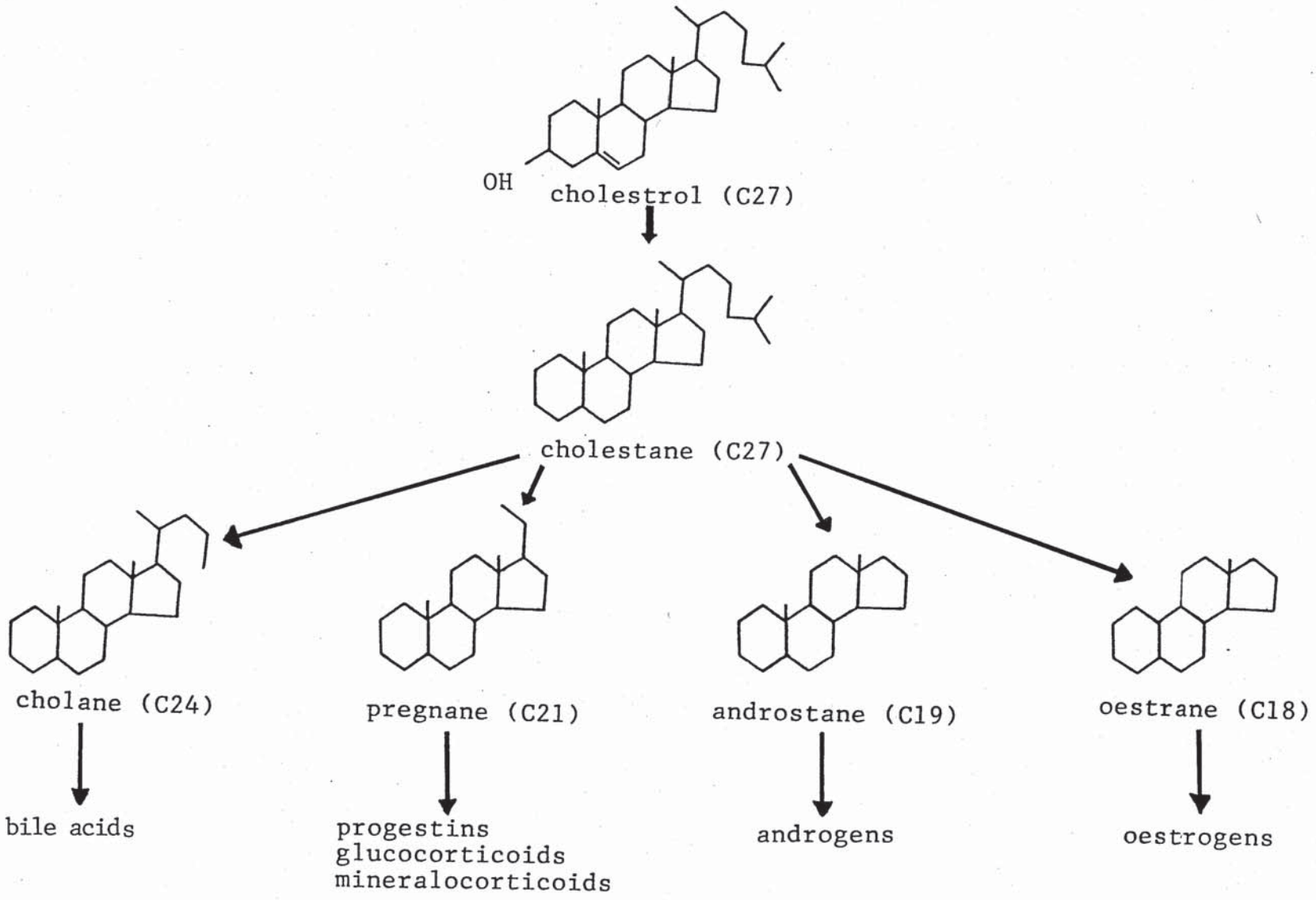

26
25
27
23
24
22
16
15
20
17
D
21
18
13
14
12
C
8
7
11
9
B
6
19
10
5
1
A
4
2
3

Figure 2: The relationship of the different classes of sterols and steroids


OH
cholestrol (C27)
cholestane (C27)
cholane (C24)
pregnane (C21)
androstane (C19)
oestrane (C18)
bile acids
progestins
glucocorticoids
mineralocorticoids
androgens
oestrogens

1.1.2 Steroid Entry into the Cell

Steroids are relatively small hydrophobic molecules and this is thought to assist in the diffusion of steroids across the cell membrane. It is generally said that the steroid enters target cells by passive diffusion (Higgins and Gehring, 1978) which explains steroid entry into both target and non-target cells (Jensen and Jacobson, 1962).

In the intact cell *in vivo* unfilled receptor may be attached loosely to the inside of the plasma membrane (Leake, 1976). Pietras and Szego (1977), using affinity chromatography, have demonstrated the existence of oestrogen binding sites on the surface of endometrial and liver cells. Similar studies had been reported by several groups (O'Malley and Means, 1974; Wittliff, 1975). Also Mueller *et al*. (1978) and Pietras and Szego (1979) demonstrated the presence of specific ER associated with uterine plasma membranes.

Terayama *et al*. (1976) demonstrated that some plasma membrane steroid binding sites are lost during malignant transformation of cells and Zanker *et al*. (1981) have demonstrated a similar loss of binding sites from the plasma membrane of neoplastic tissue compared with normal tissue. They further suggested that normal cell receptor at the plasma membrane may be serving as the modulator of intracellular hormone levels, protecting the cells from excess exposure to hormone.

Nenci *et al*. (1980, 1981) demonstrated that plasma membrane is involved in the steroid uptake. However Chamness *et al*. (1980) point out that these plasma membrane sites are type 2 binding proteins and not oestrogen receptor (which is known as Type 1)

1.1.3 Properties of Steroid Receptors

All the receptors for steroid hormones (oestrogens, androgens, progestins, glucocorticoids, mineralocorticoids, vitamin D) do have certain common characteristics. The molecular weight is in the

region of 50,000 to 110,000 with an affinity in the range 10^{-10} to 10^{-9} M for its respective ligand. They also have a tendency to aggregate *in vitro* at low ionic strength. The steroid-binding site is relatively heat labile, and they have a domain in the molecule that is able to interact with DNA and/or with the nuclear chromatin. The physicochemical characteristics of the receptor are also similar. Weigel *et al.* (1981) and Dougherty *et al.* (1982) demonstrated that progesterone receptor from chick oviduct is a phosphoprotein. The same report was given about the glucocorticoid receptor from mouse fibroblasts (Housley and Pratt, 1983) and oestrogen receptor from the calf uterus (Migliaccio *et al.*, 1982). Phosphorylation was found to modulate the activity of the steroid-binding site in oestrogen receptor from the calf uterus (Migliaccio *et al.*, 1982) and from glucocorticoid receptor (Sando *et al.*, 1979). Most of these studies utilised the property of a steroid-binding site on the receptor protein. Oestrogen receptors, at least, are substrate for tyrosine kinases (Auricchio *et al.*, 1984).

1.1.3.1 Oestrogen Receptor

The oestrogen receptor is known to exist in two states. One form predominates in animals that have been treated with oestrogen or an oestrogenic compound and can be isolated from the nuclear fraction of the target tissue. A cytosolic or soluble form of receptor (ER_c) is found in cell-free preparations in the absence of steroid (Jensen *et al.*, 1968; Shyamala and Gorski, 1969). This form of receptor can be transformed to the nuclear form (ER_n) when treated with oestrogen. The degree of transformation depends on exposure period temperature and ionic strength of the buffer. After transformation, the receptor is believed to have properties differing from the nontransformed receptor in several ways including its chromatographic behaviour (Molinari *et al.*, 1981), and an increased affinity both for ligand (Weichman and Notides, 1977), DNA (Yamamoto, 1974), and for chromatin (Gschwendt and Hamilton, 1972; Jensen *et al.*, 1972). A change in the sedimentation value from 4S to 5S was also observed in receptor from immature rat uterus and calf uterus (Jensen *et al.*, 1971; Gschwendt and Hamilton, 1972).

This change has also recently been shown in human tumour tissue (Hyder and Leake 1982). The 4S form of the cytosolic receptor is said to be a monomer in high salt extract and has the tendency to aggregate to 8S in low salt buffer.

Notides et al. (1981) and Sasson and Notides (1983), suggested that at sufficiently high receptor concentration, the affinity for ligand is due to the presence of site-site interaction within the dimer after its formation, while Puca et al. (1977) suggested that the transformation event is mediated by other proteins present in the cytosol in the form of enzymes or even through direct formation of a complex with receptor (Thrower et al., 1976; Thampan and Clark, 1981).

However, Sakai and Gorski (1984a) tested the role of the subunit-subunit interactions and nonreceptor proteins in transformation. They dissociated the oestrogen receptor into monomers by treatment with high-salt buffers and then bound these monomers to hydroxylapatite. They did not find any cooperative ligand-binding behaviour showing that adsorbed monomers cannot dimerize or interact with other adsorbed cytosol proteins. This report which suggests that dimerization does not occur for transfomation to take place, is in disaggreement with that of Weichman and Notides (1977) who postulated that dimerization is essential for the transformation of receptor to the high affinity ligand state. Other studies (Sato et al., 1979; Bailly et al., 1980; Gschwendt and Kittstein, 1980 and Muller et al., 1983, 1985) which showed the ability of monomeric 4S oestradiol-receptor complexes to bind to nuclei and DNA also suggest that transformation may occur without the process of dimerization.

Oestrogen receptor from the liver has been purified to 80% purity and has a molecular weight of 55,000 (Gschwendt et al., 1983). The sedimentation value is also in the region of 4S. It occurs in both sexes in equal concentration (Tamulevicius et al., 1982). The DNA binding site is affected by protease activity and by molybdate (Petterson et al., 1982) and is probably on the same

domain as the E_2 binding site. However, the mechanism by which molybdate acts is still unclear. Molybdate may bind directly to the DNA binding site as suggested by Lukola and Punnonen (1983), or inhibit the activity of phosphatase which is required for the binding to DNA to occur.

1.1.3.2 Progesterone Receptor

Most of the work on progesterone receptor has used chick oviduct. The protein has been found to contain two polypeptides: A and B which are dissimilar in molecular weight, 79,000 and 108,000 respectively (Sherman et al., 1970; Schader et al., 1972; Schrader et al., 1977; Schrader and O'Malley 1980). Dissociation of the subunits by pyridoxal phosphate leaves A able to bind to DNA (Hughes et al., 1981) while B appears to be the acceptor protein binding site. However, both subunits are required for receptor function. The loss of either subunit prevents the ability of the progesterone to alter RNA synthesis in vivo. Purification and antibody production on this protein had also been reported (Gasc et al., 1984; Renoir and Mester, 1984; Tuohimaa et al., 1984).

1.1.3.3 Glucocorticoid Receptor

The glucocorticoid receptor has been purified by taking advantage of its DNA-binding property (Wrange et al., 1979; Westphal and Beato, 1980), or by affinity chromatography (Govindan and Sekeris, 1978; Lustenburger et al., 1981; Grandics et al., 1982). Polyclonal antibodies (Carlstedt-Duke et al., 1982) and monoclonal antibodies (Grandics et al., 1982; Westphal et al., 1982) had allowed one to distinguished at least three distinct domains on the receptor molecule i.e. steroid-binding domain, DNA-binding domain and immunoactive domain. The steroid-binding domain comprises at most one quarter (called meroreceptor) of the monomeric receptor. Thermodynamic studies suggest that the steroid molecule is 'buried' into its binding sites (Wolff et al., 1978). Inhibition of steroid binding to the receptor by pyridoxal 5'-phosphate and 1,2-cyclohexanedione (Disorbo et al., 1980) may indicate that lysine and arginine

residues are involved in steroid binding. DNA-binding domain comprises a portion of the receptor which is about the same size as steroid-binding domain. Experiments with chemical probes of amino acid residues, using crude receptor preparations suggest that histidine, arginine and lysine residues are important for DNA-binding activity (Disorbo et al., 1980). Immunoreactive domain consists of an immunoreactive fragment of about 3nm, which is roughly half the size of the holoreceptor (Rousseau, 1984). This domain is required for glucocorticoid activity and has been called the 'specifier' domain (Vedeckris, 1983). Loss of this domain by mutation leads to an abnormal increase in DNA binding and steroid insensitivity (Gehring and Hotz, 1983; Okret et al., 1983). Most of these receptor studies are on liver from rats and involved the use of molybdate. Molybdate is said to inhibit protease activity (Sherman et al., 1981, 1983; Sherman and Steven, 1984) and may also inhibit dephosphorylation. Activation of the receptor also involves the removal of a phosphate group (Sekula et al., 1981).

1.1.4 Mechanism of Action of Oestrogen

In spite of a large number of studies using different approaches by different investigators, the mechanism of action of steroid hormones is still not completely understood.

1.1.4.1 The Two-step Model

In earlier studies most of the work was based on the use of radiolabelled steroid hormone as a marker. Jensen and Jacobson (1960) demonstrated that target tissues for the hormone could retain physiological amounts of radiolabelled oestrogens. Then, it was found that the oestrogen was retained both in the nucleus as well as in the cytosol (Jensen et al., 1968). When Toft and Gorski (1966) introduced the sucrose density gradient technique for receptor studies, the homogenised immature rat uterus extract contained almost all of the oestrogen binding protein in the soluble fraction. This protein sedimented at 4S in high salt. When the immature rats

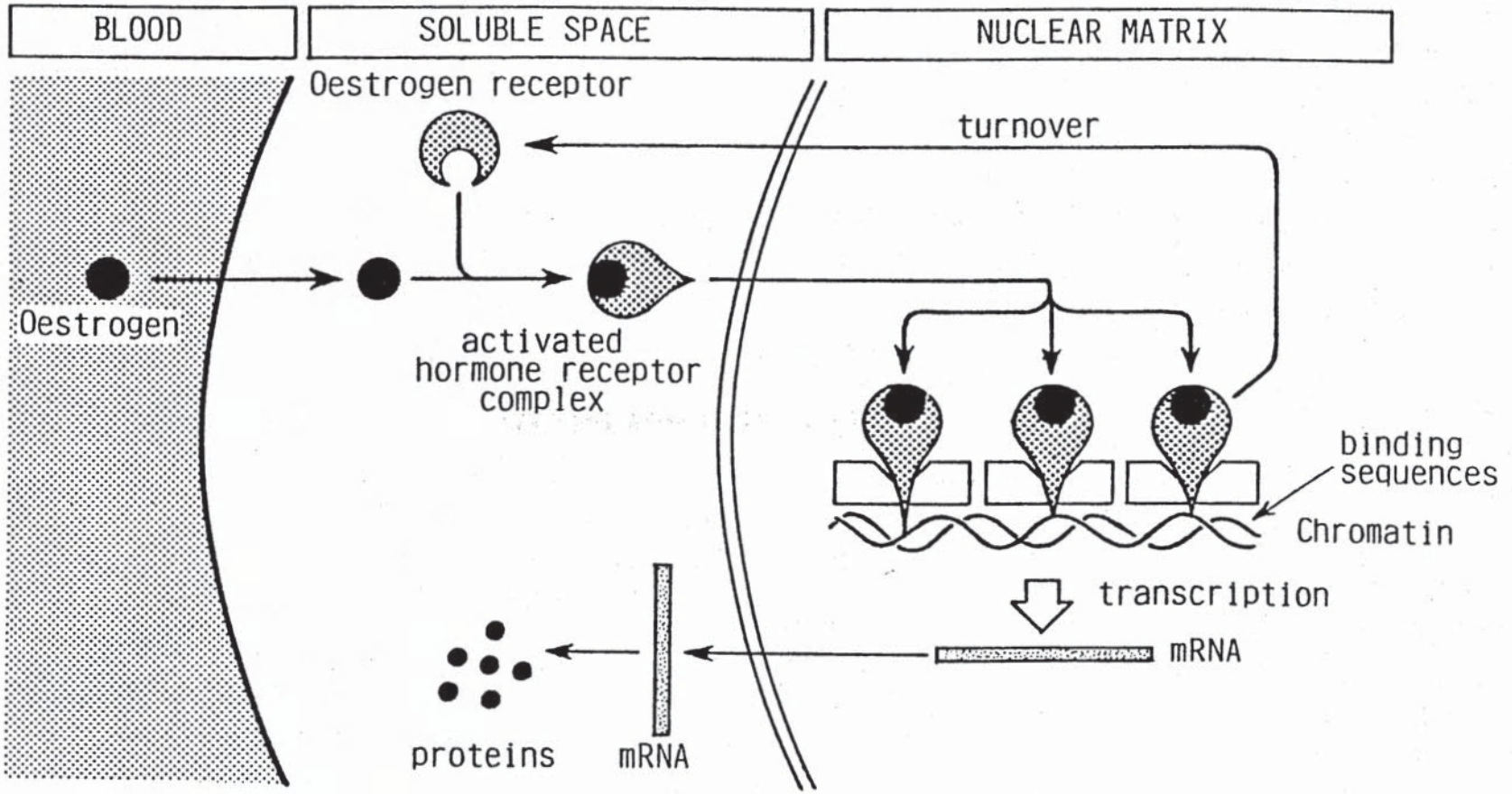
recieved E_2 prior to death, the receptor was found mainly in the nucleus and, after extraction in high salt, sedimented at 5S (Gorski et al., 1968; Jensen et al., 1968). As a result of their experiments they proposed a general pathway for the mechanism of action of oestrogen in target cells as shown in the Figure 3.

Steroid enters the cell, probably by passive diffusion, and rapidly binds to its receptor protein forming an oestrogen-receptor complex in the soluble region of the cell. Notides et al. (1975) believed that the complex might undergo activation. This was then followed by dimerization. The dimerized-form complex was then translocated to the nucleus where interaction with a certain acceptor site was followed by subsequent initiation of nucleic acid synthesis leading to the synthesis of protein, growth and cellular responses that are characteristic of the hormone response. This model is known as the classical two-step model.

The supposed translocation from the cytoplasm to the nucleus occurred after activation had taken place. This evidence for activation of the steroid-receptor complex is (1) a change in the sedimentation coefficient of the complex from 4S to 5S (in the presence of a high ionic strength buffer) when filled receptor is incubated with E_2 at 37°C and (2) the ability to bind to isolated nuclei and DNA is confined to the 5S. Hyder and Leake, (1982) could also demonstrate a similar change in sedimentation constant in human breast tumour under specific conditions. While oestrogen receptor shows a change in sedimentation value on activation, other steroid receptors do not. Progesterone receptor complexes from hamster (Chen and Leavitt, 1979), guinea pig and rabbit uterus (Saffron et al., 1976) exhibit a decrease in sedimentation value, while no change was observed in chick oviduct (Buller et al., 1975). Liao (1975) showed that activation of dihydrotestosterone complex of rat prostate also shows a decrease in sedimentation value from 3.8S to 3S.

Whether activation is a real phenomenon is debatable. A number of reports have appeared which claim to have difficulty in reconciling

Figure 3: The Two-step Model of Steroid Hormone Action
(Adapted from Leake, 1980)


BLOOD
SOLUBLE SPACE
NUCLEAR MATRIX
Oestrogen receptor
turnover
Oestrogen
activated
hormone receptor
complex
binding
sequences
Chromatin
transcription
mRNA
proteins
mRNA

practical data with the two-step model. Important amongst these is the observation that empty receptors do in fact exist in the nucleus of both normal (Jungblut et al., 1978; Carlson and Gorski, 1980; Clark et al., 1982) and other oestrogen target tissues (Sonnenschein et al., 1976; Edwards et al., 1980).

Consistent with this is the failure to observe activation in cytosol in the absence of a nuclear component. Linkie and Siiteri (1978) showed that the conversion of 4S to 5S takes place in the nucleus. They used immature rat uterus, sucrose gradient analysis and biochemical extraction using high salt buffer for nuclear receptor. After 2h incubation of the receptor extract with E_2, the 4S conversion to 5S was detected and the ratio was quantified. What they observed as the 5S was always in the nuclear pellet, while the ratio of the 5S to 4S rose from 0 to 6.41. Pietras and Szego (1979) using hypotonic saline and 0.25M sucrose buffer found that the use of these buffers led to the recovery of cytosolic (4S) empty receptor from the particulate fraction. Thus the origin of these unfilled sites is still not very clear.

1.1.4.2 The Equilibrium Model

Using autoradiography of the rat uterus, Sheridan et al. (1979) showed that nuclear uptake of tritiated oestradiol-17β([^{3}H]E_2) occurs even after short periods of in vitro incubation at low temperatures. These conditions are said to reduce translocation from the cytoplasm to the nucleus to a minimum. In another experiment Martin and Sheridan (1980) prepared the nuclei in different buffers. The first buffer contained 100% glycerol in which a frozen and freeze dried cell pellet was homogenised. The other buffer contained 10% glycerol in Tris or phosphate buffer. They found that with 100% glycerol significantly more nuclear receptor was recovered compared to 10% glycerol in phosphate buffer. The use of 10% glycerol showed that the proportion of receptor found in the cytosol, depended on the volume of buffer used in the homogenisation.

Sheridan et al. (1981), also did experiments involving

progesterone receptor. By using a thaw-mount autoradiograph technique, with [^{3}H] progesterone and [^{3}H] R5020, they concluded that the steroid was localised in or over the nucleus. But when the progesterone receptor was analysed biochemically, the receptor was mainly in the soluble fraction. Thus, after these studies Martin and Sheridan (1982) proposed that, in the steroid responsive cell, empty steroid receptors are in equilibrium between the available water space of cytoplasm and the nucleus, the equilibrium being shifted in favour of the 'cytoplasm' by tissue homogenization (Figure 4).

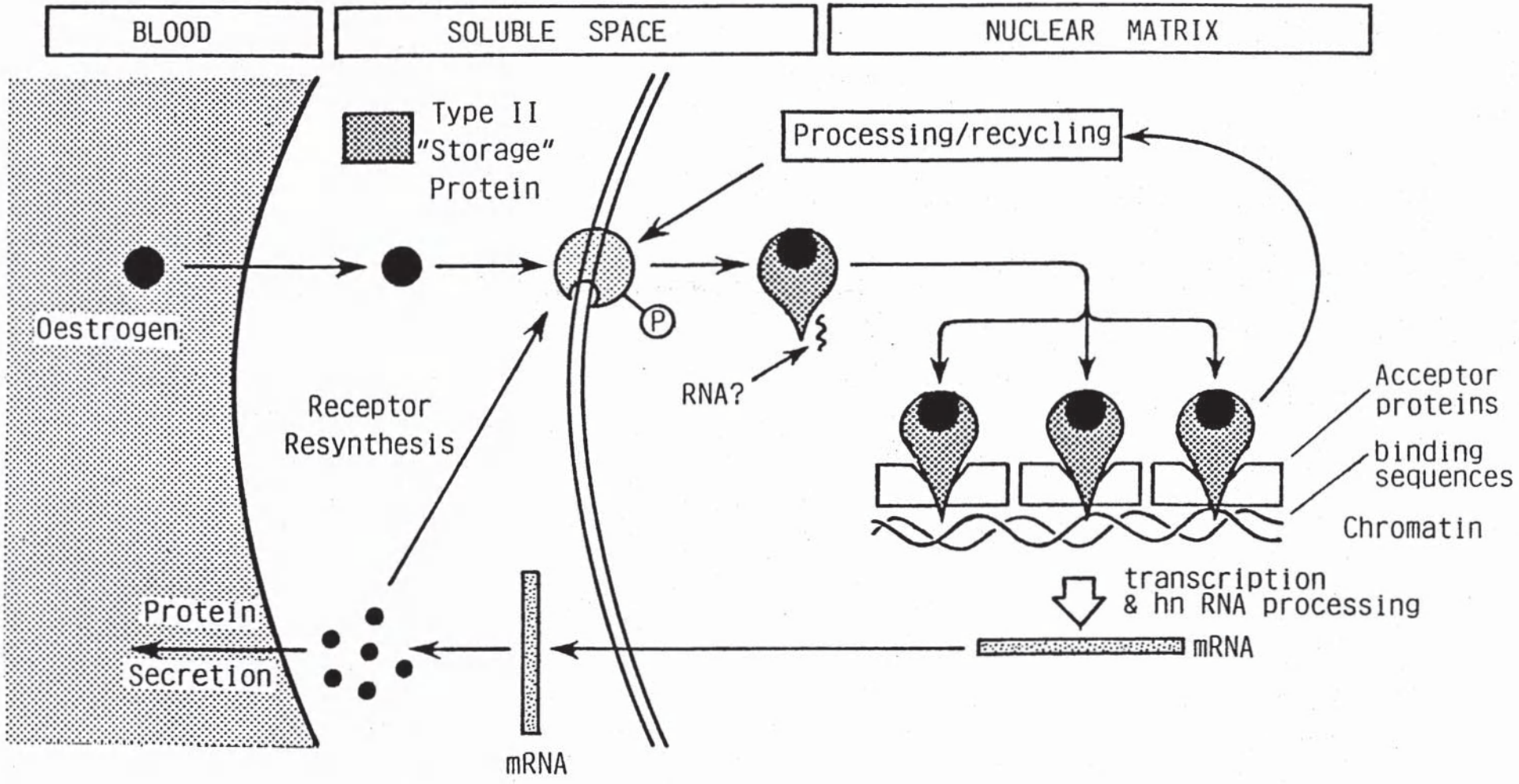
Based on this model, empty nuclear receptor assayed using conventional techniques may only represent a small fraction of the total of the cellular content of unfilled sites actually present with the bulk of unfilled sites being soluble in the cytosol during the preparation.

1.1.4.3 A New Model of Steroid Action

Given the concept that some cytosol receptor may be present due to the breaking down of the nucleus during extraction, Welsholns et al. (1984) recently described a novel procedure involving the use of cytochalasin B-induced enucleation in the preparation of cytoplast and nucleoplast fractions from receptor-rich GH_3 cells derived from rat pituitary tumour. The cytoplast fraction was found to contain little oestrogen receptor, about 5-10% of the whole cells, while most of the empty receptor resided within the nuclear fraction. Scatchard plot revealed that the K_d was about 0.3nM and there were 20,000-30,000 binding sites per cell. The total number of receptors per cell was retained when the whole cells were incubated in the enucleation medium. This finding supports the idea that empty receptors reside within or in association with the nucleus of oestrogen responsive cells.

Another important and equally convincing study was carried out independently by King and Greene (1984). They used monoclonal antibodies which are specific to oestrogen receptor. Five different

Figure 4. The Equilibrium Model of Steroid Hormone Action
(Adapted from Leake, 1985)


BLOOD
SOLUBLE SPACE
NUCLEAR MATRIX
Type II
"Storage"
Protein
Processing/recycling
Oestrogen
P
RNA?
Receptor
Resynthesis
Acceptor
proteins
binding
sequences
Chromatin
transcription
& hn RNA processing
mRNA
Protein
Secretion
mRNA

monoclonal antibodies were used individually to localise the oestrogen receptor from different tissues including fixed sections of human breast tumours, human uterus, rabbit uterus and other mammalian reproductive tissues, as well as fixed MCF-7 cultured cells. They observed that, using the immunoperoxidase technique, the specific staining was confined to the nucleus of all oestrogen sensitive tissues while staining was limited to a few cells in receptor-poor breast cancer tissues. Non-responsive tissue such as colon epithelium was negative. King and Greene incubated some of the target tissues or cells in E_2 for various times prior to staining. No increase in nuclear intensity was observed with time of exposure to E_2. From this, they concluded that both the cytosol and nuclear forms of the receptor reside in the nuclear compartment and no translocation was taking place after the formation of the oestrogen receptor complex. The distribution of oestrogen receptors as predicted from the studies of Welshons et al. (1984) and King and Greene (1984) could be viewed as only an alteration in the position of the activation and translocation step from that established in the two-step model. According to Leake (1985) the two-step mechanism of action is not totally abolished and it is only the "site of action" that must be changed. That is, steroid still diffuses into the cell down a concentration gradient, then comes in contact with the empty receptor which is loosely attached to the nucleus. This is followed by the activation of the complex which has a high affinity for a specific nucleotide sequences, located in, or close to, the AT-rich sequences whose binding of steroid-receptor complex may be helped by the non-histone chromosomal proteins. Thus, activation is merely a nuclear event, as the study of Linkie and Siiteri (1978) suggested. However, much work needs to be done before the precise cellular location of each event can be described.

1.1.5 Binding of the Steroid-receptor Complex to the Nucleus

1.1.5.1 Acceptor Site

An acceptor site is a site in the nucleus where the steroid receptor binds specifically to the chromatin to induce a biological

response (Leake, 1976). Evidence regarding the participation of non-histone chromosomal proteins in localising the sites of interaction of the activated complex with the chromatin, comes mostly from Spelsberg's group. Several groups believe that the receptor may have two independent binding sites, one for hormone and the other for interaction with chromatin (Milgrom et al., 1973; Khan et al., 1980; Myatt et al., 1982a,b)

Spelsberg and his colleagues, working on the progesterone receptor complex derived from the avian oviduct, found that a specific component of the chromosomal non-histone protein fraction is the essential protein component of the nuclear acceptor site. The acceptor protein, bound to DNA, may act as the appropriate chromosomal signal to rapidly pull the activated receptor to the general region of the DNA containing the specific polynucleotide receptor binding sequence (Leake, 1981). A similar observation has been reported for acceptor proteins of activated oestrogen receptor (Ruh and Spelsberg, 1983). Using differential extraction of chromosomal proteins, Spelsberg determined the fractions which contained the specific acceptor protein. Extraction using 3M NaCl at pH 6.0 extracts all the histones plus 10% non-histone protein. A solution of 4M GuHCl removed 80% of the non-histone and 7M GuHCl removed another 10%. None of the three fractions isolated showed any ability to bind to the receptor-protein complex. However, in complex with DNA, the last fraction retained the majority of the receptor, whilst the other two fractions still had minimal binding activity. When an undamaged, protein-free DNA preparation was used, and the condition of incubation controlled to be very close to physiological, minimal binding of activated progesterone-receptor complex occurred (Thrall and Spelsberg, 1980).

However, conflicting observations on this property of the acceptor site have also been reported. While Buller et al. (1975) and Spelsberg (1976) observed saturation in the binding of oestrogen receptor to the nuclear sites, Chamness et al. (1974) did not. This conflicting result could be due to the different experimental conditions whereby a physiological ionic strength buffer was not

used by the latter group.

The involvement of non-histone proteins in receptor-chromatin interaction has been agreed in many laboratories besides Spelsberg's (King and Gordon, 1972; Puca et al., 1974, 1975; Mainwaring et al., 1976). The role of DNA in the acceptor activity has also been concluded from several studies. Treatment with DNAase destroyed the acceptor activity (Shyamala-Harris, 1971; King and Gordon, 1972) while preheating the nuclei or treating them with RNAase did not change the acceptor activity. An agreement was reached from three different groups that both DNA and chromatin were involved in acceptor activity of the steroid-receptor complex in the nucleus (King and Gordon, 1972; Spelsberg, 1974; Leake, 1976). Activated semipurified glucocorticoid receptors have been found to bind to a DNA fragment which contains about half the sequences present in intact murine mammary tumour virus (MMTV) DNA. One sub-fragment was found to bind to the glucocorticoid-receptor with enhanced activity (Payvar et al., 1981; Govindan et al., 1982; Payvar et al., 1982).

Other important studies were carried out by Mulvihill et al. (1982). Competitive binding assays were done in which oviduct progesterone-receptor complex was incubated with DNA-cellulose. By competing with purified cloned fragments of genomic DNA from hormonally responsive genes coding for the egg white protein and comparing the homology of the various sequences in their experiments, the authors concluded that a highly AT-rich sequence is the appropriate binding region for the progesterone-receptor complex.

The confirmation of the involvement of non-histone protein-DNA complexes is important in the field of recombinant technology. The isolation and molecular cloning of certain steroid modulated genes has successfully been carried out (Dugaiczyk et al., 1978; Gannon et al., 1979; Payvar et al., 1981). For example subunit A of the progesterone receptor has been shown to bind selectively to the ovalbumin gene fragment (Compton et al., 1982). This further supports the involvement of a specific DNA sequence in the genomic

binding of steroid receptor.

Yamamoto and Alberts (1975), after observing the presence of low affinity binding of oestrogen-receptor complex to the DNA, suggested that, since the low affinity binding exists to a far greater extent than the high affinity binding, there is a possiblity of a masking effect.

1.1.6 Oestrogen Receptor Replenishment

After being translocated, the oestrogen receptor undergoes restoration or replenishment and disappearance from the nucleus. How these processes are achieved in the system has been investigated by many laboratories.

When oestrogen is injected into the oestrogen responsive system, there is a depletion of cytosolic receptor with a concomitant increase of the nuclear receptor. This phenomenon is then followed by a gradual rise in the cytosolic unfilled receptor. This is termed replenishment. Anderson *et al*. (1974) suggested that the replenishment was important before the tissue can respond to further administration of oestrogen.

1.1.6.1 Mechanism of Receptor Replenishment

The use of the protein synthesis inhibitor, cycloheximide, has provided information on receptor replenishment. However, the use of this inhibitor is time dependent. It is only effective (at inhibiting replenishment of receptor) when given 0-2h after the injection of E_2. When given at 6h later no effect was detected (Sarff and Gorski, 1971). Cidlowski and Muldoon (1976) observed a similar result when working with castrated rats. Kassis and Gorski (1983) interpreted the result to mean that the synthesis of receptor does take place but that the new receptor is the inactive form.

Contradictory results have been also reported by other groups. Using a higher dose of E_2 (1µg) Mester and Baulieu (1975) found 90% depletion of cytosolic receptors and replenishment

(50% of control value) obtained after about 6h. Replenishment of receptors was completed in 11h.

Many factors need to be considered when using this approach. Firstly the use of an inhibitor of protein synthesis which could kill the animal before the study is completed. The study of receptor replenishment is a slow process and the toxicity of the inhibitors of protein synthesis could kill the animal in 12h exposure. Secondly the stability of the receptor is still under debate. Uterine oestrogen receptor was found to be stable for up to 8h in the presence of this drug by Sarff and Gorski (1971) while Cidlowski and Muldoon (1976) noticed a conspicuous drop at this time.

Further evidence comes from the work of Horwitz and McGuire (1976) and Sica et al. (1981) who studied the effects of protein and RNA synthesis inhibitors (cycloheximide and actinomycin D) on the loss of nuclear receptor. It has been found that both the compounds inhibit nuclear receptor loss in some system. This implies that, the drugs might stabilize the receptor in the nucleus rather than inhibiting the synthesis of a new protein.

1.1.6.2 Evidence of Recycling

To shorten the time for the observation of receptor replenishment, Kassis and Gorski (1983) used a 'short acting' oestrogen (oestradiol-16α) rather than the 'long-acting' E_2. The physiological difference between the two oestrogens is: 'short-acting' oestrogen induces very early responses (water imbibition, induced protein synthesis etc.) but not DNA synthesis which is a long-term response. Injection of oestradiol-16α shows complete replenishment of receptor after 4h, whereas DES or E_2 gave no replenishment in that time. Since replenishment could be observed in such a short time, they could rule out the weaknesses of the use of the drug. They also used cycloheximide in their studies. They could not observe any reduction in cytosolic receptor replenishment. Thus they concluded that receptor recycling, rather than resynthesis, occurs after induction of, at least, the short-term

responses.

The effect of progesterone on oestrogen receptor replenishment had also been looked at. Experiments done by Hsueh et al. (1975) showed that progesterone could reduce the concentration of cytosolic oestrogen receptor. In 1976, the same group reported that progesterone inhibits synthesis of receptor but does permit re-cycling of waiting receptor.

1.1.6.3 Receptor Processing

If receptor is simply recycled, then the total receptor concentration in the system should always remain constant. However, this is only true for rats treated with oestradiol-16α (Kassis and Gorski, 1983) and not those treated with other oestrogen, (Mester and Baulieu, 1975; Zava et al., 1976; Baudendistel et al., 1977; Kassis and Gorski, 1981; Sica et al., 1981). This loss of receptor is termed processing, which means the loss of detectable binding of steroid. This might mean that there is a change in the binding activity of the receptor.

Thus processing may also be a modification of the oestrogen receptor to a form which is not able to recognise steroid. If this is the case, it will not be detected by the normal steroid binding assay. Using inhibitor studies, Horwitz and McGuire, (1978c, 1980) showed the possibility of inhibiting processing. Actinomycin D and chromomycin A, both G-C specific DNA intercalators, were very effective in inhibiting processing in MCF-7 cells. This could be due to the direct disruption of the DNA structure.

Further evidence that processing is due to inactivation of receptor comes from the work on Swiss mice. The loss of oestrogen binding in the nuclei of cells from these mice can be inhibited by molybdate, thus a phosphatase might be involved in this process (Auricchio et al., 1981). Futher, when the depleted oestrogen-binding cytosol is treated with ATP, an active binding component was recovered (Migliaccio and Auricchio, 1981). From these

studies, Auricchio et al. (1981) suggested that the receptor is processed or inactivated by dephosphorylation in the cytoplasm.

However, there is no clear indication that processing is involved in the oestrogen response pathway.

1.2 CARCINOMA OF THE BREAST

1.2.1 Oestrogens in Breast Cancer

Oestrogens have long been recognised as trophic factors for normal and neoplastic breast growth. In 1896 Beatson first observed tumour regression following oophorectomy, in premenopausal patients with advanced breast cancer. Huggins and Bertgenstal (1952) reported the same observation following adrenalectomy on postmenopausal breast cancer patients.

The first evidence that the interaction of oestrogen with the tumour could be of diagnostic value was given by Folca et al. (1961), who reported that the lesions of the patients who later responded to adrenalectomy took up more of the administered oestrogen (hexoestrol) than did the lesions of the patients who did not respond.

The administration of pharmacological doses of oestrogens will also cause tumour regressions in some patients (Stoll, 1972). It is reported that the regression in response to either of these therapies is correlated with the presence of oestrogen receptor in tumour biopsies (Englesman et al., 1973; Byar et al.,1979; Hawkins et al., 1975; Leake et al., 1981)

1.2.2 Oestrogen Receptors in Human Breast Tumours

Steroid hormone receptors occur late in the differentiation pathway of breast epithelial precursor cells. For this reason they are, in normal cells, a direct index of the highly differentiated state (Leake, 1981). Thus receptor positive tumours might be

expected to reflect slow and controlled growth with minimum invasive potential.

Another phenomenon which is said to have a role in neoplastic mammary tissues is increased androgen metabolism, leading to synthesis not only of oestrogens but also of a spectrum of C19 steroids (Couch et al., 1973) which are released into the immediate micro-environment of the oestrogen receptor in mammary cells and which may modulate E_2 binding and receptor translocation (Poortman et al., 1975; Nicholson et al., 1978; Zava and McGuire, 1978).

Refinements in laboratory technique have enabled steroid receptors to be quantitatively assayed in individual tissues. These refinements have also led to the discovery of receptors in mouse and rat mammary glands (Shyamala and Nandi, 1972; Bohnet et al., 1977), normal mammary gland at low levels (Sander, 1968; Block et al., 1975) and in malignant tissue (Jensen and DeSombre, 1972; Leake et al., 1981a)

Normal mammary cells contain specific receptors for each of the hormones known to influence growth and function of the mammary gland. These receptor sites are responsible for the initial interaction between the hormone and the cell, and trigger the biochemical events characteristic for that particular hormone. When malignancy occurs, the mammary gland epithelial cells could escape the influence of oestrogen, and could lose the capacity to synthesize oestrogen receptor. Alternatively receptor sites may be retained by only a proportion of the cells (Wittliff et al., 1972; Jensen, 1975). In the later case, the growth and function of a tumour may still be governed and controlled by the hormones as in normal cells and such tumours may be responsive to endocrine therapy, at least initially. If the receptors are lost from the tumour cells, the cells would be endocrine resistant and would not be responsive to hormone manipulation, except by some indirect mechanism.

In breast cancer, the first receptors to be identified were oestrogen receptors. There are extensive data that the growth promoting

effect of oestrogen is receptor-mediated (Wittliff, 1979). The properties of the oestrogen receptor have been demonstrated in induced hormone-dependent rat mammary tumours and in human mammary tumour cytosols (McGuire and De La Garza, 1973). The principal properties are the high affinity binding of $[^3H]E_2$ (K_d<1nM) and the sedimentation values of the oestrogen receptor i.e. 8S in low salt and 4S in high salt sucrose gradients. Parallel gradients are always run with a 100-fold excess of unlabelled diethylstilboestrol to measure any nonspecific binding component.

An indirect role of prolactin in governing the tumour growth in rat mammary tumour had been reported (Sinha et al., 1973). Prolactin had been demonstrated to be able to increase the level of oestrogen receptor in both rat mammary gland and uterus and in mammary tumours of intact ovariectomised and ovariectomized-hyphophysectomised rats (Sasaki and Leung, 1974; Vignon and Rochefort, 1976; Asselin et al., 1977). There is considerable indirect evidence for a role for prolactin in human breast cancer (Sinha et al., 1973; Henderson et al., 1975; Pearson and Monni, 1978).

Very little is known about the inhibition of oestrogen receptor synthesis. Experiments showed that prostaglandins could inhibit oestrogen receptor synthesis (Jacobson, 1974). Progesterone is recognised as a physiological inhibitor of oestrogen receptor synthesis (Bohnet et al., 1977; McGuire, 1978; Clark and Peck, 1979).

The use of oestrogen receptor as an index of hormonal involvement in growth and development of tumours has been studied by various groups (Feherty et al., 1971; Maass et al., 1975; Englesman et al., 1973). Other studies showed that 40-60% of patients with oestrogen receptor positive tumour biopsies show regression after receiving hormone therapy (Byar et al., 1979; Hawkins et al., 1980; Leake, 1981) and after taking the receptor concentration into account the possibility of response is increased (Jensen, 1975; Leclerq and Hueson, 1977) but only in premenopausal patients

(Hawkins et al., 1979).

It has been suggested that patients with receptor in both cytosol and the nuclear fractions have a much better chance of responding to hormone therapy than those with receptor in the cytosol alone (Leake et al., 1981a).

Receptor negative tumours were thought to be more aggressive (Knight et al., 1977) and growing at a faster rate (Meyer et al., 1977). Various reports showed that patients with E_2 receptor negative had earlier recurrance rates and shorter survival times compared to patients with receptor positive tumours (Kiang et al., 1978; Bishop et al., 1979; Cooke et al., 1979; Kinne et al., 1981).

Oestrogen receptors have been the most extensively studied, the following statements summarise the present knowledge of receptors in breast cancer.

1. 60 to 70% of primary breast cancers are oestrogen receptor positive. This is lower in advanced disease.

2. Premenopausal women have a lower incidence of oestrogen receptor positive tumours than postmenopausal women.

3. Receptor status may change with the natural history of the disease. Thus a primary oestrogen receptor positive lesion may recur with oestrogen receptor negative metastases, and vice versa (though this latter is unusual).

4. Individual tumours are probably heterogenous with regard to receptor concentrations, some clones of cells being receptor positive, others receptor negative.

5. Following from (4) it has been suggested that the higher the concentration of oestrogen receptor, the greater the chance of a response to endocrine therapy. A number of clinical studies support this theory (DeSombre and Jensen, 1980).

6. The risks of breast cancer are thought to increase in women with no ovulation due to an inadequate level of progesterone (Lilienfeld et al., 1975). Sherman and Korenman (1974) also reported that oestrogenic stimulation of breast cell growth in the absence of sufficient progesterone secretion could provide a favourable environment for the development of breast cancer. Thus, progesterone receptor levels are found to be important.

7. Androgen receptors are found in about 50% of breast cancers, and glucocorticoid receptors in about 30%. The therapeutic significance of these receptors remains to be assessed.

8. About 50% of ER+ and 70% of ER_c/ER_n+ or ER+/PR+ tumours will respond to endocrine therapy, whereas only 5-10% of receptor negative tumours seems to be hormonally sensitive. It has been suggested that receptor positive patients are less likely to respond to cytotoxic therapy, but this remains controversial (Lippman et al., 1978; Kiang et al., 1978).

The relationship between oestrogen receptor content of primary breast cancer and subsequent prognosis was examined with regard to nodal status (Leake et al., 1981b). They found that, within a particular nodal group, patients with tumours containing fully functional oestrogen receptor had a longer disease-free interval than those with receptor negative tumours. Also that receptor negative primary disease gave rise to distant metastases as first site of recurrence more frequently than did receptor positive patients.

The discovery of steroid receptors does not automatically explain all aspects of endocrine therapy in breast cancer, but it does offer a starting point for putting that explanation on a rational basis. Furthermore, as oestrogen receptor assays become more widely available, they offer an important guide for selecting appropriate treatments for women with breast cancer.

1.2.3 Treatment of Breast Cancer

Beatson's observation that, after oophorectomy two premenopausal patients with advanced breast cancer experienced regression of disease laid the foundation for the development of endocrine therapy, in breast cancer. Endocrine therapy was originally ablation (castration, adrenalectomy, hypophysectomy). Later additive therapy, (pharmacological doses of oestrogens, androgens and glucocorticoid) was preferred and, more recently, use of synthetic antioestrogens anti-prolactins and LHRH analogues have all been tried. In premenopausal women oophorectomy is still common whereas postmenopausal patients usually receive tamoxifen - an anti-oestrogen with minimal side effects.

Another important factor in the endocrine therapy of breast cancer is the withdrawal response. Of those women who gain a remission on oestrogen or antioestrogen therapy, about 30% will achieve a second remission merely by stopping therapy (Patterson et al., 1982).

Tamoxifen, probably acts in the long-term, through inhibition of replenishment of available, functional receptor. Biopsies taken from patients, prior to treatment with tamoxifen, have yielded significant quantities of functional receptor, but biopsies taken during treatment show no detectable receptor and receptor does not reappear until some weeks after treatment is ceased (Leake et al 1980b).

Because anti-oestrogens act initially through the receptor system, they appear to be oestrogenic in the short term. Tamoxifen, for example, will induce the first round of DNA synthesis and the characteristic oestrogenic increase in wet weight at 24h after injection. However after a longer time course, the anti-oestrogenic activity becomes apparent, presumably due to, either the loss of available receptor or to the blocking of the relevant nuclear binding sites (Leake et al., 1980b).

Thus, the current choice of endocrine therapy for patients with advanced disease is tamoxifen initially for receptor positive patients and combination chemotherapy in receptor negative patients. A similar approach has been made to adjuvant therapy for primary disease with node involvement (Bonadonna et al., 1982). It seems reasonable in theory but recent evidence suggests that adjuvant chemotherapy may be only of general value to pre-menopausal patients (Rouesse, 1984)

These findings of receptor assays and response to endocrine therapy have led to the classification of hormone dependent and hormone resistant tumours (McGuire et al., 1975).

1.2.4 Alternative Indices of Hormone Dependence

Alternative indices of hormonal-dependence have been tried, and perhaps the most successful is measurement of soluble progesterone receptor, a product of oestrogen action in normal target tissues. However the presence of progesterone receptor is not always associated with an improved clinical response. It is usually associated with the presence of fully functional oestrogen receptor and so yields a similar success rate in the identification of responders to hormone therapy (Barnes et al., 1979; Thorsen and Stoa, 1979).

1.3 UTERUS AND STEROID HORMONES

1.3.1 The Uterus

The uterine wall is mainly made up of bundles of smooth muscle fibres supported by vascular connective tissue. This is called the myometrium. The uterine cavity is lined by a mucous membrane termed the endometrium which consists of a mixture of simple columnar ciliated and secretory epithelium supported by a broad, highly cellular, connective tissue stroma. The endometrium experiences monthly cyclic changes in structure. This is in response to rhythmical variations in the secretions of steroid hormones from the

ovary.

The endometrial surface epithelium is invaginated to form numerous tubular glands called uterine glands. These glands extend into a thick lamina propia, which is referred to as endometrial stroma. It contains stellate cells embedded in a copious ground substance. The epithelium lining the glands is continuous with that of the surface and contains fewer ciliated cells. The glands subdivide in the deeper layer of the endometrium and vary in size and shape during the monthly cycle.

The uterus is supplied by a highly coiled uterine artery which arises from the internal iliac artery. Large branches pass transversely across the surface of the organ to anastomose with the vessels from the opposite side. The deepest portion of the endometrium, the stratum basale, is supplied by the basal arteries. Continuing upwards into the endometrium, the spiral arteries reach the innermost endometrial zone, where they break up into a rich network of capillaries. The region of the endometrium which the spiral arteries course is called the stratum functionalis.

1.3.2 Uterine Cycle

Hormones secreted by the ovary during the ovarian cycle result in changes involving principally the epithelium and supporting connective stroma tissue of the endometrium. This is termed the uterine cycle.

In women, the uterine cycle can be divided into three phases. The proliferative phase coincides with the growth of the ovarian follicles and their secretion of oestrogenic hormones. The proliferative phase lasts for 10-11 days. In this phase, the endometrium initially consists solely of the stratum basale, which represents only a few stumps of uterine glands embedded in a dense endometrial stroma. The surface epithelium rapidly generates from these glands and lines the uterine cavity.

In the ovary, if ovulation is to occur, the Graafian follicle (and the egg within it) must undergo further changes that can only take place under precisely controlled hormonal conditions. The level of circulating FSH remains elevated for only a short time at the beginning of the growth phase, after which the quantity of circulating gonadotrophin (FSH and LH) remains fairly constant. The onset of pre-ovulatory maturation is marked by a sudden and dramatic rise in gonadotrophins from the pituitary, especially of LH (the so-called 'LH surge'). In reality, both the FSH and LH levels rise. The LH surge (or the increase in oestrogen which causes it) is said to induce a final 'wave' of mitosis in granulosa cells so that their number reaches an optimum size for the ovulatory Graafian follicle. The oocyte is shed from the Graafian follicle by the process of ovulation at a precise time after the onset of the LH surge.

The secretory phase coincides with the period when the corpus luteum is functionally active and secreting progesterone. This phase lasts about 14 days. Under the influence of the steroid hormones the cells of the endometrial stroma enlarge. The glands dilate and finally the endometrium forms a thick, edematous, highly vascular lining. When the secretions from the ovary decline the endometrium breaks down. This gives way to the third phase of the menstrual cycle. Towards the end of the menstruation, the endometrium is reduced to about half a millimetre in thickness which is approximately a tenth of its maximum thickness.

In other mammals, changes in plasma levels of steroid hormones give rise to the oestrous cycle. A duration of 4-5 days is common in rats and mice (Johnson and Everitt, 1980). The cyclic changes are somewhat similar to those described in humans, except for the absence of bleeding at the end of the secretory phase.

1.3.3 The Effect of Oestradiol on Uterine Cells

Oestradiol-17β stimulates uterine growth by inducing cellular hypertropy and hyperplasia. The magnitude of the hyperplastic growth response is dependent upon the species and strain of animal

and its hormonal status (Martin et al., 1973; Martin, 1980; Kirkland et al., 1981).

Morphological changes in the hamster uterus during the oestrous cycle include proliferation of the luminal epithelium prior to ovulation (Ward, 1948). Similar cyclic changes occur during the oestrous cycle of the rat and mouse (Allen, 1922). In these species, proliferation occurs in response to oestrogen and is blocked by progesterone (See Sandow et al., 1979).

West et al. (1978) correlated the serum oestrogen and progesterone levels and uterine oestrogen receptor levels with the morphology of the uterine luminal epithelium in cycling and hormonally altered hamsters. Proliferation of the uterine luminal epithelium occured from dioestrus day 2 through pro-oestrous, when oestrogen was elevated and serum progesterone was low. Uterine nuclear oestrogen receptor levels were found to be elevated. Degenerative changes in the epithelium occurred during oestrous when serum oestrogen was low and serum progesterone was high. Uterine nuclear oestrogen receptor levels were depressed. The degenerative changes were symptoms of oestrogen withdrawal because they occurred within 24h after ovariectomy on the morning of pro-oestrous and could be prevented by oestrogen treatment (West et al., 1978).

Sandow et al. (1979) reported ultrastructural evidence for the degenerative process in the hamster uterus including the death of numerous epithelial cells by apoptosis, a type of cell death characterized by extensive nuclear and cytoplasmic fragmentation (Kerr et al., 1972). The ultrastructural alterations in such cells include condensation and margination of the chromatin, cytoplasmic condensation, amoeboid changes in cell shape with formation of pseudopodial extensions and nuclear and cytoplasmic fragmentation. The cell fragments, or apoptotic bodies, accumulate near the basal lamina, and are then phagocytosed by macrophages and possibly by neutrophils as well. Apoptosis appears to be a sensitive morphological end-point of oestrogen withdrawal in the hamster uterine luminal epithelium (Sandow et al., 1979).

Work on determination of oestrogen receptors in human uterus suggests that functional binding sites in human tissue have a $K_d=10^{-10}M$ (Soutter et al., 1979) and the same was reported by Zachariah and Chakmakjian (1983). The stability of the uterine receptor was checked using cytosol prepared from tissues and then immediately frozen in liquid nitrogen compared and with tissues kept for various lengths of time up to 20 months at -70°C. They found that the oestrogen receptor from cytosol prepared from frozen tissues were more stable. Soutter et al. (1979) found that the dissociation constant of the E_2 receptor of human endometrium was similar in both cytoplasmic and nuclear fractions which was in common with other workers using rat uterus and human breast tumour cell lines. They also found that inhibition of oestrogen receptor by progesterone takes place in human endometrium at late follicular phase (Soutter et al., 1979).

1.3.3.1 The Oestrogen Effect in Mature and Immature Animals

Uteri from immature and mature rodents are used extensively as model systems to evaluate oestrogen-receptor interactions and tissue responsiveness to oestrogen stimulations. However there exist important differences between the cellular responses seen in uteri of immature and mature rats. For example, in the immature rat, high doses of oestrogens stimulate cell division in all uterine component tissues: epithelia, stroma and myometria (Kaye et al., 1972; Kirkland et al., 1979). In contrast, in the adult castrated rat, this does not happen. Only the uterine epithelial cells respond to oestrogen stimulation through DNA synthesis and cell division (Clark, 1971; Tachi et al., 1972).

1.3.3.2 Effect of Oestrogen on the Mouse Uterus

The uterus of adult ovariectomized mice show two peaks of DNA synthesis occurring after oestrogen stimulation (Kerach, 1981). This raises the possibility that in the mouse uterus, DNA synthesis and cell division might be occurring in two different cell types at two different times after oestrogen treatment.

Quarmby and Korach (1984) reported that uteri from immature (21-day-old) and adult mice show different patterns of cell division in response to a physiological dose of E_2. In the immature mouse uterus, oestrogen increased the stromal and epithelial cell proliferation by shortening the cell generation time. In the adult mouse uterus, oestrogen stimulated epithelial but not stromal cell proliferation. They discovered that oestrogen stimulated uterine hyperplasia by selectively decreasing the G1 phase of the cell cycle in specific cell populations. In their developmental study, they found that the stromal cell population gradually lost its ability to divide in response to oestrogen stimulation during day 22-52 after birth. In prepubertally ovariectomised mice, the stromal cell population showed a very low mitotic response to oestrogen stimulation at all ages. Thus an ovarian mechanism may regulate the change in stromal responsiveness to oestrogen stimulation.

1.4 ANTIBODIES TO OESTROGEN RECEPTOR

Biochemical assays of oestrogen receptor require relatively large amounts of tissue. Both for investigation of oestrogen receptor in primary cell cultures and to determine oestrogen receptor in small clinical samples such as a fine needle aspirate (FNA), an antibody to oestrogen receptor would be most valuable.

1.4.1 Polyclonal Antibodies

As soon as the oestrogen receptor was accepted as the main molecular component for the mechanism of action of oestrogens, attempts at purification began in several laboratories (Jungblut et al., 1965, 1967; De Sombre and Gorell, 1975; Puca et al., 1970, 1972, 1975; Sica et al., 1973; Bresciani et al., 1978). The aim was to produce purified receptor suitable for biochemical, chemical or physical characterization. It was also hoped that the molecule would have sufficient antigenicity for antibody production. The presence of receptor protein in the cell is very low i.e. only between 10^4 and $5x10^4$ copies/cell (Moncharmont and Parikh, 1984). Therefore the difficulties lie in the preparation of homogeneous receptor

protein in amounts sufficient to immunize the animals and to produce antibodies in quantities adequate for characterization of the antigen. DeSombre and Gorell (1975) and Bresciani et al. (1979) have used kilogram quantities of calf uterine tissue to recover 1-2mg of receptor protein.

The first report of the production of a rabbit antiserum to oestrogen receptor dates back to 1969 (Soloff and Szego, 1969). They used a semipurified oestrogen receptor and the presence of the antibody was checked by looking at the shifted peak on sucrose gradients in the presence of such an antibody. Then, in 1976, Fox et al. published another report on the production of rabbit antiserum against oestrogen receptor. They suggested that the receptor be injected into the rabbit in its aggregated form in order to increase its antigenicity. They used gel filtration chromatography and immunoprecipitation as specificity tests for the antibody.

In 1977, Greene et al. prepared the antigen from calf uterus after translocating the cytosolic receptor to the nucleus and subsequent extraction in high salt. The purified antigen was injected into rabbits. For specificity tests of the antibodies they used immunoabsorbants, immunoprecipitation and sucrose density gradient analysis methods. These antibodies were found to react with cytosolic and nuclear oestrogen receptor complexes from calf uterus as well as oestrogen receptor from other mammals and also from chick oviduct. They also found that the binding of the antibodies to the antigen did not affect the hormone binding site. A similar procedure was applied to raise antibodies in goat (Greene et al., 1979). The properties of the antiserum were similar to the previous rabbit antiserum except that the affinity of the receptor for ligand was reduced if the goat antibodies were allowed to react with naked receptor protein prior to incubation with oestrogen. They demonstrated the immnochemical similarity of mammalian oestrogen receptor, since the immunoglobulin obtained from both species cross-reacted with all radioactive oestradiol-receptor complexes from several mammalian species. The immunoglobulin showed no affinity for the receptor proteins for other steroid hormones. These

antibodies recognise the oestrogen receptor when associated with antioestrogen (Garcia et al., 1982). Radanyi et al. (1979) did not use purified, translocated receptor but rather used purified cytosolic receptor from calf uterus as antigen and reported that they raised satisfactory antibodies in rabbit.

In 1973, Sica et al. introduced the affinity chromatography method in the production of highly purified oestrogen receptor from calf uterus. Since then, the application of affinity chromatography in the purification process had been widely applied. Coffer et al. (1980) and Coffer and King (1981) reported the use of human uterine oestrogen receptor, purified through affinity chromatography and by electrofocusing. Their antibodies were raised in sheep. Besides having reactivity with human oestrogen receptor, the antibodies are also reported to cross-react with other mammalian oestrogen receptor. Other antisera to oestrogen receptor have been reported by Raam et al. (1981) using gels as a means of purification. The sections of the gels, containing oestrogen receptor from human breast cancer, were sliced, pooled, and ground to a fine powder before being injected into rabbits. This group reported that the antiserum which they raised decreased the affinity of the receptor for its ligand. Al-Nuaimi et al. (1979) also used oestrogen receptor from tumour tissue but from rat mammary tumour induced by Dimethyl-benzanthracene.

Antibodies against the chick oviduct progesterone receptor have also been raised in goat, and in rabbit. Glycerol gradient sedimentation profiles showed that several immnoglobulin molecules can bind to the chick oviduct progesterone receptor, since several types of antigen-antibody complex were detected (Renoir et al., 1982). Logeat et al. (1981) immunised a goat with a semipurified preparation of progesterone receptor extracted from rabbit uterus. The antiserum obtained reacted with both cytosolic and nuclear rabbit progesterone receptor and also with progesterone receptor from other tissues. A cross-reactivity was also observed with progesterone from cytosolic receptor of rat and guinea pig uterus, human breast cancer tissue, and nuclear receptor from human endometrium. On the otherhand, no interaction with nonmammalian

receptor (chick oviduct progesterone receptor) was observed. Also no cross-reactivity with nonreceptor progestrone-binding proteins (transcortin from plasma and uteroglobin from uterine fluid) was seen.

Tuohimaa et al. (1984) injected a rabbit with the highly purified B-subunit of the chick oviduct progesterone receptor. The specificity of the antibodies obtain were tested using immunoprecipitation, immunoblotting, density gradient ultracentrifugation and Protein A-Sepharose assay methods. They found that the antibodies not only recognised the B-subunit, but also the A-subunit. Besides that, cross-reactivity also occurred with the nuclear progesterone receptor, the meroreceptor (proteolytic cleavage product) and the "non-activated" molybdate stablized "8S" receptor.

Polyclonal antibodies, though heterogeneous, may become an important diagnostic tool in small specimens or smears for the detection of the heterogeneity and localization of receptors in the tissues and cells. As far as structure and function are concerned, monoclonal antibodies are more useful and reliable.

1.4.2 Monoclonal Antibodies to the Hormone Receptor

With the development of hybridoma technology by Kohler and Milstein (1975, 1976), the production of monoclonal antibodies against the oestrogen receptor was made possible. Greene and Jensen (1982a) produced monoclonal antibodies against different sites on oestrogen receptor and used them to build a library of monoclonal antibodies for the location of determinants on the molecule of the protein (Greene et al., 1984). Monoclonal antibodies have been used to study the molecular structure of acetylcholine receptor (Lindstrom et al., 1980; Tzartos and Lindstrom, 1980; Gullick et al., 1981; Conti-Tronconi et al., 1981). Lennon et al. (1980) used monoclonal antibodies for the purification of the cholinergic receptor. The B-adrenergic receptor (Frazer and Venter, 1980), and structure and function of thyrotrophin receptor (Yavin et al., 1981)

have been similarly studied.

1.4.2.1 Monoclonal Antibodies to the Oestrogen Receptor

Monoclonal antibodies to oestrogen receptors were obtained for the first time by Jensen and co-workers (Greene et al., 1980). They immunized rats with oestrogen receptor protein purified from calf uterus. The hybridization of Lewis rat spleen cells with a mouse mutant myeloma cell line gave hybridoma cell lines which secrete anti-oestrogen receptor immunoglobulin of classes G and M. All their clones showed cross-reactivity with receptor only from calf uterus. The IgM has affinity for the nuclear receptor, while the IgG had affinity for soluble as well as nuclear receptor. The K_d for binding was 0.1nM.

Using a human breast cancer cell line, MCF-7, the same group (Greene et al., 1980) after purification of the cytosol through affinity chromatography, produced monoclonal antibodies. The production of large numbers of MCF-7 cells was necessary for this experiment. Using two mouse myeloma cell lines, fusion with the spleen cells of the immunized Lewis rat, followed by cloning from limiting dilution, gave rise to three hybridoma cell lines, two IgG2A and one IgM. One of the antibodies recognized the oestrogen receptor from human and primates only, while the others recognized calf and rat uterine receptors as well. There seems to be evidence that these three antibodies recognise three different epitopes on the human receptor molecule. Moncharmont et al. (1982) also obtained monoclonal antibodies to the native cytoplasmic oestrogen receptor from calf uterus. The fusion of spleen cells from an immunized mouse with a non-secreting mouse myeloma cell line give them five hybridoma lines, all IgG's of subclass 1 and 2A. Four of the clones showed cross-reactivity with the receptor of other mammalian and avian species, while one does not recognize human oestrogen receptor. These monoclonal antibodies show a high affinity (K_d=0.5nM) for the oestrogen receptor. They also found that even though these antibodies show differences in subclasses and cross-reactivity, they have no ability to bind simultaneously to the antigen molecule i.e.

they must all be directed against closely related sites on the receptor molecule.

1.4.2.2 Monoclonal Antibodies to the Progesterone Receptor

Monoclonal antibodies to progesterone receptor have been produced from partially purified molybdate-stabilized progesterone receptor from chick oviduct (Radanyi et al., 1983). They reported one clone which secretes IgG2B and is able to recognise progesterone receptor. This antibody, which has a high affinity for progesterone receptor from chick oviduct with K_d=1nM, does not cross-react with progesterone receptors originating from mammalian species but instead, does cross-react with other chick steroid receptors (Joab et al., 1984). Logeat et al. (1983) also reported the production of monoclonal antibodies to progesterone receptor purified from rabbit uterus. Eleven clones were obtained. Five of the clones show an affinity towards the antigen in the range of 0.1 to 0.4nM. Three of the clones secrete IgG and all the antibodies cross-reacted with mammalian receptors but not avian. No report was made on the cross-reactivity with other steroid receptors.

1.4.2.3 Monoclonal Antibodies to the Glucocorticoid Receptor

Monoclonal antibodies against the 90,000 molecular weight species of the activated rat liver glucocorticoid receptor were reported by Westphal et al. (1982). Using an immunoprecipitation assay of labelled liver cytosol receptor for screening, eight of the clones were found to react with the receptor molecule. One of the positive clones secreted IgG while the rest secreted IgM. Seven of the clones tested reacted with the 90,000 molecular weight form of the receptor which contained both the steroid and DNA binding domain. Other reports on glucocorticoid receptor monoclonal antibodies came from Grandics et al. (1982) and Gametchu and Harrison (1984). The latter group confirmed the specificity of the antibodies by sucrose density gradient analysis and Western blot analysis which showed that the antibody detected a single band with a mobility (molecular weight about 95,000) identical to that of the

[^{3}H] dexamethasone 21-mesylate-labelled rat glucocorticoid receptor.

So far, no reports have been made of the presence of common antigenic determinants between steroid receptors and other steroid-binding proteins, showing that there is no immediate genetic relationship between these proteins. However, the existence of a common site in all steroid receptors needs to be further explored. Radanyi et al. (1983), produced monoclonal antibodies to chick oviduct progesterone receptor, which could recognise not only progesterone receptor from the same tissue, but also the oestrogen, glucocorticoid and the androgen receptors (Jaob et al., 1984), all in their native forms (8S). These receptors, if present in high salt (4S), are not recognised by the antibody. This finding might suggest that the antibody may be directed towards a protein that forms a common subunit to the aggregated form of all steroid receptors in this particular tissue. The validity of such a hypothesis needs to be further investigated and also the application to the different tissues of different species has to be tested. The physiological significance of such an observation has yet to be established.

1.4.2.4 Monoclonal Antibodies and the Structure of the Steroid Receptor

Most of the antibodies to steroid receptors so far obtained have no effect on the interaction of the receptor protein with its steroid hormone. Only some of the antibodies, such as the polyclonal from goat antiserum (Greene et al., (1979) in rabbit (Raam et al., 1981) reduce the affinity of the receptor for oestrogen. Grandics et al. (1982) found a monoclonal antibody to the glucocorticoid receptor which inhibited hormone binding. Several groups have used monoclonal antibodies to investigate the subunit structure of a receptor protein. Each antibody has specificity for only one antigenic determinant, and forms stoichiometric complexes with its antigen, thus information on the number of antigenic determinants present on the molecule can be determined. Moncharmont et al. (1982) and Moncharmont and Parikh (1983) have investigated

various molecular forms of the calf uterine oestrogen receptor using these criteria. They found that the native 8S oestrogen receptor has two antigenic determinants per molecule, while the 4S high salt molecule has only one. They found that agents that induce the dissociation of the 8S form to smaller forms also dissociate the two antigenic determinants. This suggests that the homodimer of the 4S subunit is associated with another protein molecule(s) to form the large 8S form of the native receptor. Moncharmont et al. (1984) also concluded that the 4S form was the smallest form of the molecule and did not contain repetitive structures associated with the steroid binding site. The 5S nuclear receptor, which was said to be a homodimer of the 4S subunit by Notides and coworkers (Notides and Neilsen, 1974; Neilsen and Notides, 1975; Notides et al, 1975, 1981), was shown to possess two antigenic determinants for the monoclonal antibody (Moncharmont et al., 1984).

In 1984, Greene et al. collected a library of monoclonal antibodies reacting with different regions of the human oestrogen receptor molecule. Using this collection, they were able to locate roughly the position of various determinants on the molecule. They could locate an epitope on the DNA binding domain from one monoclonal antibody and found that six of the monoclonal antibodies recognized the steroid binding domain. They then realised that all these seven antibodies recognised receptor from all species while two other monoclonal antibodies, each showing specificity for mammalian or primate oestrogen receptors bind in the region between the steroid binding domain and the DNA binding domain. They used mercuripapain, chymotrypsin and trypsin which had the ability to remove selectively from receptor the determinants for each antibody. However they discovered that their results are in contrast to the findings of Carlstedt-Duke et al. (1982) who reported that the glucocorticoid receptor can be cleaved into three separate domains by limited proteolysis: a domain for binding to DNA, one for binding steroid and the third is the immunoactive site which recognises all the antibodies to glucocorticoid receptor.

1.5 DETECTION OF OESTROGEN RECEPTORS

The first essential criterion of any assay is that it should measure the biologically functional receptor in the target tissue. The assay should also be accurate, relatively simple and inexpensive to perform.

1.5.1 Biochemical Assays

Classical assays of intracellular steroid receptors require homogenization of tissues and titration of binding sites with labelled hormones. The unfilled oestrogen receptor is a soluble protein characterised by a limited binding capacity, high binding affinity and strong specificity for oestrogenic hormones. The assay should have minimal background contamination due to binding of the hormone to non-specific sites. Binding to the cellular receptor should be a saturable phenomenon reflecting a single class of binding site.

It is essential that the measured affinity of the receptor for the hormone should be in the physiological concentration of that hormone. It is also important that the receptor measured should be that which distinguishes biologically active hormone from chemically similar, but biologically inactive molecules. e.g. the oestrogen receptor will bind diethylstilboestrol (DES) (a non-steroid) and E_2 with about the same affinity, whereas its affinity for oestradiol-17α is very low (Leake, 1981).

An ideal assay should, therefore, accurately quantitate a specific receptor, determine a single binding affinity, be able to eliminate non-biological binding, and be both inexpensive and easily reproducible (Leake, 1981).

The biochemical method should separate receptor-bound hormone from both unbound steroid and that bound by plasma carrier proteins. Plasma proteins are a common contaminant of human tumour biopsies (Maass *et al.*, 1975) and have been suggested as the most likely

source of "false-positive" identification of receptor in some biopsy materials (Leake et al., 1980). Elimination of non-specific and lower affinity binding is usually done by running parallel incubations containing radiolabelled steroid alone and radiolabelled steroid plus 100-fold excess of unlabelled competitor. In the case of oestrogen, the competitor is usually diethylstilbestrol (DES) since this oestrogenic compound binds the cellular receptor with an affinity at least equal to that of E_2 but has a much weaker affinity for sex hormone binding globulin (SHBG). The use of an anti-oestrogen as competitor has also been tried with apparent success, although critics suggest that an anti-oestrogen may bind to a separate site on the receptor (Rochefort and Capony, 1977).

The assays measure unoccupied receptor sites. This was a reasonable approach since only a maximum of 30% of the receptors in the cytosol fraction of breast tumour biopsies have been found to be filled (Sakai and Saez, 1976) and no correlation has been found between high plasma oestrogen levels and reduced cytosol receptor content (Fishman et al., 1977). However, the measurement of nuclear receptor levels have been suggested as a better index of hormone dependence (i.e. translocation to the nucleus being an indication of functional receptor) (Laing et al., 1976). Exchange assays have therefore been developed and investigators have observed an increased correlation between receptor positive tumours and response to endocrine therapy (Barnes et al., 1979; Macfarlane et al., 1980; Leake et al., 1981a,b).

At the moment the two biochemical methods most widely used to measure levels of oestrogen receptor are sucrose density gradient centrifugation (SGA) and dextran-coated charcoal (DCC) analysis.

1.5.1.1 Sucrose Gradient Assay (SGA)

The SGA can be used to quantitate receptor by demonstrating the specificity of 8S and 4S binding to $[^3H]E_2$ (Chamness and McGuire, 1972). They stated that the 6S form is the physiological soluble receptor form and that the 8S and 4S forms are artifacts of

preparation in low or high salt. The 6S form associates to yield 8S molecules in low salt (0.15M KCl). The dissociation of 8S back to 6S is slow at 2°C, leaving a significant amount of 8S material on the gradient. This shows that a slight variation in conditions can affect oestrogen receptor forms considerably. The polyanion effect in particular allows production of almost any sedimentation artifact. Nevertheless this method is very popular and shows direct measurement of oestrogen receptor.

1.5.1.2 Dextran-Coated Charcoal Assay (DCC)

This method depends on the concept that DCC will bind free and loosely bound steroid but will not strip steroid from receptor. The assay yields data that can be plotted by the method of Scatchard to determine both the affinity and the number of oestrogen-binding sites. This method was first developed by Korenman and Duke (1970). After that several modifications has been proposed (Hawkins et al., 1975; Koenders et al., 1978; Chamness and McGuire, 1980). McGuire and De La Garza (1973) observed the importance of temperature and thiol reagent during incubation of labelled steroid with the receptor, as well as during tissue fractionation. To get good competition data for the Scatchard plot, DES is usually added at a hundred-fold concentration above that of oestradiol. It will effectively compete for all the high affinity receptors but will not displace labelled E_2 from low affinity sites (Leake, 1980). Even though there are several modifications of this method, the procedures are relatively similar. The advantage of this assay is that it enables the determination of total binding sites and affinity of steroid for receptor.

The data of the specific binding needs to be analysed. Most authors use the well-established Scatchard plot (Hahnel and Twaddle, 1973). However, such data can be easily misinterpreted (Chamness and McGuire, 1975; Braunsberg and Hammond, 1979) and these plots are frequently non-linear. Several alternatives to the Scatchard plot have been proposed (Braunsberg and Hammond, 1980), the Woolf plot is perhaps least subject to error of the graphical methods (Keightley

and Cressie, 1980).

These biochemical methods have the weakness that the receptor has to be prepared in homogenates. Since the procedure includes homogenization and fractionation, the specimen and its cellular components are totally disrupted. Thus this procedure does not allow the detection of receptors at their native sites in the intact cells. In addition, a relatively large amount of tissue is necessary to perform the assay which is a problem in small tumour samples and biopsies. The fact that it uses radioactive materials and expensive equipment makes its application limited to larger medical centers and commercial laboratories which obtain a sufficient number of specimens. Individual tumour cell receptor heterogeneity as well as the original sources of receptor protein, cannot be identified. This is important since benign as well as malignant tissue may contribute to total receptor content. De Sombre et al., (1978) suggested that the variable levels of oestrogen receptor in breast carcinomas may reflect varying proportions of oestrogen receptor positive (ER+) and oestrogen receptor negative (ER-) cells. If the proportion of the latter is large, then the effect of endocrine therapy may be masked by the continuous growth of autonomous cells.

The recognition of these factors and other limitations of current biochemical methods has led several groups to investigate other techniques for the identification of oestrogen receptor using other techniques.

1.5.2 Assay for Oestrogen Receptor Using Immobilized Oestradiol Antiserum

Fishman and Fishman (1974), Fishman et al. (1975) and Castaneda and Liao (1975) used antioestradiol antibody as a probe in determining the quantity and binding characteristics of materials which compete with the antibody for the same ligand. Fishman and Fishman (1979) used immobilized antibody on a polymer film. This complex is used in competition for $[^3H]E_2$ with oestrogen receptor

or antioestradiol antibody in solution. Through this method evaluation of association constant and quantification of the oestradiol-complexing species in solution is possible. Using the immobilised antibody method at 4°C, $0.8\text{-}0.12\times10^{11}$ M^{-1} was the association constant for oestradiol receptor in rat uterine cytosol, and 2.6×10^{11} M^{-1} was the association constant found for antioestradiol antibody raised in a rabbit. The antigen is E_2 linked to bovine serum albumin via a C-6 carboxymethyloxime. Rochi et al. (1979) worked on the same antibody but using E_2 17-hemisuccinate as antigen. The antioestradiol antiserum was reacted with a protein-A-bearing strain of Staphylococcus aureus. This bacterial immunoabsorbant was then used to estimate the oestrogen receptor concentration in several breast cancer specimens. Using this method they calculated the affinity constant of the immobilized absorbent for the E_2 to be $10^8 M^{-1}$ which for the breast oestrogen receptor complex are about $10^9\text{-}10^{10} M^{-1}$. Through this method it is possible to remove the free steroid from a reaction mixture containing cytosol, radiolabelled E_2, and the antioestradiol bacterial adsorbent by pelleting the bacteria by low speed centrifugation. The radiolabelled oestradiol receptor complex remains in the supernatant and can be easily counted in the scintillation counter. The data can then be analysed using the saturation curve of the specific binding.

1.5.3 Analysis of Oestrogen Receptor by Isoelectric Focussing

Several authors had reported on the use of isoelectric focussing in oestrogen receptor studies (Coffer and King, 1976; Boyd and Spelsberg, 1979; Gustafsson et al., 1979). However this technique had been found inadequate due to large sample requirements, low recoveries, long focussing times and poor resolution. Later, Thibodeau et al. (1980) reported the use of 1% agarose as supporting medium and $[^{125}I]E_2$ as labelled ligand for oestrogen receptor. After completing the focussing procedure, the gel is frozen at -70°C and subjected to fluorography. Using diisopropylfluorophosphate (DFP) and molybdate (Mo) as stabilising agents they showed the presence of at least six distinct species of

the human oestrogen receptor. Of these, four appear to be products of proteolysis. Their isoelectric points are 6.9, 6.8, 6.4, and 6.3, with a combined sedimentation coefficient of approximately 3.2S. The non-activated form of the receptor, prepared in the presence of serine protease inhibitor diisopropyl fluorophosphate (DFP) (Hartley, 1960) and molybdate, a proposed inhibitor of oestrogen receptor activation (Nielsen et al., 1977; Leach et al., 1979; Toft and Nishigori, 1979; McBlain and Shyamala, 1980), focused at pH 4.8 and had a sedimentation coefficient of approximately 9.5S. The activated form detected in the presence of DFP sedimented at 8.0S and had an apparent pI of 5.4 (Thibodeau et al., 1983)

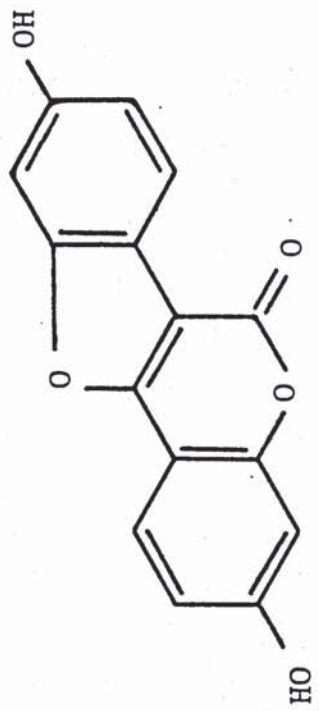
1.5.4 Gel Filtration Technique

The gel filtration technique was adapted by Godefroi and Brooks (1973). They used either Sephadex G-25 or G-100 columns. Gore-Langton et al. (1973) proposed a thin layer gel filtration procedure using Sephadex G-150 to avoid complexity. Although this approach was rapid and separated 8S and 4S forms of receptor it did not always produce results comparable with conventional techniques and therefore, it is not popular. Ginsberg et al. (1974) used a small LH-20 Sephadex column and produced results comparable with those obtained by DCC (Barnes et al., 1977).

1.5.5 Visualisation of Oestrogen Receptor Using Fluorescent Ligand

Martin et al. (1983) used four fluorescent ligands as agents for visualisation of oestrogen receptor in MCF-7 cells: coumestrol (Figure 5) and 12-oxoestradiol which are fluorescent compounds; and tamoxifen and 4-hydroxytamoxifen which become maximally fluorescent only after ultraviolet irradiation. Using photon-counting spectrofluorometry, coumestrol and 12-oxoestradiol can be detected in protein solutions down to 0.5nM. Three of these compounds have good affinity for the oestrogen receptor: coumestrol (20%), 12-oxoestradiol (12%) and 4-hydroxytamoxifen (37%), relative to E_2 (100%). Under conditions where autoradiographic experiments showed the nuclear localisation of oestrogen receptor, they could

Figure 5: Structure of Coumestrol



Coumestrol

demonstrate the nuclear fluorescence using nM concentrations of coumestrol, 12-oxoestradiol, and 4-hydroxytamoxifen. Using excess concentrations of DES, the fluorescence could be abolished.

Tobin et al. (1980) used coumestrol to detect oestrogen receptor in whole cells grown in tissue culture. To the cells, $1x10^{-5}M$ coumestrol was added for 2-180 min and the cells were then immersed in ice cold PBS and immediately examined by ultraviolet microscopy.

Coumestrol, a naturally fluorescent probe, has been shown to bind to oestrogen receptor with high affinity and specificity (Bickoff et al., 1957; Folman and Pope 1969; Perel and Lindner 1970; Shemesh et al., 1972; Shutt and Cox, 1972). Martin et al. (1978), demonstrated the coumestrol-receptor binding in MCF-7 cells by biochemical means. Pertschuk et al. (1978) showed histochemical analysis with direct visualization of nuclear translocation. They also showed the specificity of coumestrol-receptor fluorescence by the marked inhibition with DES and E_2, while progesterone, testosterone and corticosterone had no effect. They also found the coumestrol-receptor assay in breast cancer correlates closely with results of DCC.

1.5.6 Cytochemistry and Immunochemistry

The visualization of steroid binding sites at the microscopic level can, in theory, be approached either by labelling the receptor protein itself, or by labelling the steroid which binds to the receptor protein. With respect to the latter approach two methods are available: cytochemistry and immunochemistry i.e. there are two methods of labelling the steroid. The first one involves conjugating the steroid with enzymes (peroxidase, alkaline phosphatase) or with fluorescent probes (fluorescein isothiocyanate (FITC)). The other means of locating oestrogen receptor is by detecting the presence of E_2 through the use of an antibody to the steroid. The bound antibody is directly or indirectly labelled with peroxidase or fluorescein.

Different investigators have used several types of ligands for histochemical receptor assays. Dandliker et al. (1978) showed that labelling E_2 with FITC at the position 17 resulted in a compound with a higher affinity for oestrogen receptor than labelling at position 16. Barrows et al. (1980) also employed E_2 labelled at position 17. Pertschuk et al. (1978, 1979) and Lee (1979) used a BSA-FITC E_2 conjugate labelled at position 6, whereas Walker et al. (1980) used a similar molecule with peroxidase as the marker. Antibodies to E_2 have also been widely investigated in several laboratories (Kopp et al., 1979; Nenci et al., 1979).

Walker et al. (1980) demonstrated that histological techniques gave similar qualitative results to the dextran-coated charcoal assay. They noted that the only differences that they obtained in the results of both techniques was due to sampling procedures or the variation in the number of binding sites per cell. They also found that the immunocytochemical method was less satisfactory due to the small number of positive cells detected compared to the cytochemical methods. This could be due to the use of unpolymerised oestradiol whereas Pertschuk et al. (1978) used polymerised ligand which provides a larger antigen target for the antibody. Moreover they used fluorescent labelled ligand, while Walker et al. (1980) and Walker (1981) used peroxidase labelled-ligand to oestrogen receptor.

1.5.6.1 Fluorescent Ligand

In this method, the steroid is linked directly to a fluorescein moiety. For example 1-(N)-fluoresceinylestrone thiosemicarbazone is an oestrogen derivative, which binds specifically to cytoplasmic oestrogen receptor at 4°C, and to nuclear oestrogen receptor at 37°C (Nenci et al., 1980). Another conjugate which is more widely used is the 17β-oestradiol-6-carboxymethyloxime-bovine serum albumin fluorescein isothiocyanate (E-BSA-FITC); (Lee, 1979). The former can be used on intact cells while the latter is a macromolecular analogue whose size hinders it from permeating the plasma membrane. Therefore E-BSA-FITC must be used on cryostat sections,

in which almost every cell has been cut open (Lee, 1981).

1.5.6.2 Fluorescent Labelled Antibody to Oestrogen

1.5.6.2.1 Direct Method

The direct method does not require a second antibody. The primary antibody is conjugated to FITC (Coons and Kaplan, 1950). Then the conjugated antiserum is allowed to react with oestrogen receptor in the intact cells or frozen sections and the unbound antibody is then washed off. Ultraviolet (uv) microscopy is used to observe the site of attachment of the antibody which is apple green in colour.

1.5.6.2.2 Indirect Method

This method requires a secondary antibody as well as a primary antibody. The secondary antibody is labelled with FITC. It should be an antibody, raised in another species, (e.g. goat) to the γ-globulin of the animal (e.g. rabbit) which donated the primary antibody. It is called Goat anti-rabbit-IgG-FITC conjugated secondary antibody.

The primary antibody is incubated first with the cells or frozen sections, the excess washed off, and then incubated with secondary antibody. The first antibody, which is already bound to the antigenic sites, acts as a γ-globulin antigen for the secondary-FITC antibody. Again viewed under uv, an apple green colour will show positive staining.

There are several advantages in using indirect methods compared to the direct methods. The sensitivity of the reaction increases since two of the secondary antibody molecules can bind to each primary antibody molecule. It is also economical since one fluorescent second-layer antibody can be used to stain any type of primary antibody to a different antigen, as long as the primary antibody is raised in the same species as that which donated the IgG

for the secondary antibody.

The indirect method for detecting bound oestrogen was originally developed by Pertschuk et al. (1978). Polyoestradiol phosphate (PEP) was incubated with the frozen section. This is then followed by incubation with anti oestradiol-antiserum (Alonso and Brownlee, 1981). After 30 min, the washed tissue was then covered with a 1:10 dilution of fluorescein sheep anti-rabbit serum and washed with phosphate buffer saline (PBS) for 60 min. All dilutions were made in PBS containing 4% bovine serum albumin. Competitive binding was checked with antioestrogen, by incubating the sections with tamoxifen (1.0 mg/ml) in phosphate buffered saline for 60 min following exposure to PEP. Parallel sections of each tumour were incubated in PBS instead of PEP to allow for detection of E_2, bound in vivo.

1.5.6.3 Peroxidase Labelled Technique

This technique does not require an antibody. Horseradish peroxidase was coupled to 17β-oestradiol-6-O-carboxymethyloxime-bovine serum albumin, by the periodate method of Nakane and Kawooi (1974) or the two stage glutaraldelyde conjugation method of Avrameas and Ternynek (1971). These peroxidase labelled oestrogens were applied to the frozen sections and incubated for 2h at room temperature. Peroxidase was localised using the diaminobenzidine-hydrogen peroxide reaction (Graham and Karnnowsky, 1966).

1.5.6.4 Immunoenzyme Method

Nakane and Pierce (1966) first introduced the peroxidase technique. The tissues or cells are first immersed in a solution of hydrogen peroxide in buffer or methanol to 'exhaust' the endogenous peroxidase of the tissue. Alternatively, periodate and borohydride (Heyderman, 1979), sodium nitroferricyanide, or phenylhydrazine can be used (Straus, 1971, 1972). This is followed by the application of primary antibody, then by the secondary antibody which is IgG-peroxidase conjugated. Finally, the substrate for the

peroxidase is then applied.

The advantages of this method over the fluorescence method is the permanent staining, visible with an ordinary microscope and therefore easily photographed. However there are several disadvantages since it has extra steps. The slides need to be incubated with hydrogen peroxide because peroxidase might be present (Duffy and Duffy, 1977). Therefore extra washings are required, which may result in tissue being detached from the slides. Also the component of the substrate, diaminobenzidine, or 3-amino-9-ethyl carbazole is said to be carcinogenic. Extra precautions are required.

1.5.6.5 The Peroxidase Anti-peroxidase Method (PAP)

This is a further modification of the above indirect method, and has been found to be more sensitive. It has a first layer of primary rabbit antibody, a second or another antibody (but not labelled) to γ-globulin in rabbit, and a third layer which is a rabbit antibody to peroxidase coupled with peroxidase. Thus it forms a stable PAP complex. There are also several advantages to this technique. There is an increase in sensitivity of about 100-1000 times over the first indirect method. This is because the peroxidase is bound immunologically to the antibody rather than chemically conjugated. Therefore there is no loss in its enzyme activity. Due to the higher sensitivity, more dilute antibody can be used. This results in greater specificity. The PAP complex is highly specific since it will react only with the anti-rabbit γ-globulin of the second layer. It will not attach to tissue, so background staining is minimal. The only disadvantage it has is that it requires an extra reagent.

1.5.6.6 Technical Conditions of Tissue Preparation

1.5.6.6.1 Management of Tissue Samples

The management of tissue samples has played an important role

in the success of the application of this technique. Most authors do not fixed their cryostat sections but use them immediately after cutting or storage. Lee (1978) reported that tissue kept in PBS at 2-5°C for up to 24h did not significantly loose its binding capacity to the steroid. However, tissue blocks frozen at -20°C were not suitable for this kind of study.

Other authors reported that freezing techniques could affect the ability of the ligand to be stained. Van Marle et al. (1982) demonstrated that only freezing the tissues rapidly in liquid nitrogen-chilled isopentane showed the presence of nuclear fluorescence. They pointed out that only storage in liquid nitrogen maintains a satisfactory level of staining while storage over short periods at -70°C decreases the level of staining intensity quite considerably. However, this finding is in contrast with Curtin et al. (1982) who reported they had successfully stored their specimens at -70°C. They stated that freezing allows the histochemical detection of oestrogen binding. The same was reported by Walker (1981) for her specimens of breast cancer tissues.

1.5.6.6.2 Fixation

After the preparation of the cryostat sections, the next step is to consider whether fixation is necessary. Again different authors have different opinions. According to Lee (1978, 1979, 1981) sections must be air-dried (60 min at 4°C). Hanna et al. (1982) allowed the sections to air-dry only for 15-20 min at 4°C. Tominaga et al. (1981) centrifuged the sections first before staining. Curtin et al. (1982) let their preparations (aspiration-biopsy smears) dry before freezing.

Others (Nenci, et al., 1980; Danguy et al., 1981; Panko et al., 1982) apparently did not use any type of fixation. Lee (1978) reported that cold acetone, 3.7% buffered formaldehyde and 1% glutaraldehyde completely abolished staining.

Raam et al. (1982) stated that acetone, formaldehyde, 75%

ethanol and Bouin's fixative destroyed the antigenicity and oestrogen-binding capacity of oestrogen receptor to an appreciable degree. In contrast, oestrogen binding proteins have been displayed on routinely fixed embedded tissue by immunocytochemistry (Kurzon and Sternberger 1978; Taylor et al., 1981) using antibody against E_2. However, these may detect only Type II sites.

Thawing of the sections mounted on glass slides is also reported to influence the result. Curtins et al. (1982) emphasized that exposure to ambient temperatures after thawing, reduces the affinity for the ligand-conjugates. Nenci et al. (1980) briefly discussed the problem of temperature. They stated that thawing of frozen sections involves nonspecific diffusion of cytoplasmic oestrogen receptor complexes which could then appear in the nuclear compartment. Walker (1981) also reported that, if tissues thawed during cutting, this led to poor staining. The same results were obtained with tissue initially frozen in solid carbon dioxide, rather than liquid nitrogen (Walker, 1981).

Must the sections be rehydrated before incubation? Raam et al. (1982) used frozen unfixed sections which were first dehydrated gradually by 10 min exposures in graded ethanol (30, 50, 75, 90% in physiological saline) and rehydrated by reversing the dehydration steps. The authors stated that this procedure is a fixation method which retains the antigenicity and oestrogen binding capacity, as opposed to the other commonly used fixation methods or to the use of unfixed tissues. Rehydration, when used, is generally carried out by brief immersion in PBS containing bovine serum albumin. Other authors do not mention the used of rehydration.

Care in the preparation of tissue has been found to be essential. This procedure depends very much on the antigen to be tested and also on the choice of the method of detection of the steroid receptor. Walker (1981) found that detecting oestrogen receptor using peroxidase labelled ligand (peroxidase-oestradiol-BSA), using formalin-fixed or paraffin-embedded tissue were unsuitable. Rapid freezing of fresh tissue, with only brief air drying

or acetone fixation of tissue sections and a short term storage were important. They found that tissue which had been formalin fixed and paraffin-embedded consistently gave negative results. According to Walker (1981), exposure to paraffin would explain the loss of activity because of the lability of the oestrogen receptor protein. Loss of activity of receptor binding to E_2 takes places rapidly at high temperature. This resulted in loss of staining in the paraffin embedded tissues.

Treatment of frozen sections with acetone for 3 or 5 min resulted in clearly defined staining within cells but acetone treatment for any longer resulted in loss of staining. Walker also found that fixation at 4°C or at room temperature gave similar results. The use of ethanol at any time resulted in granular cytoplasmic staining in the stroma rather than in the epithelial cells. Using 4% glutaraldehyde even for 10 min caused a negative staining. Air drying for 5-10 min gives similar result to acetone fixation.

1.5.6.6.3 Incubation

This is the most constant step of all the procedures involved in the histochemical/cytochemical methods. The incubation time is usually 1 or 2h and temperature is between 20-25°C. In the present section only the cytochemical studies using BSA as the protein carrier and FITC as the fluorochrome dye for antibody to oestrogen are considered.

According to Chamness et al. (1980) the lowest concentration of E_2 conjugate which gives sufficienct fluorescence is 10^{-9}M. They suggested that at this concentration Type I binding alone is observed. At the higher concentration of 10^{-8}-10^{-7}M, Type II binding is said to occur. Accordingly, it appears that the concentration of E-BSA-FITC plays a major role in the extent of binding capacity and in the possible interpretation of the labelling. Many of the studies discussed here used E_2 concentrations of about 10^{-7}M and their findings are therefore open

to question.

Danguy et al. (1981), Hanna et al. (1982) and Panko et al. (1982) used the method described by Lee (1978, 1979), hence they used the E-BSA-FITC concentration of $5x10^{-6}$M. McCarty et al. (1979) used a lower concentration: $1-3x10^{-9}$M, as did Pertschuk et al. (1979). Tominaga et al. (1981) and Walker et al. (1980) did not specify the concentration of the ligand in the incubation solution.

1.5.6.6.4 Processing After Incubation.

Basically most authors recommended gentle rinsing in PBS followed by immersion in PBS, the latter varying from 3x5 min to 2x30 min or even 2h. Tominaga et al. (1981) used subsequent air drying of the sections, while postfixation in acetone-ethanol (for 10 min) was carried out by Pertschuk et al. (1979) and Curtin et al. (1982).

Processed slides were mounted in buffered glycerol, pH=7.0 (Curtin et al., 1982), glycerin/water, (1:1) at pH=7.4 and 80% glycerin, pH=9.0 (Tominaga et al., 1981).

1.5.6.7 A Morphological Study of Oestrogen Receptor Using Cytochemical and Immunochemical Techniques

Cytochemical and immunochemical techniques offer an alternative to biochemical techniques as a means of localizing cellular oestrogen receptor. Fluorescence may be evidenced either in the nuclei or in the cytoplasm, or in both cell compartments. The localization depends on the chilling method and the concentration of the ligand-conjugate. Some authors do not stress the location of the staining (Tominaga et al., 1981 and Panko et al., 1982). Hanna et al. (1982) observed only cytoplasmic fluorescence in their preparation. Danguy et al. (1981) reported staining in both the cytoplasm and the nuclei.

Walker et al. (1980) observed a mixture of positive and

negative cells in the same piece of tumour. The number of positive cells ranged between 25% to 85%. The staining was predominantly intracytoplasmic, with an occasional concentration at the cell periphery or the perinuclear region. They also observed granular staining in the nucleus and sometimes the nucleolus was the main site of reaction.

Pertschuk et al. (1980) found that both tissue sections and aspirates of breast cancer displayed similar patterns of fluorescence. In fact individual cell morphology was clearer, and nucleolar and cytoplasmic binding were more vivid in the needle aspirates.

Mori et al. (1984), using the indirect immunofluorescence technique with an antibody to E_2, also observed cytoplasmic staining in the epithelial cells, stromal cells and muscle cells of the vagina of the neonatal mouse.

Heterogeneity of tumour cells has been observed by many investigators. Even though Walker (1981) did not find a high variation of the number of positive and negative cells between different sections from the same tumour (only about 10%), she demonstrated the presence of oestrogen receptor positive cells lying adjacent to oestrogen receptor negative cells which she said, could be due to the clonal origin of the breast carcinomas.

The use of peroxidase labelled antibody to E_2 was also reported to have direct correlation of oestrogen receptor localization with morphology (Taylor et al., 1981). They reported that when routinely fixed embedded tissue was used, they could differentiate between types of tumours that contained a large amount of fibrous stroma from those neoplasms composed of densely packed tumour cells. Another advantage which could be observed with this method is that it permits an assessment of not only overall tumour positivity, but also of the proportion of receptor positive and negative cells within an individual tumour (Taylor et al., 1980). In addition, because most biochemical, cytosol-based methods rely on

the specific binding of $[^3H]E_2$ to empty receptor, the large amounts of endogenous E_2 in premenopausal women, produce blocking of binding of labelled hormone and thereby lower the apparent oestrogen receptor level.

Alonso and Brownlee (1981) found that the staining intensity of the cells was independent of topographic location, and they also discovered that tumour cell populations were heterogenous for oestrogen receptor as had been seen by others (Lee, 1978; Mercer et al., 1978; Pertschuk et al., 1977, 1978, 1979). The location of oestrogen receptor within malignant cells could be determined but with some difficulty. Cytoplasmic staining predominated.

Apart from the distribution of oestrogen receptor within one cell type, Mori et al. (1984) concluded that, at the age of 1-15 days postnatal life, staining occurred exclusively in the stroma of the vagina of normal mouse. They thus suggested that epithelial cells are not the actual target of oestrogen during normal development. However, they observed labelling in the mullerian vagina and cuboidal cells of the sinus vagina in neonatally oestrogenised mice. This suggests the possible existence of a difference in the nature of the vaginal epithelium between the neonatally oestrogenised mice and normal mice.

Most of the experiments done were followed by a specificity test. To make sure that the binding reflects oestrogen receptor only, the tissue sections were subjected to preincubation in an excess of unlabelled oestrogen with or without antioestrogen. Other methods include incubating the sample with bovine serum albumin without FITC or in fluorochrome dye without conjugation to oestrogen.

Nenci et al. (1976) also reported the absence of staining in human spleen, mouse liver and non-target tumour cells. Cell suspensions from breast tissue of a pregnant woman and from other premenopausal breast cancer patients displayed a moderate cytoplasmic and faint nuclear fluoresence, without exposure to E_2.

Nenci et al. (1980) tested the receptor specificity of the E_2 binding by blocking experiments. They compared the hormone uptake in the presence and also absence of blocking compounds (Nafoxidine, N-ethylmaleimide). They found the E_2 bound specificically to the receptor.

1.5.6.8 In Vitro Translocation of Oestrogen

There was a shift from predominantly cytoplasmic to nuclear binding after treatment with coumestrol (Pertschuk et al., 1980). They observed in their experiments that there exists two groups of patients: a larger group that exhibits nuclear translocation and a smaller group that does not. The phenomenon could be related to the success of breast cancer hormone therapy since a significant proportion of women with ER+ breast cancer fail to respond to endocrine therapies. These could, at least in part, reflect patients whose tumours contain an abnormal oestrogen binding protein which is unable to undergo activation and so cannot induce genetically mediated responses.

Nenci et al. (1976) used the immunocytochemical indirect technique with FITC labelled antibody to E_2 in E_2 target cells. They used an E_2 concentration of $2x10^{-7}M$. The first experiment on in vitro translocation used temperatures of 4°C, 20-22°C and 37°C. They observed three general patterns depending on the treatment. The cells incubated in the cold showed a homogeneously diffuse fluorescence in the cytoplasm. The nuclei were unstained. In many breast tumour samples negative cells were also observed.

The second pattern of fluorescence was shown by cells incubated at room temperature. They observed nuclear staining together with cytoplasmic staining.

The third staining pattern was seen after the slow post-incubation warming up to 37°C. In addition to cytoplasmic fluorescence, increasing nuclear staining occurred which, later, filled the whole nuclear area. In some breast tumours they did not

observe any nuclear labelling after warming, despite the clear cytoplasmic fluorescence. They referred to this as a double cell population.

The translocated staining was concentrated in nuclear chromatin in small fluorescent dots after 5-6h. These dots disappeared after 12-15h, leaving only one or rarely two roughly spherical, bright nuclear bodies still stained. Phase contrast microscopy and cytological staining showed that these bodies are the nucleoli. It is interesting to note that Raam et al. (1982) reported the same observation but using an antibody to oestrogen receptor protein.

Mori et al. (1984) also investigated the in vitro temperature-dependent nuclear translocation. Vaginal sections were incubated in 10^{-6} M E_2 for 1h at 4°C. Other sections were incubated with oestrogen for 1h at 4°C and then postincubated in medium without oestrogen for 1h at 37°C. For competitive binding, the vaginal tissue was incubated with 10^{-4}M diethylstilboestrol (DES), followed by exposure to 10^{-6}M E_2 for 1h at 4°C. After incubation, vagina sections were placed on brass tissue holder and frozen by liquid nitrogen. Sections were cut at 6um by a cryostat and were kept in a desiccated slide box at 20°C. In tissue exposed to oestrogen at 4°C and postincubated without oestrogen at 37°C, fluorescence was diffusely distributed over the cytoplasmic and nuclear area. This may indicate the occurance of temperature dependent nuclear translocation of oestrogen. They also found that the staining was effectively blocked by prior incubation with excess DES. However, the levels of steroid used cast doubt on the nature of the binding proteins studied.

1.5.6.9 Interpretation of the Results

The interpretation of the results had not been standardised and therefore reported according to different authors' chosen criteria. Pertschuk et al. (1980) classified a tumour as negative when the tumour specimen showed only 10% positive cells. Hanna et al. (1982) too used the same criteria. Tominaga et al. (1981)

recommended a threshold value of less than 20% positive tumour cells before the tumour be considered negative. Walker et al. (1980) apparently did not find positive tumours exhibiting staining less than 30% (in other words all positive tumours yielded at least 30% of positively stained cells).

Some investigators related the findings with hormone therapy. Mercer et al. (1980) stated that hormone therapy of breast cancer was found to be active only when 90% or more of the cells exhibit positive staining.

The percentage of labelled cells is not the sole parameter to investigate. Hanna et al. (1982) emphasized that the intensity of fluorescence is as important as the percentage of stained cells. Many tumours contain a certain number of cells that fail to translocate the hormone into the nucleus despite normal cytoplasmic uptake (Nenci et al., 1980). Since nuclear translocation is a prerequisite for the functionality of oestrogen receptor, nuclear staining after 37°C incubation would appear to be the important finding.

1.5.6.10 Comparison with the Biochemical Assay

Pertschuk et al. (1979), detected oestrogen receptor through the use of a fluoresceinated bovine serum albumin-oestradiol conjugate linked to E_2 at position 17 and containing 5 mol fluorescein and 4 mol E_2 per mol albumin. From 120 specimens of breast cancer, they compared their results with the biochemical dextran coated charcoal method and found a 92% correlation, compared to their earlier work (Pertschuk et al., 1978) which showed 89.4% correlation. They reported that divergent results most often occurred in specimens sparsely populated with malignant cells.

Walker (1981) compared the cytochemical results with those of biochemical results done on parallel tissue sections. She found a good qualitative correlation between the two sets of results.

Taylor et al. (1980) found a 60% correlation between the histochemical method and the biochemical technique. They reported that in most cases which were negative by the biochemical method, they found the tumours consisted of cords and nests of tumour cells interspersed with predominantly stroma or adipose tissue, the carcinoma cells constituting only a minor proportion of the tumour tissue. They found that the peroxidase technique could identify those small numbers of positive tumour cells, surrounded by large amounts of fibroadipose tissue.

Alonso and Brownlee (1981) reported that the use of immunofluorescent steroid antibody techniques showed a 67% correlation with the DCC method in the detection of oestrogen receptor levels on 21 cases of breast cancer. Both direct and indirect fluorescent antibody assays were used.

1.5.6.11 Validity of Histochemical Method in the Detection of Oestrogen Receptor

Almost all immunocytochemical studies reported have used rabbit anti-oestradiol antiserum which has been absorbed with bovine serum albumin (BSA). The purpose of the adsorption step is to prevent the 'unadsorbed' antiserum cross-reacting with the cellular antigen and albumin from the culture medium or prepared specimen thus giving a false positive staining. The elimination of E_2 binding with anti-oestradiol is to prove its immunocytochemical specificity for oestrogen. Usually a marked reduction or total elimination of fluorescence staining is observed. This indicates specificity for E_2 (Mercer et al., 1981).

In order to prove that the antibodies are able to recognize receptor associated E_2, one must be able to show the presence of the immune complexes of antibody-oestradiol-receptor. Mercer et al. (1981) failed to detect the existence of this complex, as did Castaneda and Liao (1975). Both groups used sucrose density gradient analysis (SDGA). Using radioimmunoassay (RIA) to detect

competition between cytosol oestrogen receptor and antibody for E_2, they could not show that a complex of antibody to oestrogen receptor existed (Fishman and Fishman, 1974; Castaneda and Liao, 1975; Fishman et al., 1975). These experiments involved the use of insolubilized antibodies to E_2 which were incubated with $[^3H]E_2$ and cytosol from breast tumour preparations. It was observed that the antibody would bind to free $[^3H]E_2$ and $[^3H]E_2$ bound to nonspecific sites but not to the oestrogen receptor complex.

Mercer et al. (1981) found that at least 3 types of oestrogen-binding components in breast cancer cells can be detected i.e. Type I binding (the oestrogen receptor itself), Type II and Type III.

Type I has the highest affinity for E_2. The dissociation constant (K_d) is in the order of 1nM.

Type II has a lower affinity for E_2 but it has a higher capacity for the hormone. Clark et al. (1978) and Eriksson et al. (1978) refer to this receptor as Type II oestrogen binding sites in the rat uterine cells. Below 10nM few of these sites are occupied but as the E_2 increase to 1μM most of the binding sites are occupied.

Type III sites are the other oestrogen-binding molecules within the cells, albumin and other membrane associated molecules which have low affinity for the hormone, but have a high total binding capacity. These sites will be occupied at a very high E_2 concentration. They are also called nonspecific oestrogen binding sites.

Therefore during the immunocytochemical assays which use very high levels of steroid (>10nM) detection of oestrogen binding protein (EBP) other than Type I must occur. Mercer et al. (1981) discovered that, using 10nM E_2, there was no detection of E_2 specific immunofluorescence in the cells. Mercer and co-workers also mention that in the rat uterus the sites detectable in the cell nuclei

increase greatly after oestrogen treatment. However the cytosol sites are not depleted by this oestrogen treatment. Since Type II nuclear sites are independent of Type II cytosol sites, they conclude that this technique measured the Type II sites in the MCF-7 cell lines. They even calculated the binding sites of Type II by subtracting the total binding (incubated with 10x10nM E_2) from oestrogen receptor (Type I) binding (incubated with 100nM E_2). It was estimated that nearly half of the total E_2 binding was due to lower-affinity but competible E_2 binding sites.

The use of antioestrogens or DES in excess in the immunofluorescent detection should have eliminated only the specific staining since it would fill the high affinity receptor sites. However the DES concentration that was commonly used (1μM to 1mM) was far too high to saturate only the specific binding sites. Besides, E_2 solubility decreases in the presence of high concentrations of DES. These observations weaken the value of immunofluorescence techniques in routine assays.

Nenci (1981) disputed the idea of Type II sites as contributing to the positivity of oestrogen receptor in the intact cells. He showed that no residual cytoplasmic staining was observed in these systems after nuclear translocation has taken place and noted that cytosol Type II binding sites do not translocate (Clark et al., 1978; Eriksson et al., 1978). However, even if it is Type II binding sites that are measured, this does not form a limitation in the use of the technique if the growth response of target tissues elicited by oestrogen requires both nuclear receptors and cytosol Type II sites (Nenci, 1981).

Pertschuk et al. (1978) reported that no immunohistochemical staining of oestrogen receptor was observed after the specimen was treated with the anti-oestrogen (Cl-628). Dandliker et al. (1978) observed that there was no effect of excess competitor, DES or excess E_2, on the staining by fluorescent oestrogen in the incubated sections. This makes it impossible to differentiate definite binding from nonspecific binding.

Histochemical methods of oestrogen receptor detection also face several minor weaknesses which need to be overcome before they can be of potential clinical value. Firstly, the fact that oestrogen receptor is a component of the soluble part of the cell may cause it to 'leach out' into aqueous processing media if the cells are not fixed. Secondly, when the cells are fixed, this might adversely affect the ability of the receptor to bind to E_2. Thirdly, the modifications of the E_2 molecule, such as conjugating it to tracers (peroxidase or fluorescence substances) might disturb the quantification of results as, at present, there is no standardardization of the data collected (Alonso and Brownlee, 1981).

1.6 TECHNIQUES

1.6.1 Purification of Oestrogen Receptor

1.6.1.1 DNA-Cellulose Chromatography

Many proteins that function in association with intracellular DNA recognise purified DNA as a substrate and bind tightly to it at physiological ionic strengths *in vitro*. When DNA-free crude extracts are passed through a column consisting of DNA adsorbed onto an inert cellulose matrix ("DNA-cellulose"), these proteins are specifically retained. After an extensive wash to remove unbound proteins, the DNA-binding proteins are recovered in active form by elution with buffers containing either an elevated salt concentration, competing nucleic acids, or specific biological inducers. With a proper choice of conditions, the binding of non-DNA-related proteins to DNA can be avoided. This type of procedure, discovered by Litman (1968), has been successfully used in the purification of glucocorticoid receptor by Westphal (1982).

1.6.1.2 Ion-exchange Chromatography

Ion-exchange chromatography is a type of adsorption chromatography in which the interactions between the chromatographic medium and the solute are based primarily on ionic charge.

The development of ion exchangers based on cellulose (Peterson and Sober, 1956) or other polysaccharides were found to be suitable for protein separation. The most common types available are:

(a) DEAE (diethylaminoethyl)-cellulose, Sephadex or Sepharose; an anion exchange resin used primarily for neutral and acidic proteins.

(b) CM (carboxylmethyl)-cellulose, Sephadex or Sepharose; a cation exchanger used primarily for the separation of neutral and basic proteins.

In general, however, the major attractive force between proteins and these adsorbents is believed to be electrostatic (Wofsy and Burr, 1969). The adsorbed protein could be eluted by altering the pH such that either the protein or the adsorbent loses its net charge.

1.6.1.3 Affinity Chromatography

Affinity chromatography represents the ultimate extension of adsorption chromatography since it has the complete set of forces: Van der Waal's, hydrophobic, steric and electrostatic. These forces are involved in the specific binding of substrates and other ligands to proteins.

In affinity chromatography the ligand is attached to an insoluble support which is packed into a chromatographic bed. In principle, if a mixture comprising several proteins is applied to the column, only that protein which possesses appreciable affinity for the ligand will be retained or retarded; others which show no recognition of the insolubilized ligand will pass through the bed. The specifically adsorbed protein can subsequently be eluted by altering the composition of the solvent to permit dissociation from the insoluble ligand.

Generally speaking, the volume of the sample applied to an affinity adsorbent is not critical if the substance of interest has

a high affinity for the immobilised ligand. On the other hand, weakly-bound proteins should be applied in a relatively small volume, to circumvent co-elution with the inert proteins in the void volume. The adsorption equilibrium between the immobilized ligand and the macromolecule to be purified is often reached at a very slow rate.

1.6.1.3.1 The Effect of Temperature

In general, the strength of adsorption to an affinity gel decreases with increasing temperature. Furthermore, the use of different temperatures for adsorption and elution can have very beneficial effects on the subsequent purification. Tight binding may be effected at 4°C and subsequent elution achieved under mild conditions by raising the temperature to 25°C or above (Harvey et al., 1974).

After inert proteins have been washed off the column the composition, pH, ionic strength or temperature of the buffer is changed and elution effected by passing the new buffer through the column.

1.6.1.3.2 Non-specific Adsorption

More often than not, despite the careful design of the affinity adsorbent, the adsorption and elution of the desired macromolecule does not achieve the expected specific activity. It is now widely recognised that this is due to non-specific adsorption of inert proteins to the affinity adsorbent (O'Carra et al., 1974). The presence of ionic groups on any chromatographic adsorbent will affect the elution behaviour of polyelectrolytes such as proteins. These interactions may generate problems of non-specific binding in affinity chromatography. Incomplete attachment of ligands to preformed matrix-spacer arm assemblies can introduce extraneous ionic groups into the adsorbent.

1.6.1.3.3 The Solid Matrix Support

The solid matrix support must be given careful consideration. The matrix should form a loose porous network which allows the uniform and unimpaired entry and exit of large macromolecules throughout the matrix. It must possess chemical groups which can be activated, under conditions which do not modify its structure, to allow the covalent linkage of a variety of ligands. Also the solid support must be physically and chemically stable in the conditions selected for sampling, adsorption and elution.

The water-insoluble beaded derivatives of agarose have many of the properties of an ideal solid support matrix. They have now been used successfully in numerous purification procedures. They have a very loose structure which allows ready penetration by substances of molecular weight up to the order of several million. The uniform spherical shape of the gel particles is of particular significance. Also these polysaccharides can undergo substitution reactions by activation with cyanogen halides e.g. cyanogen bromide readily. They are also very stable and have a moderately high capacity for the substitution.

Agarose is a linear polysaccharide consisting of alternating residues of D-galactose and 3,6-anhydro-L-galactose (Lowe and Dean, 1974). The agarose gels are stabilized by hydrogen bonding. Thus there is very little denaturation or adsorption of sensitive biochemical substances because of their hydrophilic nature and nearly complete absence of charged groups.

1.6.1.3.4 Spacer Molecules and Steric Considerations

For a successful purification by affinity chromatography, there must exist a sufficient distance between the chemical group (ligand) and the matrix backbone to avoid the effect of steric hindrance. This exists if the ligand is attached directly to the matrix backbone. The problem can be solved by placing the ligand at the end of a long chain or "arm", so that it extends into the solvent.

Cuatrecasas (1970) suggested that the effect of extension arm length is due to the relief of steric restrictions imposed by the matrix and in part by the increased flexibility and mobility of the ligand as it protrudes further into the solvent. The flexibility and folding of the extension arm could be the reason for the clear decrease in binding observed with longer extension arms.

However, the use of an extension arm may not be the only consideration involved in their use (O'Carra et al., 1973). Spacer units may interfere with the affinity of a ligand for a macromolecule. i.e. by generating a local steric interferance.

1.6.1.3.5 Batchwise Adsorption

This is applicable when a small amount of protein is to be extracted from a large amount of inert protein with an adsorbent of high affinity. The purification is made possible by adding a slurry of the specific adsorbent to the crude mixture. It may be preferable to apply the sample to a product column, thoroughly wash it, then proceed with the elution by dismantling the column and incubating the matrix in an appropriate buffer.

1.6.1.3.6 Elution of Specifically Adsorbed Macromolecules

The principal of elution is to drive the adsorption equilibrium of the adsorbed solute from the stationary to the mobile phase. This can be done using either of the following methods:

(1) Displacement analysis, whereby a buffer containing a displacing agent is applied to the column and is so strongly adsorbed that it expels the already adsorbed macromolecules;

(2) Extremes of pH or protein denaturants such as guanidine-HCl or urea can be used. Restoration of the native protein may be affected by neutralization or dialysis. An alternative approach to eluting the specifically bound protein is to cleave the the matrix-ligand bond selectively. This method

means the removal of the intact ligand-protein complex. This is applicable in cases where the ligands are attached to Sepharose by susceptible bonds such as those involved in azo, thiol or alcohol ester linkage. Where the ligand itself is charged, reduction of the ligand affinity for the complementary macromolecule can be achieved with increasing ionic strength. In many cases, the elution of a specifically adsorbed protein with a buffer containing a high concentration of competing ligand results in a greater elution of the protein (Lowe and Dean 1974). If this is done together with an increase in temperature, a decrease in affinity adsorption takes place improving the final percentage recovery still further (Harvey et al., 1974)

Sica et al. (1973) have shown that effective adsorbents for oestrogen receptors could be obtained by coupling oestradiol-17β-hemisuccinate to agarose derivatives containing diaminodipropylamine, serum albumin, poly-L-lysine or poly-L-lysine-L-alanine copolymer. Between 60 and 80% of the E_2 binding protein from a calf uterine supernatant was adsorbed by these columns. Also, the adsorbents could be washed with large volumes of buffer and with 1M KCl without release of receptor. Elution was effective during incubation with 0.2 to 2µg per ml E_2 for 15 min at 30°C. With the best adsorbents the E_2 binding protein was purified from 10,000 to 100,000 fold with a recovery of about 50%.

1.6.1.4 Gel Filtration

Gel filtration (Porath and Flodin, 1959) is a technique of partition chromatography in which the partitioning is based on the molecular size of the substances to be separated.

The chromatographic gels used in gel filtration comprise macromolecules with a high affinity for the solvent. The gels are allowed to swell in the solvent and, in so doing, imbibe large amounts of the liquid. Gel filtration separates substances according to their molecular size; large molecules emerge from the bed first

followed by the smaller molecules. Large molecules cannot penetrate into regions close to the cross-links in the gel lattice because of steric obstruction. In contrast, small molecules can approach these regions more closely and thus have access to most of the space between the chains of the gel matrix. As a result small molecules are distributed fairly evenly between the free solvent and the solvent present within the gel matrix, whilst large molecules are more restricted within the gel. The partition coefficient of large molecules is thus shifted in favour of the liquid outside the gel particles with the consequence that large molecules emerge from the gel bed earlier than small ones.

The solvent in a chromatographic column packed with swollen gel beads may be regarded as being in two phases (Flodin, 1961); in the spaces between the gel beads is the void volume, Vo, and entrapped within the gel matrix the internal volume, Vi. The Vo, may be determined by chromatography of a substance that is completely excluded from the gel beads and so is its elution volume. In practice, a polysaccharide with an average molecular weight of $2x10^6$, Blue Dextran 2000 is commonly employed.

1.6.2 Antigens and Antibodies

Antibodies are characterised by certain physicochemical and biological properties. Salting-out procedures combined with column chromatography or Sephadex filtration have made it possible to isolate five main classes of antibodies usually called IgA, IgG, IgM, IgD and IgE depending on their antigenic properties. The IgG immunoglobulins have a molecular weight of 150,000-160,000 and are composed of two identical halves each of which contain two polypeptide chains: a heavy chain of molecular weight 55,000-60,000 and a light chain of molecular weight 20,000-24,000 which are held together by disulphide bridges and non-covalent interactions. When an antibody combines with its complementary antigen, the binding energy is derived from the relatively weak non-specific attractive forces which exist between atoms, ions and molecules. This energy is strengthened by the effect due to the interaction

between solvent molecules which push the antibody and antigen together. However, these attractive forces are effective over short distances only and require that the combining region of the antibody fit closely over the antigen to produce sufficient binding forces to hold the molecule together. Therefore, the specificity of the antigen-antibody interaction is very important for a strong interaction. In addition, the distribution of groups on the antigen must be matched by complementary groups on the antibody.

It is essential in understanding immunological reactions to grasp the fact that a particular preparation of antibody globulin, even that from a single animal immunised with 'a single antigen' is a mixture of closely similar molecules and not a preparation of identical molecules. This is explained by the fact that most 'pure' antigens contain multiple sites (or chemical groups) every one of which gives rise to specific antibody formation.

1.6.2.1 Antigen-antibody Reaction

The quantitive nature of antigen-antibody reaction was first exploited by Heidelberger and Kendel (1929, 1935) who found that it is possible to isolate and estimate the immunoprecipitate formed by adding increasing amounts of an antigen to a fixed amount of antibody. The amount of the precipitated complex increases with rising quantities of antigen up to a certain point. This region of increasing precipitation in referred to as the interval or zone, of antibody excess. Above this point there is no further increase in precipitated complex as long as some degree of equivalence exists between the two reactants (zone of equivalence). With increasing quantities of added antigen, a so-called zone of inhibition of complex formation is reached (antigen excess). Here, there is a progressive decrease in the amount of antigen-antibody complex precipitated.

The antigen-antibody reaction shows temperature dependence of the rate of association of the two reactants between about 0-40°C. The reaction was maximal at a low salt concentration (Clausen,

1970). Finally, the antigen-antibody reaction is dependent on pH, showing an optimum at 7.0 (Kleinschmidt and Boyer, 1952). The immunoprecipitate is soluble below pH 4.5 and above pH 10.0.

Small changes in the thermodynamic constants and a positive entropy change, which may be partly related to displacement of water from the antibody site and partly to conformational changes in the reactants, occurs after establishment of the antigen-antibody complex. These changes may be related to formation of hydrophobic bonds (Clausen, 1970).

The physicochemical event during the antigen-antibody reaction depends on the type (horse and rabbit) of antibody used. The antigen-antibody complex formed is usually characterised by being soluble in excess of antigen and also, in the case of horse type antibody only, in excess of antibody.

1.6.2.2 Immunoblotting

Several method have been applied to determine the immunological reactivity of proteins after being separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Burridge, 1976; Renart et al, 1979; Towbin et al., 1979). Proteins of interest can be transferred to a nitrocellulose membrane (NCM) from polyacrylamide gels (Towbin et al., 1979). The polypeptides that are bound to the NCM can be incubated with antisera and can be localised by antibody labelled with a staphylococcal protein A followed by autoradiography of the NCM (Batteiger et al., 1982). These authors also report on the use of nonionic detergent Tween 20 to block the unoccupied protein binding sites on NCM. Other proteins such as bovine serum albumin (BSA) or gelatin are generally used for this purpose (Towbin et al., 1979; Burnette, 1981; Cohen and Falkow, 1981; Vaessen et al., 1981).

1.6.3 Biopsy Techniques.

The aim of any biopsy is to make a definitive histological

diagnosis for treatment. It would be advantageous to both the patient and the surgeon if a diagnosis could be made preoperatively by either needle biopsy or aspiration cytology.

1.6.3.1 Frozen Sections

It has been widely accepted that frozen section histology is an accurate diagnostic method. In a review by Lessells and Simpson (see Crawford and George, 1984), they reported an accuracy of 99.4% in 2,197 frozen section biopsies. There were 13 false negative diagnosis, 7 due to sampling errors, and 6 due to errors of interpretation. One false positive diagnosis was subjected to an unnecessary radical mastectomy.

1.6.3.2 Fine-needle Aspiration (FNA)

The technique of FNA is simple and requires no special equipment. An ordinary 10ml syringe and needle are used. Constant suction is applied as the needle is passed through the lesion several times in several directions to allow for tumour heterogeneity. The suction is then released before the needle is withdrawn and the contents of the needle are expelled on to glass slides for cytological examination. Despite its simplicity the technique requires considerable practice before good quality samples can be produced consistently (Crawford and George, 1984).

The ability to interpret smears accurately required a considerable period of learning. The accuracy rate in the diagnosis of both malignant and benign lesions has been reported to be in excess of 95%. Like frozen sections, FNA false-positive diagnoses are also reported. For this reason some surgeons would not be prepared to consider mastectomy on a cytological diagnosis alone.

However, some reports of FNA cytology must be interpreted with caution. Disadvantages include unsatisfactory smears which lead to false positives. Also the need for an experienced cytologist might be the reasons why the technique is not in more widespread use

in this country.

1.6.3.3 Tru-cut Needle Biopsy

This is a biopsy of discrete breast lesions with a large-bore needle. A tru-cut needle biopsy is a simple, safe procedure and it has the advantage of high diagnostic accuracy with no false positive results. Its disadvantage is false negative results often reported which could be due to sampling errors but this can be reduced with experience. It also requires a local anaesthetic and can leave significant bruising.

1.6.4 Modes of Application

Immunohistochemistry can be carried out on cytological smears, frozen and paraffin sections, cell and tissue cultures. Frozen sections are used mainly for fluorescent labelled antisera, but the results are not permanent and peroxidase techniques on frozen sections or imprints often give a heavy background stain. This is reported to be reduced by treating the slides with 20% acetic acid solution prior to incubation in the primary antiserum (Colman et al., 1976). In smears and frozen sections both cytoplasmic and surface immunoglobulins are demonstrated.

In tissue sections, cytoplasmic, nuclear and interstitial antigens can be demonstrated. The morphological detail seen in paraffin sections is one of the advantages of the method.

1.6.5 Technical Aspects

1.6.5.1 Fixation

It is thought that fixation and processing may denature or alter the antigenicity of the tissue components, so that specificity and sensitivity are impaired. Prompt fixation is essential for consistent results, since poor fixation causes loss of antigenicity or diffusion of the antigens into the surrounding tissue. A wide

range of fixative may be used to demonstrate various antigens. Many of the antigenic determinants may be lost during fixation, but enough may remain to enable them to be visualised using the PAP technique. There is not one fixative that can be said to be ideal for the demonstration of all antigens. Some may still require fresh frozen sections. However, in general, many antigens can be demonstrated after fixation in formal saline, formal mercury, Zenker's or Bouins'. (Some antigens can be localised after osmium fixation. Periodate-lysineparaformaldehyde fixative gives good preservation of antigens and can be used for both light and electron microscopy).

Certain fixatives can be used only if enzyme digestion is carried out on the section prior to immunostaining, e.g. isotonic formaldehyde solutions. Other fixatives gives good results without the use of enzymes, e.g. formal mercury, Bouins' and Carnoy's. Very good results are obtained if 2 - 10% acetic acid is added to formal saline.

1.6.5.2 Processing

In some cases the tissues may be washed in buffer to reduced background staining.

Cutting

The thickness and quality of the sections is important, the average thickness for immunohistochemistry being 4µm Adhesives are not always necessary, but slides coated with chrome-gelatine or egg albumin solutions may be used.

Endogenous Peroxidase Blocks

Peroxidase is found under normal conditions in red blood cells and granulocytes. At first this endogenous peroxidase was stained red using alpha-napthyl pyronin, distinguishing it from the brown antigen/antibody complex. It is now usually inhibited before

staining. There are a number of methods available: 0.5% H_2O_2 in methanol 30 min, 3% H_2O_2 in water, 3-10 min periodic acid, followed by sodium borohydride, 1% acid alcohol and methanol/picric acid. Probably the most commonly used blocking method is hydrogen peroxide/methanol. Some tissue antigens may be sensitive to blocking agents in which case it is best to leave the sections blocked.

Antisera and Dilution

To obtain the best possible results the antisera must be used at the correct dilution. This is found by carrying out a range of dilutions on the same tissue, with all other steps being constant. A compromise is made between the staining of the positive cells and background, to get a clear picture. Each time a new batch of antisera must be titrated to find its optimum dilution. Too strong a solution will result in a false negative - a prozone effect. Most antisera are incubated on the sections for 30 min at room temperature. However, some antisera may require longer periods of incubation, in which case they are left at 4°C for anything up to 24h. It is possible that incubating the sections at 37°C will shorten the incubation time.

Controls

Any immunological staining will be only as good as the antibodies used. Many antisera contain impurities causing cross-reactions. However, with most other antisera controls must be carried out as follows, to obtain a meaningful result:

1. Replacement of the primary antiserum with a normal serum of the same species.

2. Replacement of the primary antiserum with another primary antiserum directed against a different antigen.

3. Absorption of the primary antiserum with purified antigen.

The last is the most useful specificity control if the purified antigen is available. The antigen must be pure enough to prevent it absorbing out both wanted and unwanted binding, giving a false impression of monospecificity.

1.7 OBJECTIVES

Studies utilizing radiolabelled E_2 had demonstrated that the soluble oestrogen receptor found in oestrogen responsive tissue bound the hormone with high affinity and transported it to the nucleus (Muldoon, 1980). Steroid conjugated to fluorescent compounds or anti-oestradiol antibody are all tools that have been applied for studying the intracellular distribution and translocation of the oestrogen binding proteins (Nenci et al., 1976, 1981; Pertchuk et al., 1978, 1979, 1980; Lee 1978, 1979, 1981). However these tools are still in the investigational stage and their relation to physiological receptor is unclear (Morrow et al, 1980; Mercer et al., 1981). Antibodies that are specific to the oestrogen receptor are the most suitable means of determining the distribution and concentration of the receptor in the cells.

The main objective of this study was to obtain anti-oestrogen receptor antibodies and use these antibodies as a tool in investigating the receptor distribution, quantification and translocation at cellular level. The antibodies are produced from a rabbit immunized with affinity purified oestrogen receptor of human origin. The next step in the study was to find out the specificity of the antibodies towards oestrogen receptor. The aims of the study were:

1) to investigate how far the antibody could provide information on the oestrogen receptor distribution, quantification and translocation,
2) to investigate the correlation of the antibody technique with the standard biochemical method (DCC),
3) to investigate the heterogeneity of the oestrogen receptor in the tumour specimen,
4) to assess the clinical value of the antibody in studying

breast tumour fine needle aspirates (FNAs) taken from breast cancer patients at out-patient clinic.

2.1 REAGENTS

2.1.1 Fine Chemicals

Acrylamide and Bis-acrylamide	Sigma, London
Ammonium persulphate	Sigma, London
Ammonium sulphate	Sigma, London
Bovine Serum Albumin (fraction V)	Sigma, London
DEAE-Cellulose	Pharmacia, Sweden
DEAE-Sepharose	Pharmacia, Sweden
Deoxyribonucleic acid (DNA)-Cellulose (calf thymus type V sodium salt, highly polymerized)	Sigma, London
Dextran blue	Sigma, London
Dextran T70	Pharmacia, Sweden
Dimethyl Formamide (DMF)	Sigma, London
Dithiothreitol (DTT)	The Boehringer Corporation (London) Ltd.
Ethanolamine	Sigma, London
Freund complete and incomplete adjuvant	Difco Laboratories, Detroit
Human-γ-Globulins (fraction II)	Sigma, London
Methanol, ethanol, ethylene glycol, acetone, isopropanol, isopentane	Fisons, England
Norit A activated charcoal (untreated)	Sigma, London
Oestradiol-17β 17-Hemisuccinyl-Albumin	Sigma, London
Sephadex G25	Pharmacia, Sweden
Sepharose-4B	Sigma, London
Sodium molybdate (AnalaR)	BDH chemicals Ltd., England
Sodium thiocyanate	Sigma, London
Sucrose (AnalaR)	Sigma, London
TEMED	Sigma, London
Trichloroacetic acid (TCA)	Koch-Light Laboratories

Unless otherwise stated, all other chemicals used were of AnalaR grade, supplied by BDH chemicals Ltd., England.

2.1.2. Buffer reagents

Hydroxypiperazine-N'-2-ethane Sulphonic acid (HEPES) was obtained from The Boehringer Corporation (London) Ltd.
Tris (hydroxymethyl) aminothane was obtained from Sigma, London.

2.1.3 Steroids

Steroids prepared in 95% alcohol were stored at -20°C for up to 6 months.

Oestradiol 17β, progesterone, and cortisol were obtained from Sigma, London.

2.1.3.1 Radioactive Ligands

[2,4,6,7-^{3}H] Oestradiol-17 , specific activity 101 Ci/mmol
[^{3}H]-dehydrotestosterone, specific activity 105 Ci/mmol
[^{3}H]-ORG 2058, specific activity 45 Ci/mmol

All these radioactive ligands were obtained from the Radiochemical Centre, Amersham, now Amersham International.

2.1.3.2 Competitor for Receptor Analysis

Diethylstilboestrol (DES) was obtained from Sigma, London.

2.1.4 Radioactive Sedimentation Markers

[^{14}C]-labelled BSA (4.6S), molecular weight 69,000 and specific activity 58-60 μCi/mg was obtained from the Radiochemical Centre, Amersham.

[^{14}C]-labelled human-γ-globulin (7.1S), molecular weight 150,000 and specific activity 11.5-26.9 μCi/mg was obtained from New England Nuclear, Southampton, U.K.

The sedimentation constants were taken from Fazekas and MacFarlane (1980) and S represents Svedberg Units (1S = 10^{-13}sec).

2.1.5 Scintillation Materials

The following materials were obtained from Koch-Light Laboratories, Colnbrook, England.

Toluene (AnalaR grade)
2,5-diphenyloxazole (PPO)
1,4-di-[2-(5-phenyloxazolyl]-benzene (POPOP)
Triton X-100 was obtained from Rohm and Haas, Croydon, England.

2.1.6 Livestock

Livestock used were mature (250-280g) and immature (16-21 days old) female Albino Wistar rats (Glasgow University Colony), and mature (3 - 6 months old) New Zealand white rabbits.

2.1.7 Human Tissue

2.1.7.1 Normal Uterine Tissue

Normal human uterine tissue was kindly supplied by the following hospitals:

Victoria Infirmary, Glasgow
Western Infirmary, Glasgow
Royal Infirmary, Glasgow
Stobhill General Hospital, Glasgow
Southern General Hospital, Glasgow

2.1.7.2 Human Breast Tumour Tissue and Fine Needle Aspirates (FNA)

Human breast biopsies and FNAs were obtained from the Western Infirmary and the Royal Alexandria Infirmary, Paisley.

2.1.8 Immunochemicals

Normal goat serum was obtained from the Scottish Antibody Production Unit (SAPU).

Goat anti-rabbit IgG was obtained from Miles.

Fluorescein conjugated Goat anti-rabbit IgG was obtained from Miles.

2.1.9 Buffers

2.1.9.1 Phosphate Buffered Saline (PBS-A) (without Calcium and Magnesium), Tris-EDTA-DTT (TED) and Hepes-EDTA-DTT (HED)

For PBS, these salts were dissolved (w/v) in distilled water:
NaCl 0.8%, KCl 0.02%, Na_2HPO_4 0.115%, KH_2PO_4 0.02% , pH=7.2.
For TED, these chemicals were dissolved in distilled water:
Tris-HCl 10mM, EDTA 1mM and dithiothreitol (DTT) 1mM, pH=7.5.
For HED, the following chemicals were dissolved in distilled water:
HEPES 10mM, EDTA 1.5mM and DTT 0.5mM, pH=7.4.

2.1.10 Supplements

2.1.10.1 Bovine Serum Albumin (BSA)

A stock solution of BSA 10 % (w/v) in PBS-A was prepared. The pH was readjusted to 7.2 and stored at -20°C, in 1ml aliquots.

2.1.11 Serum

Foetal calf serum (FCS) was purchased from either GIBCO or Flow. This was either used as supplied, or was treated with dextran coated charcoal (DCC) (see below)

2.1.11.1 Heat Inactivated Charcoal Stripped Foetal Calf Serum (HIDCCFCS)

FCS was heat inactivated at 56°C for 30min, during which the temperature was monitored continuously. This heat inactivated

serum was then cooled to 4°C.

Norit-A charcoal 2.5% (w/v) and dextran T-70 0.025% (w/v) were added to PBS-A and stirred gently at 4°C for about 16h. The DCC was pelleted by centrifugation (1000xg, 10min, 4°C) and the supernatant discarded. The pellet was resuspended in 10 times its original volume of precooled heat inactivated FCS, then stirred continuously for >16h at 4°C. Most of the DCC was removed by centrifugation. The remainder was removed by millipore filtration: 0.8, 0.45 then 0.2μm filters, filter sterilised and stored at -20°C.

2.1.12 Fixatives and Staining

2.1.12.1 Trichloroacetic Acid (TCA) for PAGE

A solution of TCA 50% (w/v) was prepared in distilled water.

2.1.12.2 Staining Solution

Coomassie brilliant blue solution (0.1%) was made up fresh in 50% TCA. The destaining solution contained 7.5% acetic acid and 5% methanol

2.1.12.3 Fixatives for Cytological Specimens

Acetone (100%) was kept at -20°C.
Methanol:Acetone (1:1) made before use and kept in ice.
Ethanol 100% was kept at 4°C

2.1.12.4 Hoechst 33258

Hoechst 33258 was obtained from Sigma, London. A stock solution of 1mg/ml was made in PBS and kept at -20°C. A working solution of 1μg/ml was made just before use.

2.1.13 Miscellaneous

Photographic film 'Ectachrome' daylight was obtained from Kodak.

Polystyrene tubes used in the receptor assay, and specimen jars used for human tissue collection were obtained from Sterilin Ltd., Teddington, England.

Cellulose nitrate tubes for sucrose density gradient analysis were supplied by Beckman, RIIC Ltd., High Wycombe.

Glass microfibre filter discs (GF/C 2.5cm diameter) were obtained from Whatman Ltd., England.

Glassware was washed and rinsed in glass distilled water, as the presence of divalent metal ions have been reported to affect the receptor measurements (Laing, 1980). All glassware which came into contact with human tissue was treated overnight with Kirbychlor, obtained from Kirby Pharmaceuticals, Suffolk, England.

All solutions were made in glass distilled water.

2.2 DEVELOPMENT LEADING TO THE FINAL PROCEDURE OF OESTROGEN RECEPTOR PURIFICATION

2.2.1 Tissue Handling

2.2.1.1 Collection

Tissue was collected fresh from the operating theatre and transported on ice in a sterile container.

2.2.1.2 Storage

The fresh uterine and breast cancer tissues, if not used straight away were either stored in sucrose/glycerol at -20°C (Crawford at al., 1984) or chopped up into fine pieces, frozen in liquid nitrogen and stored in -70°C until used.

2.2.2 Assay of Oestrogen Receptor in Uterine Tissue

2.2.2.1 Cytosol Preparation

The tissue stored in sucrose/glycerol at -20° was rehydrated in TED for 15min at 4°C and then dissected free of mucus and mesentry. Frozen tissue was thawed prior to use. Five gm of tissues was then homogenized in 5 volume TED (section 2.1.9), 2x15sec burst with Ultra-Turrax at a setting of 150, with 1min cooling periods. This rough homogenate was then further refined using a glass/glass homogenizer (Leake et al., 1981a). The temperature of the homogenate was maintained below 8°C at all times, as warming the homogenate results in receptor loss (King, 1979). The homogenate was then centrifuged at 105,000 x g in a Beckman 60 Ti rotor for 30min at 4°C. The clear supernatant was carefully decanted, avoiding the thin layer of fat. The protein content of the cytosol was measured according to the method in section 2.2.2.7 and the final protein concentration was usually in the range of 3-8mg/ml.

2.2.2.2 Preparation of [^{3}H]-Oestradiol-17β Solutions ([^{3}H]E_2)

Stock [^{3}H]E_2 was prepared to a concentration of 10^{-7}M in absolute alcohol and stored at -20°C. Appropriate amounts of the stock solution were aliquoted into small bottles in two batches to give the range of 4 - 40 x 10^{-10} M. Into the second batch of aliquots were added an equal volume of DES (10^{-5}M stock solution) in absolute alcohol. The alcohol was evaporated in a stream of compressed air or nitrogen and the [^{3}H]E_2 and [^{3}H]E_2+DES redissolved in the TED buffer. The batch containing the 100-fold excess DES, is for determining the non-specific binding.

2.2.2.3 Incubation of Soluble Fraction (Cytosol) with Steroid

Fifty µl of each of the E_2 solutions prepared as above were mixed with 150µl aliquots of cytosol. This gave a final range of concentrations of steroid in the tubes of 1x, 1.5x, 2x, 4x, 6x, 8x10^{-10}M and 10^{-9} M [^{3}H]E_2±DES. A set of control tubes to give total

values was also set up for the cytoplasmic assay which were identical to those above except that TED buffer was substituted for cytosol. The mixtures were incubated at 4°C for 16-18h.

2.2.2.4 Dextran Coated Charcoal Solution (DCC)

DCC solution was prepared by resuspending Norit A charcoal and dextran T-70 in TE containing 0.25M sucrose to final concentrations of 0.15% (w/v) Norit A charcoal and 0.0015% dextran T-70. A DCC solution containing 0.5% (w/v) Norit A charcoal and 0.005% dextran T-70 was also made.

2.2.2.5 Separation of Unbound from Bound Steroid by DCC Adsorption

At the end of the incubation 900µl of buffer was added, 200 µl from the 'totals' tubes were counted for total activity and 500 µl DCC solution, as prepared in section 2.3.2.4, was added to each of the other tubes. After leaving at 0°C for 15min with periodic mixing, the charcoal was pelleted at 2x1000g for 5min at 4°C. One ml of the supernatant counted was in 10ml Triton toluene scintillant.

2.2.2.6 Radioactivity Counting

Radioactivity was measured in a Searle Mk III liquid scintillation spectrometer. Scintillant contained 1400ml toluene/PPO (5g/l)/POPOP (0.24g/l):600ml Triton-X100:200ml absolute alcohol. The counting efficiency of a 1ml aliquot mixed with 10ml of scintillation cocktail was 30%.

2.2.2.7 Protein Determination

Protein concentrations were estimated with bovine serum albumin as the standard using the BIORAD assay based on the method of Bradford (1976). The standard curve was set up using duplicate standards of 0, 10, 20, 40, 60 and 80 µg/ml.

2.2.2.8 Expression of Results

The receptor level and the dissociation constant were calculated using Scatchard plot analysis (Scatchard, 1949) and the final receptor concentration was expressed as fmol/mg cytosol protein.

2.2.3 Polyacrylamide Gel Electrophoresis

The first attempts in the purification of the oestrogen receptor used polyacrylamide gels. Protein separation were done on 4 % polyacrylamide tube gels using the continuous Tris-borate-EDTA buffer system (Raam et al., 1981).

2.2.3.1 Preparation of the Gels and Buffers

The gel/electrode buffer was the Tris-borate EDTA buffer (0.09M Tris, 0.09M Boric acid, 0.0025M EDTA pH=8.9). A 4% gel was prepared from a stock solution of 16% by weight of acrylamide and 0.8% of N,N'-bis-methylene acrylamide prepared in borate buffer. Insoluble material was removed by filtration through Whatman No.1 filter paper. The stock solution was kept in the dark at 4°C.

To polymerise the gel, TEMED and ammonium persulphate were added. Two hundred µl TEMED and 140mg ammonium persulphate were dissolved in 100ml buffer. From the above solution 4% gels were made in the following proportions, gel stock solution:TEMED:ammonium persulphate (1:1:2).

2.2.3.2 Preparation of Samples

Oestrogen receptor positive cytosol, from breast cancer biopsies, as determined in the laboratory by routine DCC analysis, were used. The cytosols were either labelled with E_2 or $[^3H]E_2$ at a concentration of 10nM. Before the sample was loaded onto the gel, 3 parts of cytosol was mixed with 1 part of sample buffer (50% sucrose in HED buffer pH=7.4 and 0.1% bromophenol blue). A 100µl aliquot was layered onto the surface of each gel and electrophoresis was run at 2mA/gel. Human uterine cytosol and known molecular weight markers (240K, 68K, 45K) were also run.

2.2.3.3 Preparation of Immunogen

One gel was sliced horizontally into 2mm discs. These were counted to locate the labelled protein (oestrogen receptor). The corresponding position from the other eleven gels was then removed. These portions were pooled, frozen in liquid nitrogen and ground to a fine powder which was mixed with complete Fruend's adjuvant for primary injection and incomplete adjuvant for booster injections. In this experiment Balb c mice were used. Due to the small amount of antigen, the experiment was not successful.

2.2.4. Procedure for DNA Cellulose Chromatography

Chromatography was then used in the next attempt at purification. Five gm of DNA cellulose (4.1mg DNA/gm DNA cellulose) was equilibrated in TED buffer overnight. It was packed into a column (1x10cm) and the column was washed with 2x50ml TED. Samples (100ml cytosol) prepared in this buffer (as described in section 2.2.2.1) were labelled by incubating with 10nM E_2 overnight and the unbound steroid was removed by DCC (section 2.2.2.4) pelleted from 0.15% DCC solution. The sample was heated to 30°C for 30min and applied to the packed columns using a peristaltic pump, followed by a 200ml wash with TED buffer. Buffer containing 0.2M KCl (50ml) was passed through to salt extract the DNA bound receptor. Fractions (2ml) were collected at 10ml/h, and 0.1ml of each fraction was used for radioactivity measurement and a graph of cpm versus fraction number was constructed. Protein concentration was measured using absorbance at 280nm and protein under the radioactive peak was measured using the dye method (section 2.2.2.7). The fractions under the peak (20ml) were pooled, dialysed overnight in 0.01M phosphate buffer pH=7.4 at 4°C and applied to the ion-exchange column, as prepared in the next section (2.2.5).

2.2.5 DEAE Sepharose Ion Exchange Chromatography

Microgranular DEAE sepharose was equilibrated in TED buffer overnight and packed into a column (1x4cm). Pooled, dialysed sample

(from section 2.3.4) was applied to the packed column followed by a 150ml wash with the TED buffer. Buffer containing 0.13M KCl was then introduced to extract the receptor. Fractions (2ml) were collected at 20ml/h, and 0.1ml of each fraction was used for radioactivity measurement. Protein concentrations were measured at 280nm absrobance and proteins under the radioactive peak were measured using the dye method (2.2.2.7). A graph of cpm versus fraction number was constructed and the fractions under the peak (20ml) were pooled and subjected to phenyl sepharose hydrophobic chromatography.

2.2.6 Hydrophobic Chromatography

The phenyl sepharose was equilibrated in TED overnight and transfered to a column (1x5cm). The fractions containing the oestrogen receptor from the DEAE sepharose column was passed through the phenyl sepharose column at the rate of 10ml/h with the help of a peristaltic pump. This was followed by a 500ml wash with TED. The receptor was eluted in 2ml fractions using ethylene glycol:TED:glycerol (40:30:30) at the rate of 10ml/h. A 0.1ml fraction was counted in triton-toluene scintillant for radioactivity to calculate the degree of purification of the receptor as well as to determine the position of the peak. Protein concentrations were measured at 280nm absorbance and the proteins under the peak were measured using the microassay of the dye method (section 2.2.2.7). The overall purification procedure is shown in Figure 6.

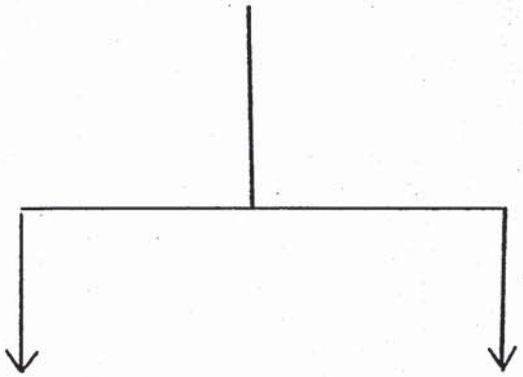
2.2.7 Affinity Chromatography

2.2.7.1 Preparation and Washing of Oestradiol-17β 17-hemisuccinyl-albumin-Sepharose 4B

Oestradiol-17β 17-hemisuccinnyl-albumin-sepharose 4B (E_2-Succ-Alb-Sepharose) the structure as shown in Figure 7, was prepared according to the method described below (Figure 8). All the procedures were carried out at room temperature unless otherwise stated. Cyanogen bromide-activated Sepharose-4B (1.5gm) was swollen

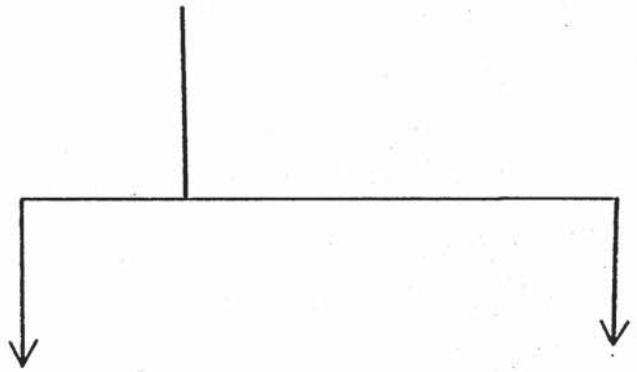
Figure 6: Flowsheet for the Overall Purification Procedure of Oestrogen Receptor Involving DNA Cellulose, DEAE Sepharose and Phenyl Sepharose

Activated [^{3}H]E_2-receptor from human uterine cytosol was applied to DNA-cellulose column. The column was washed with 2x50ml TED. Bound receptor was eluted with 0.2M KCl and subjected to the next purification step



The receptor containing fractions from DNA-cellulose were loaded onto a DEAE Sepharose column. Bound receptor was eluted with 0.13M KCl

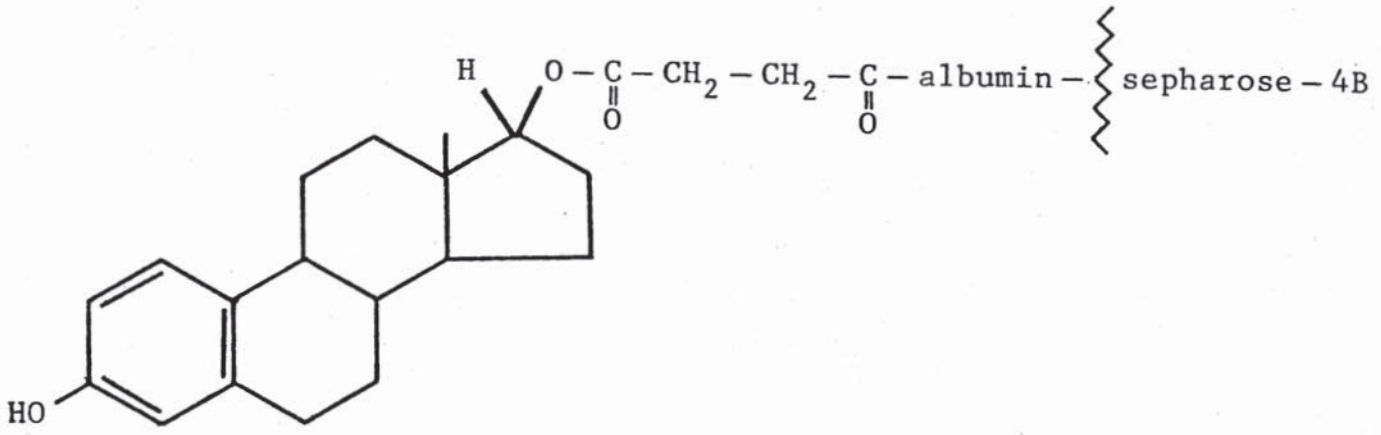
Protein concentration and radioactivity determined



Fractions eluted from the DEAE sepharose were transferred onto a phenyl Sepharose column. Bound receptor was eluted with ethylene glycol:TED:glycerol (40:30:30)

Protein concentration and radioactivity determined

Figure 7: Structure of Oestradiol-17β 17-Hemisuccinyl-Albumin-Sepharose 4B



Oestradiol-17β 17-Hemisuccinyl-Albumin-Sepharose 4B

Figure 8: Flowsheet for the Preparation of the Affinity Resin.

1.5g CNBr-Sepharose 4B + 2mg E_2-Succ-Alb in 10ml 0.2M $NaHCO_3$ and 8M urea at pH=9

Mixture rotated end-over-end overnight at 4°C

Filter gel and filterate assayed spectrophotometrically at 280nm. Protein bound was measured as the difference of input and filtrate

Wash gel with 200ml 1 M NaCl and 200ml H_2O

Inactivate the remaining active sites by washing in 50ml of 1M ethanolamine in 0.2M $NaHCO_3$ buffer overnight at 4°C

Wash gel with 300ml 1M NaCl and 200ml H_2O

Resulting affinity gel was washed thoroughly with 2.5L of 80% methanol

Prior to use the affinity gel was washed with TED (100ml/ml gel)

in 500ml of 1mM HCl (to preserve the reactive groups) for 30min. Using a scintered glass filter, the resin was washed with the acid solution and once with coupling buffer [(0.2M $NaHCO_3$ and 8M urea pH=9.0) (5ml/gm gel)]. The washed gel was immediately transferred to the solution containing 2mg of oestradiol-hemisuccinyl-albumin (E_2-Succ-Alb) dissolved in 20ml of coupling buffer. The higher pH is chosen as the coupling reaction proceeds most efficiently in the pH range 8-10 where the amino groups on the ligand are predominantly in the unprotonated form. Coupling at low pH is less efficient. The mixture containing ligand and the swollen gel was rotated end-over-end overnight at 4°C. The complex was filtered through the scintered glass and the protein reading of the eluate was taken to ensure more than 90% binding. The resulting gel (4ml) was washed with 1M NaCl (100ml). The remaining active groups were blocked by incubating the gel with (50ml) 1M ethanolamine overnight at 4°C.

The excess adsorbed protein and the blocking agent were washed off with coupling buffer followed by 300ml of 1M NaCl. The resulting gel was washed thoroughly with methanol (2.5-31) to remove the loosely bound steroid and kept in methanol (80%) until use. The concentration of E_2-Succ-Alb is 0.3-0.5 µmol/ml of gel. Prior to use the affinity resin was washed with TED (100ml/ml gel).

2.2.7.2. Stability Testing of Affinity Adsorbent

Breakdown of the E_2-Succ-Alb-Sepharose used during chromatography could lead to leakage of E_2-Succ-Alb or E_2 into the incubation medium. Checks on hormone leakage after incubation with cytosol were carried out as follows:

Twenty five ml human uterine cytosol, as prepared in section 2.2.2.1, was incubated with the gel (1.5ml gel). The gel was separated from the cytosol by centrifugation at 1000g for 10min. An aliquot (5ml) of the supernatant was extracted with ether (2x10ml). Evaporation of the ethereal extract resulted in a residue which was soluble in 0.2ml of dimethylformamide:water (1:1, v/v). Aliquots (20µl) of this solution were added to 0.5 ml of fresh rat uterine

cytosol prepared in the same way as the human cytosol but incubated with $[^3H]E_2$ (1nM) prior to use. Control incubation consists of rat cytosol only incubated with the same concentration of $[^3H]E_2$. Binding inhibition was measured by comparison of the binding in both cases. If a significant quantity (more than 10% inhibition) of free hormone was detected, the affinity resin was washed again with large volumes of methanol.

2.2.7.3 Affinity Chromatographic Procedure

2.2.7.3.1 Preparation of Human Uterine Cytosol and Ammonium Sulphate Precipitation

Homogenates from 100g of human uterus were prepared (as described in section 2.2.2.1). To the high speed supernatant, potassium chloride was added to a final concentration of 0.4M, followed two hours later by calcium chloride to a final concentration of 4mM. The solution was incubated for 45 min at 4°C to convert the oestrogen receptor to the 4.5S form (Sica et al., 1973). An aliquot of the supernatant was assayed for oestrogen receptor while treating the rest with ammonium sulphate.

Ammonium sulphate precipitation was carried out by slowly adding 0.144 g of finely powdered salt per ml (25% saturation) with continuous magnetic stirring. The suspension was centrifuged (4°C) for 20min at 18,000xg. The precipitate was dissolved in a volume of $TEDK_{0.2}$. Undissolved material was removed by centrifugation for 30min at 105,000 xg in 60 Ti rotor in Beckman L2-65B ultracentrifuge.

The clear supernatant (100ml) was mixed with the affinity resin and the suspension was either rotated end-over-end at the speed of 1rpm or applied into a column and recirculated overnight at 4°C using a peristaltic pump. The column was then washed with 0.5L of TED containing 0.01M KCl ($TEDK_{0.01}$), with 0.5L of TED with 0.4M KCl ($TEDK_{0.4}$) and 0.5L of TED containing 1.0M KCl ($TEDK_{1.0}$) the order as shown in Figure 16 (section 3.2.2). The bound receptor was eluted from the

column with 10μM DES in 8ml (2xvolume of affinity resin) TED buffer containing in 0.4M KCl,0.5M NaSCN and 10% dimethylformamide. The elution was carried out either at 4°C overnight or 30°C for 15min. In three experiments the receptor was eluted from the column by using 1μM [^{3}H]E_2 (Figure 16). Aliquots were pooled and desalted through Sephadex G-25 (fine), lyophilized and analysed by SDS-PAGE, heat exchange assay (section 2.2.8) and for protein concentration.

2.2.8 Heat Exchange Assay (Assay of Oestradiol Receptor Binding Activity)

The purpose of this assay was to check the binding activity of the above eluted proteins by exchange assay i.e. by incubating with [^{3}H]E_2. This assay was based on the separation of oestradiol-receptor complexes from free E_2 by gel filtration on Sephadex G-25 (Puca et al., 1971). The sample to be tested (0.1 to 0.3ml) was adjusted to a volume of 1ml by the addition of 10 mM Tris-HCl, pH 7.5, containing 10mM KCl, 1mM EDTA, 10nM of [^{3}H]E_2, and a trace of blue dextran (as a marker). The mixture was incubated at 4°C for 6h, and the free and the macromolecular bound E_2 components were separated by passing the mixture over a Sephadex G-25 column (diameter 1.0cm; total volume, 10ml and flow rate, 0.5ml/min). The protein-bound hormone appeared in the first macromolecular peak together with the excluded dextran blue Vo, while the free hormone appeared very late as a peak which emerged after the included volume of the column. The excluded peak was collected, pooled, and examined for oestrogen receptor content. Under these conditions of gel filtration, nonspecific binding of E_2 was negligible (Puca et al., 1971). Nonspecific binding of E_2 was the relatively low affinity E_2 binding by macromolecules other than receptors. The binding of E_2 to such molecules was directly proportional to the concentration of E_2 and was not experimentally saturable with respect to the steroid.

The specific binding of E_2 to receptor proteins is destroyed by exposure to a temperature of 65°C, whereas nonspecific binding is actually enhanced by this treatment (Puca et al., 1973). All

oestrogen-binding proteins eluted from affinity columns were therefore examined for thermolability properties. The sample, in 0.1 to 0.3ml of water, was heated at 65°C. The sample was quickly cooled to 4°C, and the oesradiol-binding activity was determined by gel filtration.

2.2.9 Checking the Purity of the Affinity Eluate on Acrylamide Disc Gel Electrophoresis

2.2.9.1 Preparation of Gels

The method as described by Laemmelli (1970) was followed with some modifications. The stacking gel (3% acrylamide) and the separating gel (5% acrylamide) were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N,N'-bis-methylene acrylamide. Insoluble material was removed by filtration through Whatman No. 1 filter paper. The solution was kept at 4°C in a dark bottle. The gels were polymerized chemically by the addition of tetramethylethylenediamine (TEMED) and ammonium persulphate.

In the separation gel the final concentration of Tris was 0.375M Tris-HCl (pH 8.8) and 0.1% SDS (w/v). The 3% stacking gel of 1cm, contained 0.125M Tris-HCl (pH 6.8) and 0.1% SDS and was polymerized chemically in the same way as for the separating gel. The electrode buffer (pH 8.3) contained 0.025M Tris, 0.192M glycine and 0.1% SDS.

The samples (0.2-0.3ml) contained a final concentration ("final sample buffer") of 0.0625M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue as marker dye and 0.01mg of loaded protein per gel. The proteins were completely dissociated by immersing the samples for 1.5min in boiling water.

The glass gel tubes were 15cm long with an inner diameter of 6mm. Before use they were soaked in cleaning solution, rinsed, and

oven-dried. For a typical run of 12 gels, 15ml of gel buffer was deaerated and mixed with 13.5ml of acrylamide solution. After further dearation, 1.5ml freshly made ammonium persulphate solution (15mg/ml) and 0.045ml of TEMED were added. After mixing, each gel tube was filled with 2ml of the solution. Before the gel polymerised a few drops of water were layered onto the top of the gel solution. After 20 to 30min an interface could be seen indicationg that the gel had polymerised. Gels with a normal amount of cross-linkage remained clear, those with double cross-linkage turned opaque. Just before use the water layer was sucked off, and replace by gel buffer. The tubes were placed in the electrophoresis apparatus. The two compartments of the electrophoresis tanks (made by Mr. Harvey, Glasgow University) were filled with electrode buffer.

2.2.9.2 Running the Gel Electrophoresis

Usually 0.01mg protein was applied per gel. The amount could be lowered if the gel was stained for a longer period. If 0.1mg was applied, although the trailing edge of the band was diffuse, the leading edge was still very sharp and the molecular weight could still be found very accurately. For each standard solution/purified receptor, 3µl of tracking dye (0.05% Bromophenol blue in water), and 50µl of sample buffer was mixed in a small test tube or eppendorf tube. Then 10-50µl of the protein solution were added (final concentration of protein of 0.01mg/50µl). After mixing, the tubes were immersed in boiling water for 1.5min. Fifty µl of the sample solution was carefully applied on the gel.

Electrophoresis was carried out at a constant current of 4mA per gel with the positive electrode in the lower chamber. Under these conditions the marker dye moved three-quarters of the way through the gel in approximately 4h.

After electrophoresis, the gels were removed from the tubes by squirting water from a syringe between the gel and the glass wall and using a pipette bulb to exert pressure. The length of the gel and the distance moved by the dye were measured. The proteins were

fixed in the gel with 50% trichloroacetic acid (TCA) for 1h before staining for 1h at 37°C.

2.2.9.3 Staining and Destaining

The staining solution was prepared by dissolving 1.25 g of Coomassie brilliant blue in a mixture of 454ml of 50% methanol and 46ml of glacial acetic acid and removing the insouble material by filtration through Whatman No. 1 filter paper. The gels were put into test tubes filled with staining solution and stained for 2h at room temperature. The gel were removed from the staining solution, rinsed with distilled water, and placed in destaining solution (75ml of acetic acid, 50ml of methanol, and 875ml of water) for 2h. The length of the gels after destaining and the positions of the blue protein zones were recorded. The gels were stored in 7.5% acetic acid solution.

2.2.9.4 Calculation of Molecular Weight

The gels swell some 5% in the acid solution used for staining and destaining (Weber and Osborn, 1975). Gels with a lower amount of cross-linkage showed more swelling (low % gel). Therefore the calculation of the mobility has to include the length of the gel before and after staining as well as the mobility of the protein and of the marker dye. Assuming even swelling of the gels, the mobility was calculated as,

$$\text{Mobility} = \frac{\text{Distance of protein migration}}{\text{Length after destaining}}$$

$$\times \frac{\text{Length after staining}}{\text{Distance of dye migration}}$$

For slab gel, the same procedure as disc gel was carried out except that the amount of solutions used were adjusted according to the size of the glass plate.

The mobilities were plotted against the known molecular weight markers expressed on a semi-logarithmic scale. The standard proteins of known molecular weight used were: 90,000, 68,000, 45,000, 30,000, 20,000 obtained from Boehringer Mannheim.

2.3 IMMUNIZATION

The purified oestrogen receptor was then ready to be used as an antigen in New Zealand white rabbits. Rabbits were chosen because they are relatively inexpensive, are easy to handle, and more than adequate volumes of serum may be bled from them on a weekly basis.

The principle of immunization is the administration of a water-in-oil emulsion containing the immunogen intradermally over a wide area on a particular part of the experimental animal. The purpose is to stimulate as many lymph nodes as possible in the stimulation of antibody production (Vaitukaitis, 1981).

The water-in-oil emulsion to be injected is prepared as follows. The purified oestrogen receptor complex (20-30µg) was first dissolved in PBS at pH=7.2. An equal volume of that solution is combined with Freund's complete adjuvant for primary immunization. Freund's complete adjuvant contains 2mg/ml of heat-killed tubercle bacillus with Mycobacterium. The Mycobacterium is usually present as a precipitate in a small spot on the side or bottom of the vial. It may be suspended with gentle tapping. For subsequent booster injection, the immunogen was injected in the same volume of Freund's incomplete adjuvant. This does not contain Mycobacterium but 5mg of heat-killed tubercle bacillus per 2ml of the emulsion. To form a good emulsion, an Ultra-Turrax was used at its maximum setting for 5x15sec with intermittent cooling (1min) until the reaction mixture thickens and forms peaks when stroked with a glass rod or spatula. The aqueous and oil phases of the preparation should not separate on standing. Usually the syringe-transfer technique does not result in good emulsification of the reagents.

The plunger of a syringe was removed and the barrel filled with the emulsion. The plunger was then re-inserted. The calibrated syringe facilitates the injection of 0.1-0.2ml administration per site. A total of 2ml of the emulsion was prepared to be injected at multiple sites (12-17 sites), subcutaneously usually with a 21-gauge needle.

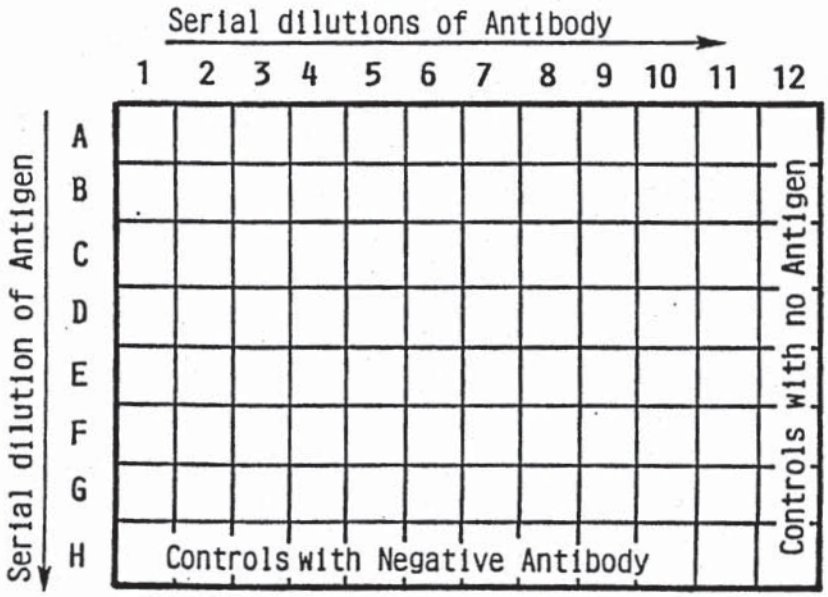
The primary injection was followed by six subsequent booster injections over a period of 8 months (October 1983 - May 1984). Three animals were injected. The sera were screened at 6-8 weeks after the primary immunization. When the antibody was detectable, the animal was bled forthnightly and the antisera assessed for titre (antibody concentration) and sensitivity (affinity). Two rabbits gave high reactivity with cell components (reacted with both positive and negative oestrogen responsive tissue) but very low specificity towards the oestrogen receptor (determined by the immunoprecipitation method). Only one animal (rabbit number 13) had a significant antibody titre and specificity towards the oestrogen receptor. This was first evident 3 months (January 1984) after the initial immunization. Blood was collected at 14 day intervals from the marginal ear vein.

2.3.1 Screening of Antisera

Screening the antisera can be done using ELISA solid-phase assays(Enzyme Linked Immuno Sorbent Assay). The chequerboard ELISA is a useful initial test of the sera against the antigen which has been used for immunisation. The ELISA method for soluble antigen has been applied for oestrogen receptor antibody screening.

Antigen (2.4ml) was prepared in PBS at a concentration of 1µg/ml. A series of 6 doubling dilutions in small tubes was made by adding 1.2ml of antigen solution to 1.2ml PBS. The final dilution was 1/64. A 96-well microtitre plate (Dynatech) was prepared as follows: 100µl of the most concentrated antigen solution was put into all 12 wells of row A (Figure 9), 100µl of the first dilution into all 12 wells of row B and so on to row G. PBS (100µl) was

Figure 9: ELISA Chequerboard


Serial dilutions of Antibody
1 2 3 4 5 6 7 8 9 10 11 12
Serial dilution of Antigen
A
B
C
D
E
F
G
H
Controls with Negative Antibody
Controls with no Antigen

put into all 12 wells of row H. The plate was incubated overnight at 4°C. The next day, the contents of the plate were discarded by shaking off the antigen solution with a sharp wrist movement and washing 4x with PBS plus 0.5% Tween 20. The plates were knocked dry by hammering them face down on paper towels. Aliquots, 150µl BSA (50 mg/ml in PBS) were added to every well and the plate incubated for 30 min at room temperature. The plate was washed 3x with PBS containing 0.05% Tween 20 and knock dried. Antisera was diluted 1 in 25 in PBS containing 0.5mg/ml BSA and doubling dilutions (0.4ml antiserum into 0.4ml buffer) again made such that the last tube has a 1 in 64,000 dilution of the antibody. Control serum (1.2ml) was also made to a 1 in 25 dilution. To each well in column 1, 100µl of the 1/25 dilution of control antiserum was added. To each well in column 2, 100µl of the 1/50 and so on until in column 12 a 1/100 dilution of the negative control was added. The plate was incubated at room temperature for 1-2h. At the end of the incubation the plate was washed 3x in PBS containing 0.05% Tween 20 and knocked dry. Aliquots (100µl) of goat anti-rabbit conjugated to horseradish peroxidase were added to every well at a dilution of 1/500 (in 0.5mg/ml BSA and 0.05% Tween 20) and incubated for 2h at room temperature. After washing the plate 3x in PBS, 100 µl of substrate in a solution of 0.4mg/ml of freshly prepared Orthophenylene diamin (OPD) dissolved in 0.1M sodium citrate, 0.2M sodium phosphate pH=6 containing 0.32 µl/ml H_2O_2 (0.01%) was added. The plate was covered with foil and incubated at room temperature for 30min. To stop the reaction, 50µl of 4N sulphuric acid was added. The plate was inspected by eye. The titre of the antisera was estimated by finding the highest dilution at which the antiserum gives more colour than the negative serum and the wells without antigen.

2.3.2 Isolation of Serum from Blood

Antibodies (IgG) are present only in the serum component of the blood. Therefore, serum should be separated from the blood cells as soon as possible after the collection. If this is not done, the cells lyse and release contaminating proteins including proteolytic enzymes which will degrade the antibodies. Blood was first allowed

to clot at room temperature for about 1h. The clot was allowed to contract at 4°C overnight. Glass containers were used as blood clots more quickly in glass than in plastic containers. The clot was detached off the wall of the container and the clot-free liquid was centrifuged at 1500xg at 4°C for 20min.

2.3.3 Storage of Antibody

Polyclonal antisera are usually stable (Campbell, 1984). This could be due to a large number of disulphide bonds which are present on immunoglobulins. Bacterial inhibitor, sodium azide 0.1%, was added and the serum stored at 4°C or straight away aliquoted and kept at -20°C or -70°C.

2.3.4 Purification of Antibody to Oestrogen Receptor (IgG-ER) from the Immune Serum

In many cases purification of an antibody is not done since all that is required is specificity and each batch of antibody can be tested for titre and used directly. However, in SDG experiments partially purified immunoglobulin gave better results.

Ammonium sulphate (12g) was added to 50ml of rabbit serum to give a desired 40% saturated salt solution which was stirred at 4°C in the cold room until all the salt dissolved and then incubated for 30min at 4°C. The mixture was then centrifuged at 3000xg for 30min at 4°C. The precipitated protein was resuspended in 15ml water and dialysed against 1L of 10mM phosphate buffer (pH=7.2) overnight. An 8ml column of DEAE Sephacel in the barrel of a 10ml pippete was prepared by first plugging the barrel with glass wool and then adding the resin which was equilibrated in 50ml 10mM phosphate buffer. The 8ml column was supposed to have the capacity to bind 1g albumin. The column should not be overloaded with more than 1g protein. The dialysed sample was then run through the column and washed with 10ml of the equilibrating buffer and 1ml fractions were collected at the rate of 30ml/h. Once the sample had fully entered the column, the IgG was then eluted with 100ml of 10mM phosphate

buffer containing 40mM NaCl. The absorbance at 280 nm was read. The early fractions under the peak are the purest and the fraction in the tail region was discarded.

2.4 THE SPECIFICITY OF THE ANTISERUM

2.4.1 Preparation of Human, Rat and Rabbit Uterine Cytosol

Uterine soluble (cytosolic) oestrogen receptor was prepared according to the methods described in section 2.3.1.1. Five gm of the tissue (uterus of human, rat or rabbit) were homogenised at 4°C in five volumes of TED buffer, using an Ultra-Turrax 181W homogenizer with 5x15sec burst, with 1min interval cooling periods. The homogenate was centrifuged at 600xg for 10min. Cytosols were obtained as the supernatant fraction after further centrifugation at 105,000xg for 60min in a L2-65B Beckman ultracentrifuge using SW50.1 rotor. The high-speed supernatant fraction was incubated with 5nM E_2 for 60 min at 4°C. The excess E_2 was removed by incubating the cytosol (4°C, 15min) with a pellet of DCC (final concentration 0.25% DCC)

2.4.2 Antibody-receptor Interaction

2.4.2.1 Immunoprecipitation Assays

The principle of immunoprecipitation is the use of a second antibody directed against the first (primary antiserum). The original method required the addition of an exact amount of second antibody to cross-link and form a lattice with the first to induce precipitation. Since an immunoprecipitate will not always form when an antibody encounters an antigen, the relative concentrations of antibody and antigen should be adjusted. The limited range of their relative concentration over which immunoprecipitation will occur is normally referred to as 'equivalence'. Outside of this range conditions of either antibody or antigen excess exist and complexes of antigen-antibody are small and soluble (Roitt, 1980). A maximum of 50-60% of the antigen-antibody complex at low dilution can be

precipitated out. For the titre, either the antisera or semipurified IgG-ER [prepared as ammonium sulphate precipitate (as in section 2.3.4) and dialysed against PBS] was used. The amount of IgG required to give half the maximal precipitation indicates the titre of the antibody.

Antibody specificity for rabbit IgG to oestrogen receptor (IgG-ER) was determined by a double antibody precipitation technique, using crude cytosol (350fmol/ml), semipurified (25pmol/ml) or purified receptor (10µg) labelled with 5nM [^{3}H]E_2. For rabbit IgG-ER specificity determinations, solutions containing labelled receptor from the above preparations were adjusted to contain 0.4pmol/ml PBS. Serial dilutions of rabbit IgG-ER (250, 50, 5, 0.5, 0.05µg) from a stock solution of 5mg/ml was made in a volume of 250µl in PBS containing 10mM EDTA. Each incubation mixture contains 0.1pmol oestrogen receptor, (0.25ml), 0.1mg (0.1ml) of normal rabbit IgG-N and each of the concentrations of the IgG-ER (0.25ml). The final volume was 1 ml and the mixtures incubated overnight (14-20h) at 4°C in an eppendorf tube. Controls for determining nonspecific binding, contained only 0.1pmol receptor, 0.1mg normal rabbit IgG-N to replace the IgG-ER and PBS. At the end of the incubation 25µl goat antiserum to rabbit (as partially purified IgG) was added to precipitate all the rabbit IgG.

After an additional 4-5h at 4°C, the tubes were centrifuged at 2000xg for 20min and the pellets were washed twice with PBS containing 1mg/ml BSA. The pellets were then dissolved in 100µl of 0.2M NaOH and neutralized with 1M HCl. Radioactivity was measured in 4ml of scintillation mixture containing Triton X-100, as in section 2.2.2.6. (efficiency for [^{3}H] was 30%).

2.4.3 Detection of IgG-ER Complexes by Sucrose Gradient Ultracentrifugation

To detect the anti-oestradiol receptor antibodies, the migration of this protein bound to [^{3}H]E_2 receptor complex was studied using density gradient ultracentrifugation. For these studies, oestrogen receptor complexes were prepared from cytosols as

in section 2.4.1. The cytosol was incubated for 2h at 4°C with [^{3}H]E_2 (final concentration 5nM). Nonspecific binding was determined by including, in parallel, incubations containing 100-fold excess of DES in TED buffer. The incubation mixture was transfered to pre-formed DCC pellets (from 0.5 % DCC suspension), and the tubes were shaken intermittently by hand for 15min at 4°C and spun at 2300xg for 10min. About 0.2-0.5pmol of oestrogen receptor in 100-200µl cytosol was incubated with IgG-ER or IgG-N (50µg) at 4°C for 5h in a final volume of 220 µl in $TEK_{0.01}$(low salt) or $TEK_{0.4}$ (high salt) depending on the gradient to be used.

The gradient was layered by hand. Five, 10, 15 and 20% sucrose solutions were prepared in the appropriate buffer. Into 5ml SW50.1 centrifuge tube 1.15ml of 20% sucrose solution was first layered. The tube was kept in the -70°C freezer for 15min, sufficient to freeze the sucrose solution. The next layer will be the 15% sucrose, followed by 10% and finally 5% each time followed by freezing at -70°C. Before use, the gradient was left to equilibrate overnight at 4°C.

A 200µl aliquot of the cytosol was layered onto 4.6 ml of 5-20% hand layered sucrose gradients in TEK i.e. either $TEK_{0.01}$ or $TEK_{0.4}$. The gradients were centrifuged for 16h at 4°C at 230,000xg in a L2-65B Beckman ultracentrifuge using a SW50.1 rotor. Two drop fractions were collected from the bottom of tube and the radioactivity determined in 4ml of aqueous counting scintillant in Triton X-100. In separate experiments [^{14}C] bovine plasma albumin (4.6S) and human globulin (7.1S), were sedimented in parallel gradients to serve as external markers.

2.4.4 Effect of IgG-ER on the Progesterone and Androgen Receptors

Progesterone receptor from rat uterine cytosol was labelled with [^{3}H]ORG 2058 and androgen receptor from rat prostate was labelled with [^{3}H]DHT. Both were incubated with IgG-ER (section 2.4.3) and sedimented through a sucrose gradient.

2.4.5 Specificity of IgG-ER-Complex Interaction Using Immunoadsorbents

2.4.5.1 Preparation of Sepharose Bound IgG-ER for Immunoadsorption Chromatography

Cyanogen Bromide activated Sepharose (1.5g) was activated with 400ml of 1mM HCl. The use of HCl preserves the activity of the reactive groups which hydrolyse at high pH. The gel was left to swell for 30min, the ligand 15mg IgG-ER or IgG-N diluted in coupling buffer. The activated resin was washed once in 8ml of coupling buffer (0.2M, pH 9.0) containing NaCl (0.5M) and immediately transferred to the solution containing the IgG-ER. The coupling buffer solution should have a high salt content to minimise protein-protein adsorption caused by the polyelectrolyte nature of proteins.

The mixture containing IgG-ER and swollen gel is rotated end-over-end overnight at 4°C. At the end of the incubation the remaining active sites were blocked in 8ml 0.2M glycine overnight at 4°C. To remove the excess uncoupled ligand that remained after coupling, the adsorbent was washed four times in alternately high and low pH buffer solutions. Acetate buffer (0.1M, pH 4) and coupling buffer (pH 8.3) each containing 0.5M NaCl are suitable. This is important as protein adsorption occurs only when the pH is changed. The protein content of each solution was determined before and after the first step in the coupling procedure in order to ensure that more than 90% of the proteins were coupled to Sepharose. The immunoadsorbent was then incubated with cytosol containing 25pmol receptor and the mixture was incubated end-over-end overnight. The immunoadsorbent with the bound receptor was transferred to a column and was washed with 200ml of $TEDK_{0.01}$, 200ml of $TEDK_{0.4}$ and 300ml of $TEDK_{0.01}$ to remove nonspecific binding. The bound receptor was eluted using $TEDK_{0.4}$ containing 0.5M NaSCN, 10% dimethylformamide and 10µM DES and the eluate was desalted on Sephadex G-25 (10ml). The Vo was lyophilized and run on native PAGE as described in section 2.2.2.

2.4.5.2 Time Course Study: Interaction of Immobilized Antibody with Oestrogen Receptor

The immunoadsorbent loaded with ligand-free oestrogen receptor, (section 2.4.5.1) was re-suspended in 6ml TED. Two batches of six portions (50µl) of the receptor-loaded immunoadsorbent were placed in polypropylene test tubes. They were incubated for 1.5h at 4°C with 500 µl aliquots of buffer containing labelled ligand (5nM) with or without a 100-fold excess of unlabelled E_2 and aliquots taken at 0, 30, 60, 90, 120, 150min. Untreated Sepharose-IgG was used as a control. At the end of the indicated time period the resin was washed three times with buffer (3x2ml), radioactivity bound to the adsorbent was measured in Triton-X-100 scintillation mixture with a counting efficiency of 30%.

2.4.5.3 Western Blot: Protein Transfer and Antibody Overlay

A Western blot is the electrophoresis of the antigen followed by its subsequent transfer to nitrocellulose paper and then incubation with a specific antibody which is recognised by a labelled second antibody. Western blotting was originally developed by Towbin et al. (1979) with polyclonal antiserum.

Incubation/Washing buffer was prepared as follows: 20mM Tris-HCl (pH 7.2), 0.15M NaCl, 0.5mg/ml NaN_3, 0.5% Tween 20 (w/v). This solution may be used as blocking buffer with 30mg/ml BSA.

The transfer buffer or tank buffer was prepared as follows: 25mM Tris, 192mM Glycine, 20% w/v Methyl Alcohol (Analar), 0.02% SDS and the pH was adjusted to 8.3 with concentrated HCl. The antibody solution (1:100) plus carrier protein (normal horse serum) and ^{125}I-Protein-A solution ($3x10^6$cpm) were prepared in incubation buffer.

Nitrocellulose paper of 0.4µm diameter and 0.1% Amido Black (Naphthalene Black) in 45% MeOH (v/v) and 10% Acetic acid (v/v) were also prepared. Using a shallow tray and gels with protein already separated as in the method described in section 2.2.7 but using a

slab gel in place of disc gel, a sandwich of gel/nitrocellulose was made as follows:

Scotch Brite pad
Filter paper
polyacrylamide gel
Nitrocellulose paper
Filter paper
Scotch Brite pad

Two sandwiches were prepared from two gels. The sandwich should be totally submersed in tank buffer, all air bubbles eliminated and the sandwich carefully positioned into the transfer tank such that the nitrocellulose paper was nearest the anode. The transfer was carried out at 40mA overnight (o/n) in LKB Transfer apparatus with the cooling system on.

At the end of the transfer, the nitrocellulose papers were removed and to check the efficiency of the transfer, one was stained in 0.2% Amido black solution for 1-2 min, then destained with a solution of 45% MeOH, 10% Acetic acid. The remainder of the nitrocellulose blot was placed in blocking buffer o/n at 4°C with shaking.

The next day the blocking buffer was removed and the blot washed with several changes of incubation buffer with shaking over a period of 10-15min at room temperature. The blot was then incubated for 90min at 37°C with the antibody in incubation buffer containing 5% inactivated carrier serum. The blot was further washed with 5 changes of incubation buffer, with shaking, over a period of 30min.

Finally the blotwas incubated for 1h at 37°C in ^{125}I-Protein-A solution with shaking (100µl ^{125}I-Protein-A, $3x10^6$cpm in 20ml incubation buffer) and washed with 5 changes of incubation buffer at 37°C with shaking within 30min and then dried between two filter papers. Autoradiography of the blot was then obtained after 6 days exposure at -20°C.

2.5 THE ANTI-ER ANTIBODY AS PROBES FOR OESTROGEN RECEPTOR DETECTION AND DISTRIBUTION

2.5.1 The ZR-75-1 Cell Line

Two cell lines: ZR-75-1 and MCF-7 resemble those human breast tumours in vivo which are ER+ and regress following ovariectomy or oestrogen therapy.

Both cell lines possess specific, high affinity, low capacity receptors for E_2 demonstrable by Scatchard analyses of competitive binding assays performed by the dextran-coated charcoal or protamine sulphate method, and in sucrose density gradients (Brooks et al., 1984; McGuire and De La Garza, 1973; Chamness et al., 1974; Horwitz et al., 1978; Lippman et al., 1976; Lippman et al., 1977).

The MCF-7 cells contain 60-100fmol of cytosol oestrogen receptor per mg cytosol protein with a K_d for E_2 which has been reported by different investigators to range from 0.06-2.5nM (Brooks et al., 1984; Lippman et al., 1976). The ZR-75-1 cells possess approximately 30fmol/mg cytosol protein with a K_d=0.06-2.5nM, same range as MFC-7 cells (Engel et al., 1978).

The ZR-75-1 and MCF-7 cells survive and grow slowly for approximately 5 days under completely serum-free conditions in Eagle's minimal essential medium (MEM). Hence some serum-derived factors are essential to long-term cell survival. Strobl and Lippman, (1978) found that ZR-75-1 and MCF-7 cells can be grown indefinitely in Eagle's minimal essential medium supplemented with 10^{-7}M porcine insulin and 5% calf serum stripped of most endogenous steroid by treatment with DCC. Growth under these conditions is considerably faster than that observed under completely serum-free conditions, yet much slower than that occurring in MEM plus 10% foetal calf serum which is enriched in many growth promoting substances including steroid hormones.

The proliferative effect of oestrogen on the ZR-75-1 cells is

demonstrated upon the addition of as little as 10^{-11}M E_2 to cells in serum-free media, and maximal at 10^{-9}M to 10^{-8}M E_2 (Lippman et al., 1976; Lippman et al., 1977). The ZR-75-1 cells do not grow when E_2 is omitted from the defined medium. Exponential growth is elicited by the addition of E_2 to the medium.

2.5.1.1 Medium and Hormones

The ZR-75-1 cells were grown in Ham's F10/Dulbecco's modification of Eagles medium, 1:1 (v/v) buffered with 20mM HEPES (pH 7.4). The medium was supplemented with L-glutamine 2mM, 0.375g/dm^3 sodium bicarbonate, 10% (v/v) FCS to which insulin (5µg/ml) was added. All medium which was oestrogen free was prepared in the Preparation Unit at the Department of Oncology, Glasgow University.

Progesterone and E_2 stock solutions in ethanol were stored at -20°C until use. Final concentrations in the medium were 10^{-9}M progesterone and 10^{-9}M E_2. The final concentration of ethanol was 0.1% and this concentration had no effect on the growth of the cells (Lippman et al., 1976).

Cultures with or without steroid were seeded at $2x10^4$ cells/16mm well or $4x10^6$ cells/Winchester and maintained at 37°C in a humidified incubator in an atmosphere of 2% CO_2 (v/v) in air.

2.5.1.2 Oestrogen Receptor Measurement

Cytosol was prepared as described below. About 10 million cells were harvested from 2 Winchesters and scraped off the wall with a rubber policeman into TED buffer at 4°C. The cells were washed twice in buffer to remove all the medium present. Four ml of TED was added and the cells homogenised in a glass-glass homogeniser at 0-4°C with 1min interval cooling period. The tube was rotated on ice in order maintain uniform cooling. To make sure that the cells (about 75%) had been fully disrupted, a drop of the homogenate was placed onto a slide and examined under a phase contrast microscope. The high

speed supernatant was obtained by spinning the cell homogenate in a SW50.1 rotor using a L2-65B Beckman ultracentrifuge at the speed of 105,000xg for 60min. Scatchard plot analysis was carried out to check the level of receptor available (section 2.2.1).

2.5.1.3 Oestrogen Receptor Detection by the Immunofluorescence (IF) Technique and In Vitro Translocation Studies

The ZR-75-1 cells were grown on coverslips in 4 x 6 wells multi-well plates in an oestrogen-free medium as prepared in section 2.5.1.1. and maintained for 6 days prior to growth in:

1. E_2 for 1, 24 or 48h.
2. Progesterone for 1,24 or 48h.
3. Charcoal treated, heat inactivated serum (56°C) (section 2.2) for 24 or 48h.

At the end of each growth period, each group of cells was transferred into fresh medium containing 1nM E_2 or 10nM DES and maintained at different temperatures (4, 22, 37°C) for 45-60min as shown in Table 1.

At the end of the incubation period, the medium was discarded, the coverslips washed and fixed in acetone (-20°C) for 30sec and were processed for detection of oestrogen receptor. The receptor is detected using an IF technique which used an antibody to oestrogen receptor and a second antibody conjugated to fluorescein. The technique is described in the section 2.5.1.4.

For the in vitro translocation studies, quantification of extranuclear and nuclear oestrogen receptor in ZR-75-1 was carried out using MPV compact linked to a fluorescence microscope. It could measure the intensity of staining in the extranuclear (E) space and in the nucleus (N). The ratio of nuclear to extranuclear staining (N/E) was taken as a translocation index of the oestrogen receptor in the ZR-75-1 cells.

2.5.1.4 IF Technique Using an Anti-Receptor Antiserum.

After the cells are fixed (Section 2.5.2.2) the coverslips are allowed to dry for 10min at room temperature. The cells are then immersed in PBS, pH=7.2. The cells should not be allowed to dry out. Sufficient normal goat serum (i.e. diluted 1:30 in PBS) to cover the cells is applied to the coverslip. Normal serum from the species supplying the second antibody is always used. This is essential to block non-specific binding.

Prior to application, the primary antibody, the antiserum, was diluted 1:100 in 0.1% BSA in PBS. This pre-absorption with albumin prevents non-specific attachment of immunoglobulins to tissue components. The cells were incubated with the diluted antiserum (100µl) at room temperature for 1h, after which they were rinsed 5x in PBS (3min each rinse) before the second antibody was applied. FITC-conjugated anti-rabbit IgG made in goat was the second antibody. It was diluted 1:50 in PBS. Fifty µl was added onto the slide and incubated for a further 1h at room temperature. The coverslip was rinsed 5 times in PBS and then counterstained with Hoechst 33258 and mounted in Citi-Fluor to prevent fading. The edges of the coverslips were sealed with PBX to prevent drying of the cells and the fluorescence was examined under the U.V. microscope linked to the MPV photometry. The intensity of specific staining for individual cells was characterized qualitatively as not present (-), weak but definitely detectable (+1), strong (+2), and very intense (+3). Quantitatively the intensity of the fluorescence was measured using a suitable window. More than 200 cells were measured. For translocation studies the window was adjusted to fit the size of the nucleus and any area of the extranuclear space. The ratio of the fluorescence intensity of the nuclear (N) to extranuclear (E) area was taken as an index of translocation of oestrogen receptor.

2.5.2 Tissue Preparation for IF Techniques

2.5.2.1 Frozen Sections

Tissues (breast tumour biopsies, human uteri, rat uteri, rat liver, rat spleen) were cut into 0.5cm cubes. The cut tissues were then transfered onto a 1cm square board containing a drop of OCT and the tissue was quickly plunged into a beaker of isopentane surrounded by liquid nitrogen. Frozen sections, 6 μm thick, were kindly prepared by Mr Hector Cairns, Microbiology Department, Western Infirmary, Glasgow and Mrs Sheila Byres, Physiology Department, Glasgow University. The prepared sections were then stored at -20°C for short term use (2-4 days) or at -70°C for a long term use (5-10 days).

2.5.2.2 Fixative for the Frozen Sections

Pilot experiments were conducted to choose a suitable fixative. They showed that the most commonly used fixatives such as acetone (100%) for 30sec at -20°C, acetone:methanol (1:1) for 10min at 4°C, and ethanol (100%) for 10min at 4°C did not destroy the antigenicity to any appreciable degree and were found to give a satisfactory clear staining. Of the three fixation method, the one with acetone gave slightly the better results. Use of unfixed tissues was found to be inadequate as the tissues appeared to become detached from the slides rather easily. Also access of antibody to intra-cellular antigens was impossible due to absence of perforation usually made by the fixatives.

The sections were then assessed by the IF technique (section 2.5.1.1). A few of the slides were processed for haematoxylin/eosin staining.

2.5.2.3 An *In Vitro* Oestrogen Receptor Translocation study in Human Uterine Frozen Sections

Method 1

A portion of the uterus was frozen in dry ice for biochemical estimation of oestrogen receptors. The rest of the tissue was cut into pieces of approximately equal size (0.3cm cube) and each piece was put in a vial containing 1ml of either plain PBS or PBS containing 1.0nM of E_2, DES, or progesterone. The incubation lasted for 1h. At the end of the incubation period the liquid was drained from the vial, and the tissues were frozen in liquid nitrogen and treated the same way as in section 2.5.2.1 and stored at -70°C until use.

Method 2

Frozen sections which had been air-dried for 10 min were used. Two slides, each with two sections, were processed without exposure to steroid to determine the native distribution and intensity of IF in the tissue (unincubated control). Four sections were treated with plain PBS or PBS containing one of the following ligands: E_2, DES, or progesterone at 1.0nM concentration. To expose the cells in the unfixed frozen sections to steroid solutions without any appreciable loss of cytoplasmic proteins, the following steps were carefully performed. To the air-dried section, 20µl of the ligand solution or plain PBS were added to cover the sections. All the liquid was immediately aspirated back into the pipette tip leaving only a thin layer of the ligand solution on top of the frozen section. The immediate draining of the excess liquid from the air-dried section was important to prevent the loss of cells which may become detached from the glass if too much liquid was left. The slides were transferred to a moist chamber kept at 37°C, and incubated for 1h. The sections were then fixed in methanol:acetone and processed as usual for IF studies. The sections were examined for the intensity and intracellular distributions of IF in cells.

2.5.3 Fine-needle Aspirates

Cell smears from needle aspirates or body fluids were performed by Mr. Derek Crawford, Department of Surgery, Western Infirmary, Glasgow. They were transported to the laboratory. The smears were air-dried for 10min and then fixed with methanol/acetone (1:1) at 4°C for another 10min. The fixed slides were either kept at -70°C or immediately processed. For all patients from whom FNA were obtained, biopsy material was taken from the tumour during surgery and used for the standard laboratory assay (DCC method) of oestrogen receptor.

2.5.4 Experimental Controls

Specific staining is defined as staining that was present in oestrogen positive tissues when the antibody to oestrogen receptor was used alone but the staining was absent when the antibody was replaced with normal rabbit IgG or pre-adsorbed with oestrogen receptor-containing cytosol.

To check the specificity of the staining, various controls were set up. The cells on coverslips as prepared earlier (section 2.5.1.1) were treated as follows.

(A) Reagent Controls

The acetone fixed ZR-75-1 cells were:

(1) Treated with the serum obtained from the rabbit prior to immunization (pre-immune serum) and diluted 100-fold in PBS and used in place of the anti-receptor anti-serum.

(2) Treated with FITC-conjugated goat anti-rabbit IgG, omitting the primary antiserum in order to assess the non-specific adherence of this reagent.

(3) Treated with antiserum preadsorbed for 16h at 4°C with

cytosol containing oestrogen receptor (>500fmol/mg protein) and used in place of antiserum.

(4) Treated with antiserum preadsorbed for 16h at 4°C with negative cytosol pooled from oestrogen receptor negative breast cancer biopsies and

(5) Treated with antiserum preadsorbed for 16h at 4°C with normal rabbit serum diluted 100-fold and used in place of antiserum.

(B) Tissue Control

(1) Oestrogen Receptor-Negative Tissue

Tissues, known to lack oestrogen receptors were pre-checked by the DCC assay to confirm the lack of oestrogen receptors and used as negative controls. Frozen sections of rat spleen and human mammary fibroadenoma (biochemically oestrogen receptor negative) were used.

(2) Oestrogen Receptor-Positive Tissue

In addition to human uterus, rat uterus and human breast tumour tissues rich in oestrogen receptors were also tested.

3.1 PURIFICATION OF OESTROGEN RECEPTOR

A variety of methods to purify oestrogen receptors were tried. This includes disc PAGE, DNA cellulose, DEAE sepharose and phenyl sepharose chromatography.

3.1.1 Purification of Oestrogen Receptor by Disc-PAGE

Using disc-PAGE continuous Tris-borate-EDTA buffer system on human uterine cytosol two peaks A and B were observed (Figure 10). Peak A appeared at 240,000 molecular weight while peak B 65,000. Cytosol prepared from ER+ human breast tumours yields a single peak which had a migration equivalent to peak A (Figures 11a and b). The sections under the peak were taken, ground to a fine powder and used as an antigen. However, antibody raising using this technique of purification was not successful.

3.1.2 Purification of Oestrogen Receptor by Chromatography Technique

A single, sharp peak of specific binding to E_2 was obtained by using the DNA cellulose column, eluted with 0.2M KCl (Figure 12). The binding activity of the pool fractions under the peak was 6-9 times greater than that of the starting material. However, a peak of specific binding to E_2 was also obtained by using a DEAE sepharose column, eluted with 0.13M KCl (Figure 13). One peak of specific binding to E_2 was obtained by using phenyl sepharose columns eluted with TED:ethylene glycol:glycerol (30:40:30) (Figure 14).

A summary of the extent of purification after each step is shown in Table 1. The procedures involved in the purification were rather complicated and the yield was very low. The purification fold was only 258 times therefore a better technique of purification was sought.

Figure 10: PAGE analysis of oestrogen receptor from human uterine cytosol using Tris-borate-EDTA continuous buffer system. Cytosol was prepared from human uterine tissue (section 2.2.2.1) and then incubated with 10nM [^{3}H]E_2 alone (o) or in the presence of a 100-fold excess DES (•). After removal of unbound steroid (section 2.2.2.5), 150µg of cytosol protein was analysed by the PAGE. Known molecular weight proteins were run in separate gel. Electrophoresis was carried out at 2mA/gel (4 C). At the end of the electrophoresis, the gels were cut into 2.5mm thick slice and radioactivity in each slice was counted. Gel with protein markers was stained and destained as described in section (2.2.9.3) The arrows indicate the position of molecular weight proteins. Two peaks were observed and designated as peak A and peak B. Peak A appeared in the region of 240,000 molecular weight protein while peak B in the 65,000, just slightly below 68,000 molecular weight protein. This experiment revealed the presence of two types of oestrogen receptor in this particular sample, a higher molecular weight and a lower molecular weight species.

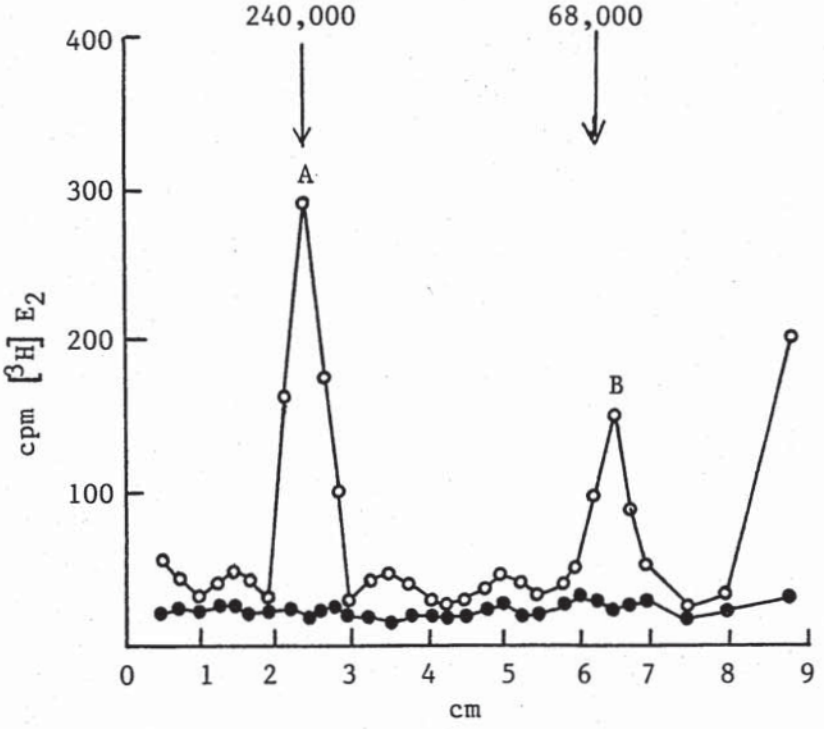
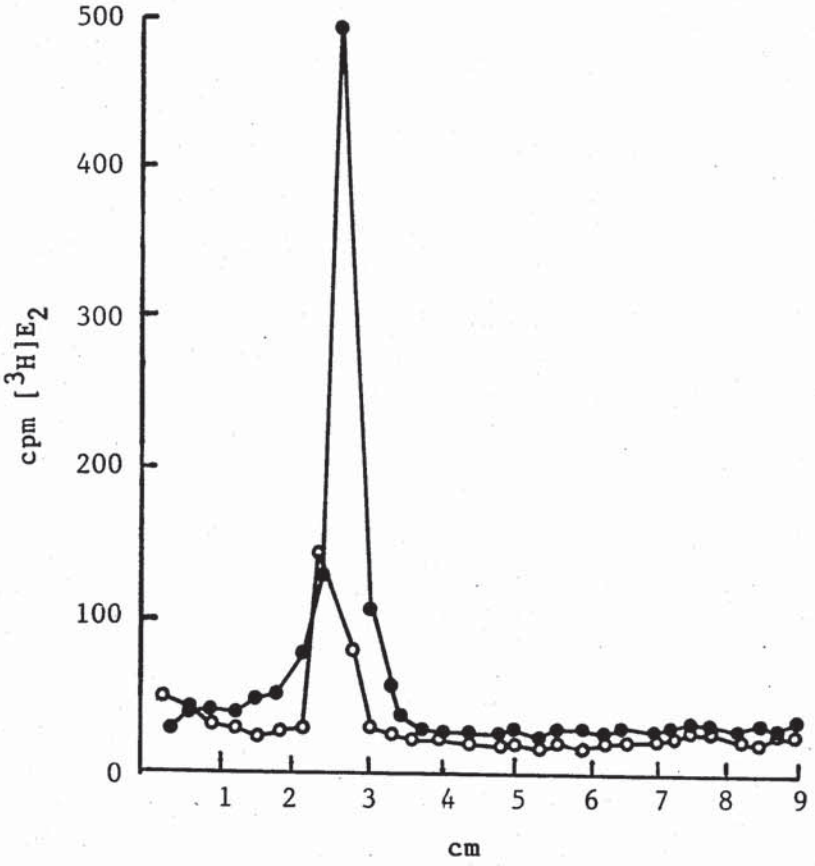

240,000
68,000
A
B
400
300
200
100
cpm [3H] E2
0
1
2
3
4
5
6
7
8
9
cm

Figure 11: PAGE of oestrogen receptor from the cytosol of ER+ breast cancer biopsy. The receptors were either labelled with $[^3H]E_2$ (o) or $[^3H]E_2$ + DES a 100 fold excess (•). Electrophoresis was carried out at 2mA/gel. At the end of the electrophoresis, the gels were cut into 2.5mm thick slices and radioactivity in each slice was counted. The portion of each gel corresponding to the radioactive peak was cut out, pooled, frozen, ground to a fine powder and injected into an experimental animal for antibody production.

(a) female patient with breast cancer
(b) male patient with breast cancer.

Only one peak was observed from this sample of ER+ breast cancer biopsy. The peak corresponds to peak A of Figure 10 thus with a molecular weight of 240,000. It was the protein that was used as an antigen. Calculation based on this molecular weight from 100fmol receptor gave only 24ng protein.

(a)



(b)

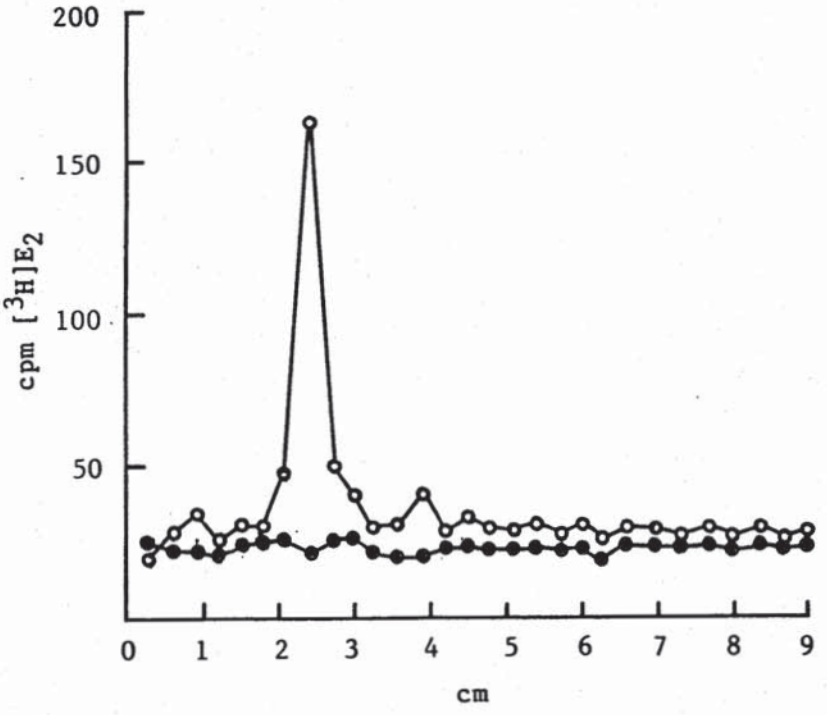


Figure 12: DNA cellulose elution profile of human myometrial cytosol. Aliquots of cytosol labelled at 4°C with 10nM [^{3}H]E_2 in the absence (o) and presence (•) of DES a 100 fold, were heated to 30°C for 30 min and applied to DNA cellulose column. Two ml fractions were collected and 100µl from each fraction was counted for radioactivity. The peak of specific binding to E_2 was obtained by 0.2M KCl elution. The fractions under the peak were pooled, protein concentration was determined (Bradford, 1976) and applied onto DEAE sepharose column. The purification using DNA cellulose gave only 80 purification fold and resulted in significant loss of receptor protein.

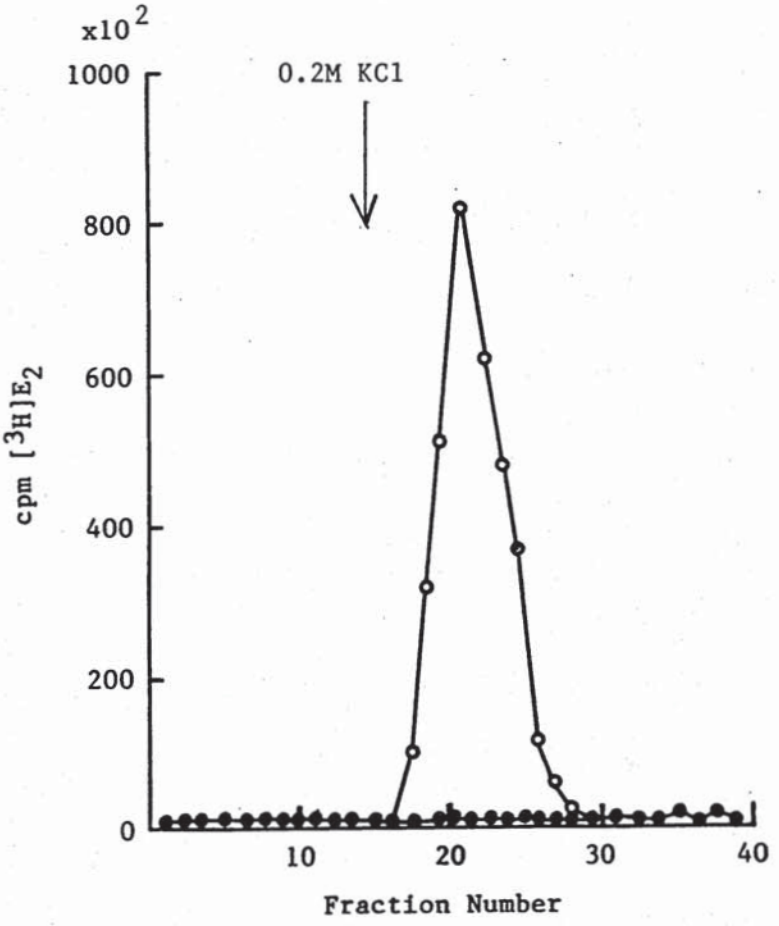

x10 2
1000
800
600
400
200
0
0.2M KCl
cpm [3H]E2
10
20
30
40
Fraction Number

Figure 13: DEAE Sepharose elution profile of human myometrial oestrogen receptor. The fractions from the DNA cellulose purified receptor preparation were dialysed against 0.01M phosphate buffer and applied onto a DEAE sepharose column. After the sample had run into the sepharose, the column was washed with TED. The bound receptor was then eluted with TED containing 0.13M KCl. Radioactivity from each 2ml fraction was counted. Fractions 13-22 were pooled, the protein concentration assayed using the method of Bradford (1976) and further purified. This step of purification gave 105 purification fold and 28% yield.

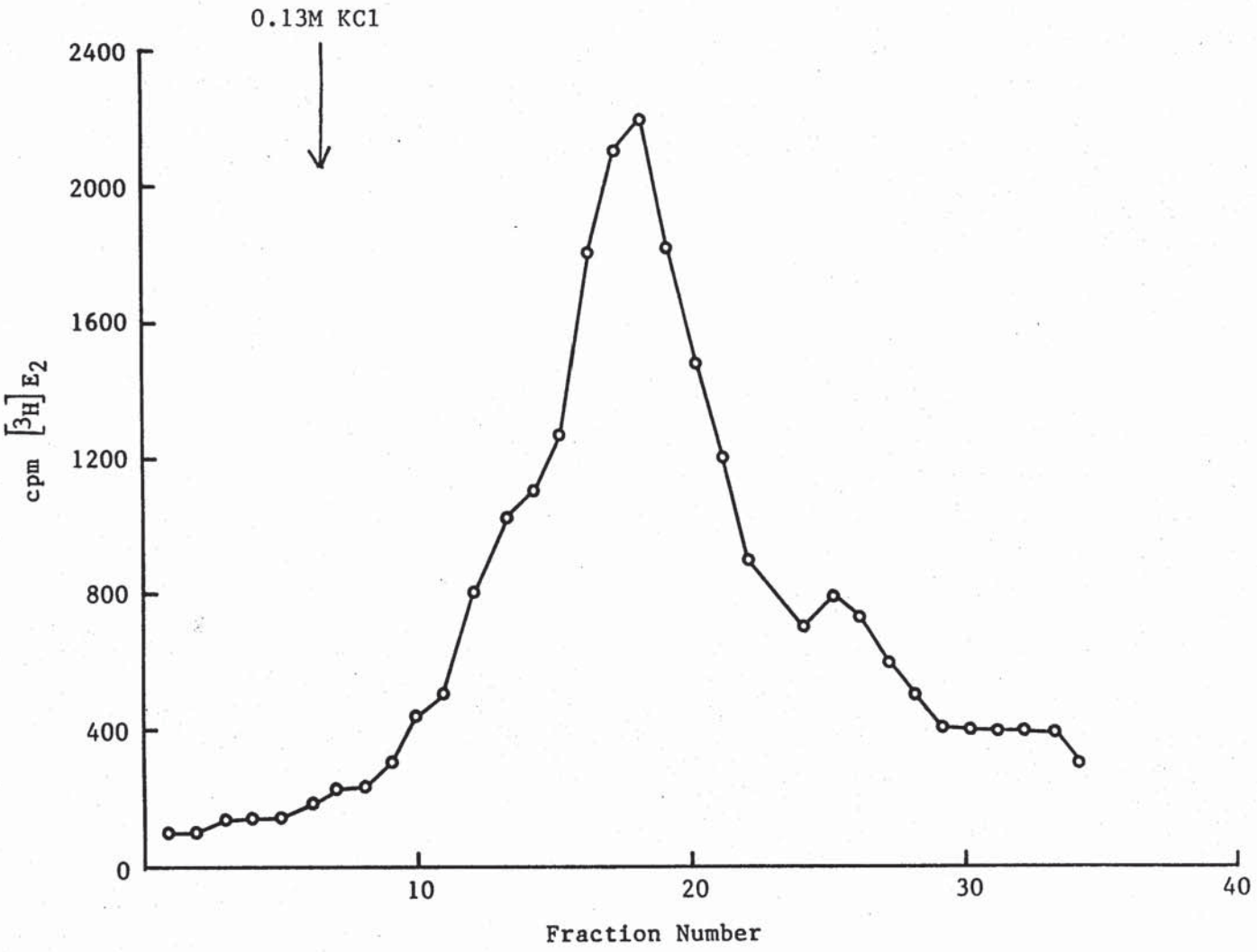

0.13M KCl
2400
2000
1600
1200
800
400
0
cpm [3H]E2
10
20
30
40
Fraction Number

Figure 14: Phenyl Sepharose elution profile of human myometrial oestrogen receptor. The purified receptor preparation from the DEAE sepharose column was applied directly to a phenyl sepharose column. After the sample had run into the gel, the column was washed with TED. Radioactivity (100µl) and protein content were determined from the two ml fractions collected. A single radiolabelled protein peak was eluted using ethylene glycol:TED:glycerol (40:30:30). However only 258 purification fold was obtained with only 9.7% recovery.

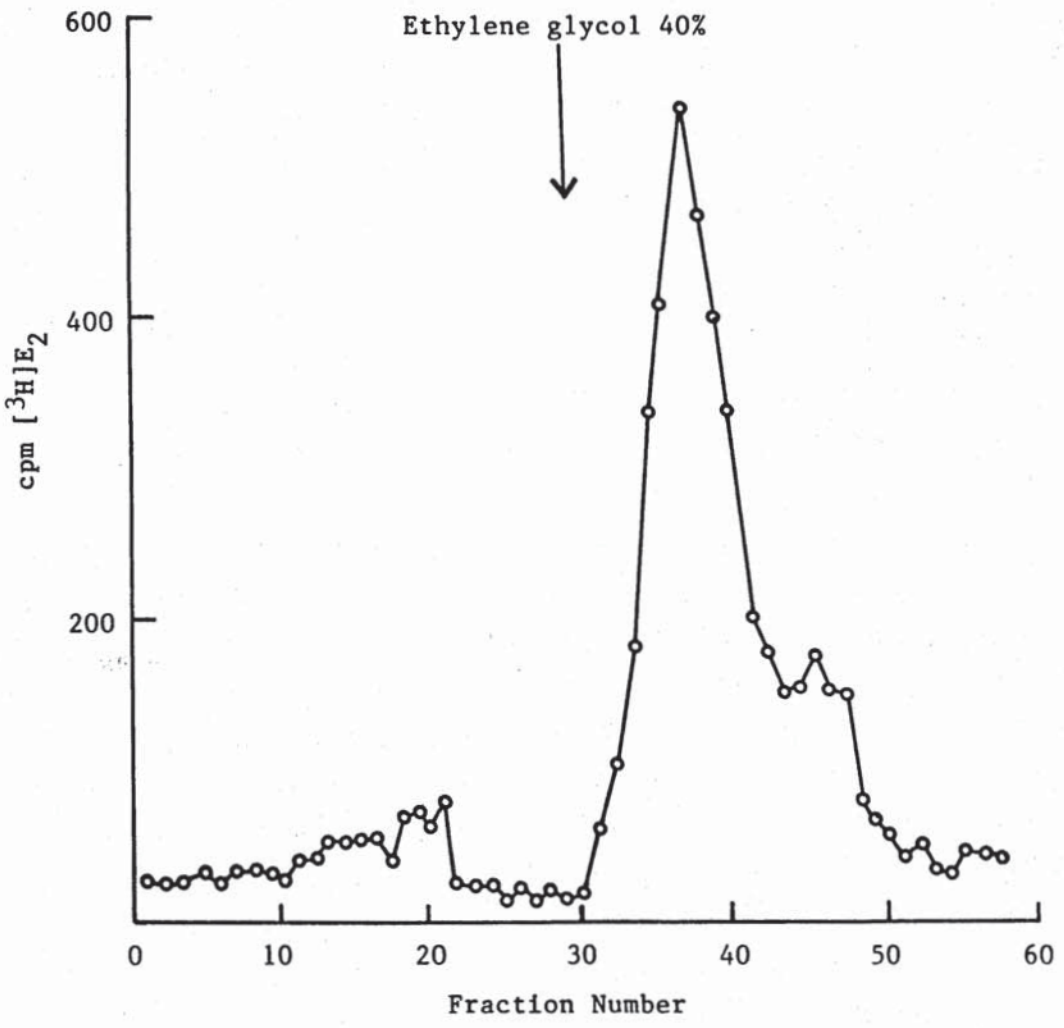

Ethylene glycol 40%
600
400
200
cpm [3H]E2
0
10
20
30
40
50
60
Fraction Number

Table 1: Extent of Purification of Oestrogen Receptor Using Chromatography Technique

Method of purification	Total receptor* (ng)	Total Protein (mg)	$\frac{\mu g \text{ receptor}}{\mu g \text{ protein}} \times 100$	Purification fold	Recovery (%)
Cytosol	1040.0	400.0	0.26×10^{-3}	1	100
DNA Cellulose	416.0	2.0	20.8×10^{-3}	80	40
DEAE Sepharose	300.0	1.1	27.3×10^{-3}	105	28
Phenyl Sepharose	100.5	0.15	67.0×10^{-3}	258	9.7

* Assuming oestrogen receptor molecular weight 65,000

3.2 AFFINITY CHROMATOGRAPHY

3.2.1 Selectivity of the Oestradiol-hemisuccinate-Albumin-Sepharose 4B Column

As shown in Table 2, an average of 81.2% of the receptor in the initial fractions was adsorbed by the column. The affinity column was clearly very selective since the protein concentration of the flow-through remained essentially unchanged.

Since the affinity resin material used here was known to bind oestrogen receptor non-specifically, this possibility was eliminated in this study by including 10μM E_2 or DES in the tissue cytosol. Progesterone at 10μM had no effect on the binding of oestrogen receptor to this affinity column (Table 2 and Figure 18)

3.2.2 Elution of Receptor

The washing procedure used to remove the noncovalently bound E_2 from the affinity column include the use of organic solvent. The column was washed with 2000 to 3000ml of 80% methanol over a period of 12 to 15h at 24°C. After this washing procedure, a sample (1ml of gel) of the affinity column was removed to determine whether any free or loosely bound E_2 remained. This sample was incubated with mature rat uterine cytosol. This incubated cytosol was then extracted with ether. This extract was assayed for free E_2 (Figure 15).

Next the conditions for the elution of receptor from the affinity resin were defined. Weakly adsorbed protein was removed most effectively by washing with 0.4M KCl. Several combinations of washing buffer were attempted in trying to obtain the best recovery and purification. The following washing steps were performed for the best results: $TEDK_{0.01}$, $TEDK_{0.4}$, $TEDK_{1.0}$, $TEDK_{0.4}$ and $TEDK_{0.01}$ (Figure 16). Washing with $TEDK_{1.0}$ helped to reduce washing volume and time. Increasing the final yield of receptor by adding 0.5M sodium thiocyanate into the 10μM DES elution buffer was not very effective but 0.5M sodium thiocyanate and 10% dimethylformamide

Table 2: Selectivity of the Oestradiol-17β 17-Hemisuccinyl-Albumin-Sepharose 4B Affinity Resin

Preparations	Column efficiency (%)
Cytosol of human uterus	81.2 ± 3.3(10)
Cytosol + diethylstilboestrol (10^{-6}M)	11.2 ± 1.1(5)
Cytosol + oestradiol-17β (10^{-6}M)	10.5 ± 1.3(5)
Cytosol + progesterone (10^{-6}M)	85.0 ± 4.0(3)
Cytosol + Sepharose 4B	9.5 ± 1.0(3)

Figure 15: Detection of free E_2 in the eluate from the E_2 -Succ-Alb-Sepharose column. Detection of free E_2 from the affinity resin was carried out as described in section 2.2.7.2. The etherial extract of cytosol not incubated with affinity resin was incubated with fresh rat uterine cytosol. The E_2 binding capacity was measured. This is considered as 100% (I) The E_2 binding capacities were measured in various etherial extracts:

(II) affinity column flow-through was incubated with fresh rat uterine cytosol and the E_2 binding capacity was measured.

(III) affinity column eluate, after passing through a Sephadex G-25 column was incubated with fresh rat uterine cytosol and the E_2 binding capacity was measured.

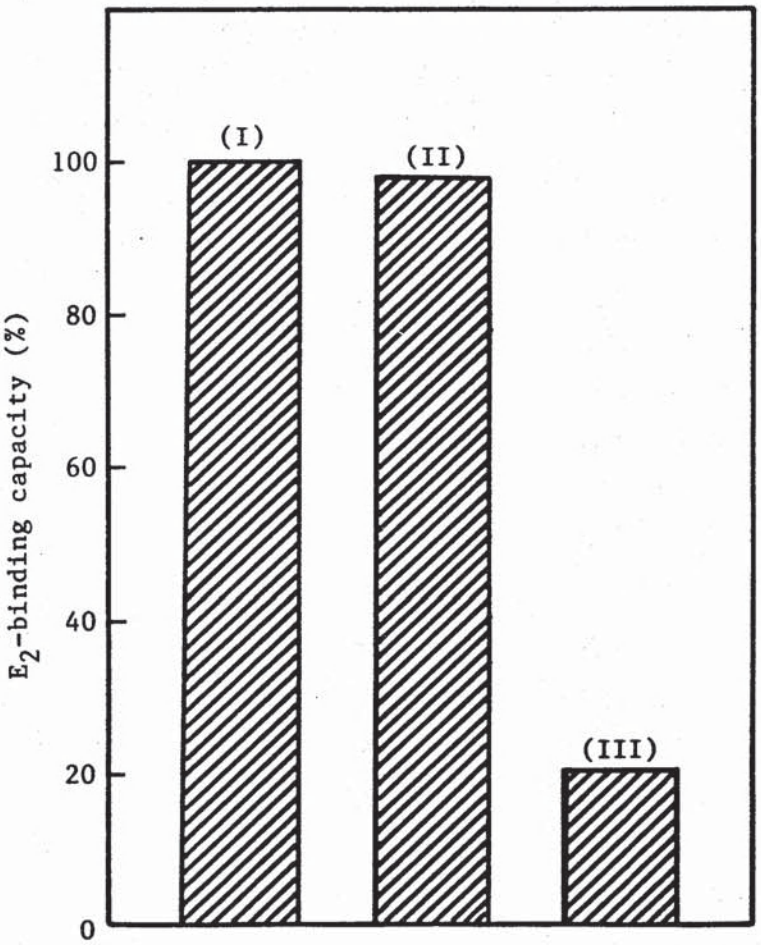
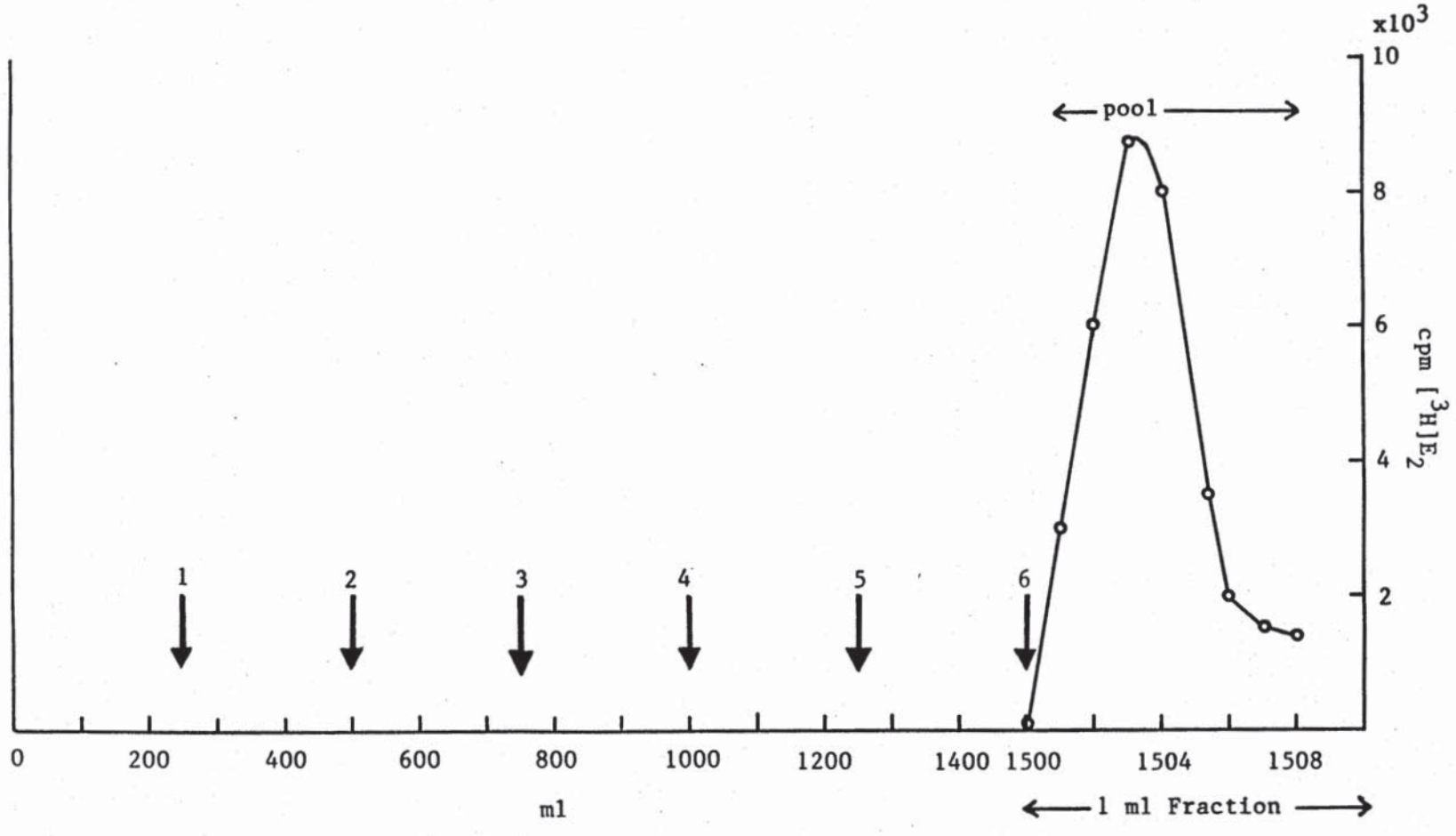

(I)
(II)
(III)
100
80
60
40
20
0
E_2-binding capacity (%)

Figure 16: Elution profile of the human myometrial oestrogen receptor preparation. The semipurified ammonium sulphate precipitate receptor as prepared in section 2.2.7.3.1 of materials and methods was passed through a 4ml affinity column. In this pilot experiment the bound receptor was eluted with E2 and not DES. The purpose was to detect the presence of the E_2 binding protein. The elution was done after a cycle of washings with different concentration of KCl in TED. The numbered arrows indicate the fractions where changes in the washing cycle occurred. The washing sequence is as follow:

1. start 0.01M wash at approximately 60ml/h;
2. 0.4M wash;
3. 1.0M wash;
4. 0.4M wash;
5. 0.01M wash;
6. 0.4M with elution buffer containing 10^{-6}M E_2. One ml fraction was collected and the protein bound E_2 was separated from free E_2 by gel filtration technique (section 2.2.8).


x10^3
10
8
6
4
2
cpm [3H]E2
pool
1
2
3
4
5
6
0
200
400
600
800
1000
1200
1400
1500
1504
1508
ml
1 ml Fraction

(DMF) seemed to be effective (Greene et al., 1980). Eluting at 30°C for 20min or 4°C for 16h was found to have no significant difference in the yield (Table 3). Only 6-8% of the activity initially added to the column can be recovered by the DES elution procedure, but the application of 10% DMF increased the yield (Table 3).

3.2.3 Purification

Purification was performed with a column containing 3ml of the resin. About 80% of the oestradiol-binding activity of a supernatant was adsorbed onto the column. Elution was carried out by using the basic procedure as described in section 3.2.2. The purification achieved in one experiment was about 46,000-fold (Table 4).

3.2.4 Characterization of Receptor Protein Eluted from Affinity Column

Typical electrophoresis patterns of oestrogen receptor and of reference proteins run in parallel are shown in Figures 17 and 18. A single band of receptor protein was observed, which was positioned slightly below the BSA. By interpolation in the semilogrithmic plot of molecular weights of reference proteins against their R_f values, an apparent molecular weight of 64,400±600 (S.D) is found for the denatured oestrogen receptor.

When the purified receptor was analysed by SDG it was found to have a sedimentation coefficient of about 4S, essentially the same as that of the native receptor in crude preparation (Figure 19). The oestrogen receptor purified by the affinity chromatography procedures described here thus appears to be the same macromolecular species as in the crude homogenate.

Experiments were performed to estimate the binding activity of the purified oestrogen receptor for E_2 (Figure 20). The free and the macromolecular bound E_2 components are separated by filtration on Sephadex G-25 at 4°C. The protein-bound hormone emerged in the first macromolecular peak in coincidence with Vo.

Table 3: The Effect of Temperature and Dimethylformamide on the elution of Bound Receptor from the Affinity Column

Column Number	Elution Condition		Yield	
	Time (min)	Temperature (^{o}C)	Without DMF (%)	With DMF (%)
1	960	4	8.1±0.7(3)	19.1±1.8(5)
2	20	30	6.4±0.5(3)	17.8±1.2(3)

Table 4: Extent of Purification of Human Myometrial Oestrogen Receptor using Affinity Chromatography

Purification Step	Total Protein (mg)	Total Receptor Sites ($x10^{-6}$ dpm)	Specific Activity* (dpm/mg protein)	Yield (%)	Purification Fold
Cytosol	2173.00	104.40	4.80×10^4	100	1
Ammonium Sulphate Precifitation	73.10	39.90	5.47×10^5	38	11
Affinity Elute	0.01	22.00	2.20×10^9	21	46,000

Maximum specific activity is estimated to be $3.4x10^9$ dpm/mg protein on the basis of molecular weight 65,000. Based on this figure the receptor is about 65% pure.

* calculated on the basis of competition assays using $^3H\ E_2$ (S.A. 101 Ci/mmol)

Figure 17: SDS-PAGE of purified oestrogen receptor. The single protein band on this electrophoresis is evidence that oestrogen receptor has been purified to at least 60% homogeneity.

Lane 1: A protein standard, BSA molecular weight 68,000
Lane 2: A semipurified receptor preparation. Treatment with 25% ammonium sulphate precipitation as in section 2.2.7.3.1.
Lane 3: Oestrogen receptor purified by affinity chromatography.

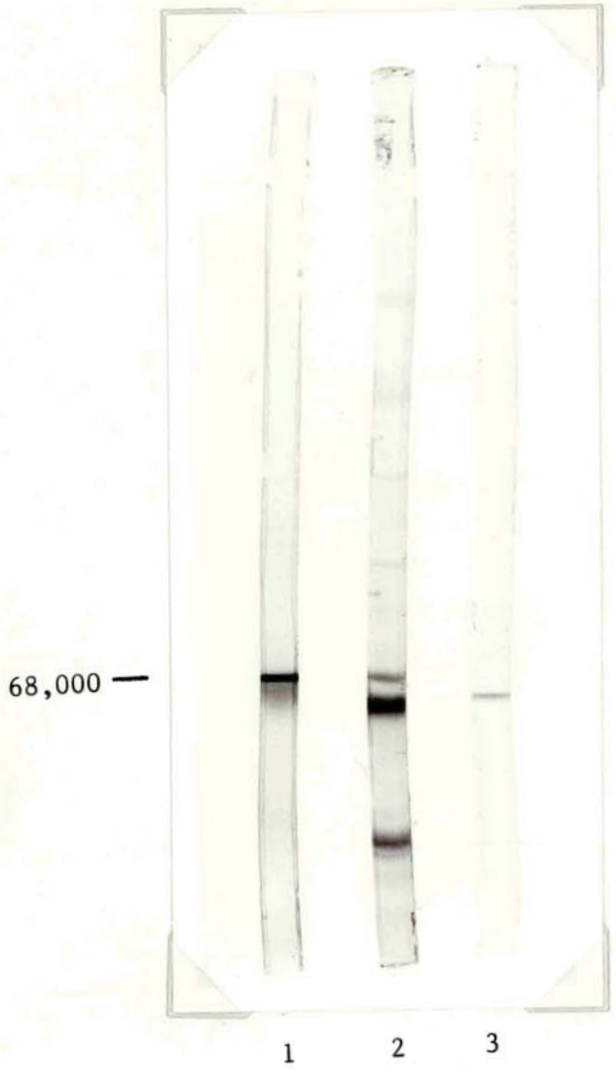

68,000
1
2
3

Figure 18: Determination of the capacity and selectivity of E_2-Succ-Alb-Sepharose for oestrogen receptor. Fifty ml of semipurified human myometrial oestrogen receptor was incubated with 10μM E_2 overnight at 4°C. It was then applied to a 2 ml affinity column. One 50ml fraction of ammonium sulphate treated cytosol was incubated overnight with 10μM progesterone and was passed through another column containing the same amount of affinity resin. After washing with 250ml $TEDK_{0.4}$ the affinity column was eluted with 10μM DES in $TEDK_{0.4}$, 0.5M NaSCN and 10%DMF. The eluate was subjected to 5% SDS-PAGE. Only the column with progesterone treated cytosol shows the presence of 65,000 molecular weight protein.

Lane 1. E_2 treated cytosol,
Lane 2. reference proteins, 94,000, 67,000, 43,000, 30,000 and 20,000 molecular weight,
Lane 3. progesterone treated cytosol.

The effect of washing is also evident in this experiment. Lane 3 shows protein eluted from a column which was washed with only $TEDK_{0.4}$ The presence of higher molecular weight proteins was clearly observed. But when a complete washing sequence (as in Figure 16) was carried out, no other protein (Figure 17) was found. This indicates that these proteins have no affinity towards E_2

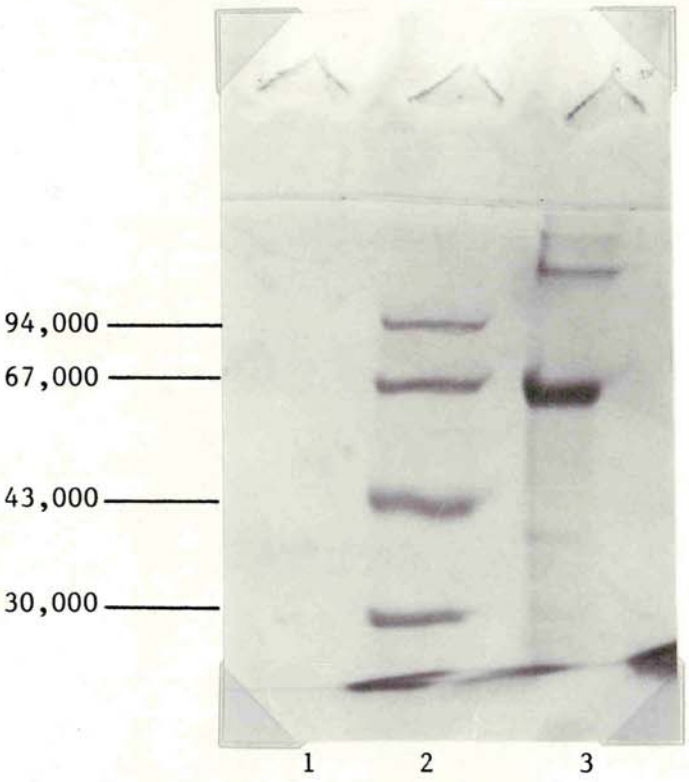

94,000
67,000
43,000
30,000
1
2
3

Figure 19: Sucrose density gradient profile of the affinity gel eluate. Gradients of 5-20% sucrose were made in $TEDK_{0.4}$ Purified receptor (200µl) was layered on a 5-20% sucrose gradient and centrifuged at 230,000xg for 16h at 4°C in a SW50.1 rotor using an L2-65B Beckman ultracentrifuge. The gradient were fractionated at 4°C and each fraction was assayed for radioactivity. The arrow indicates the sedimentation of [^{14}C] BSA (4.6S).

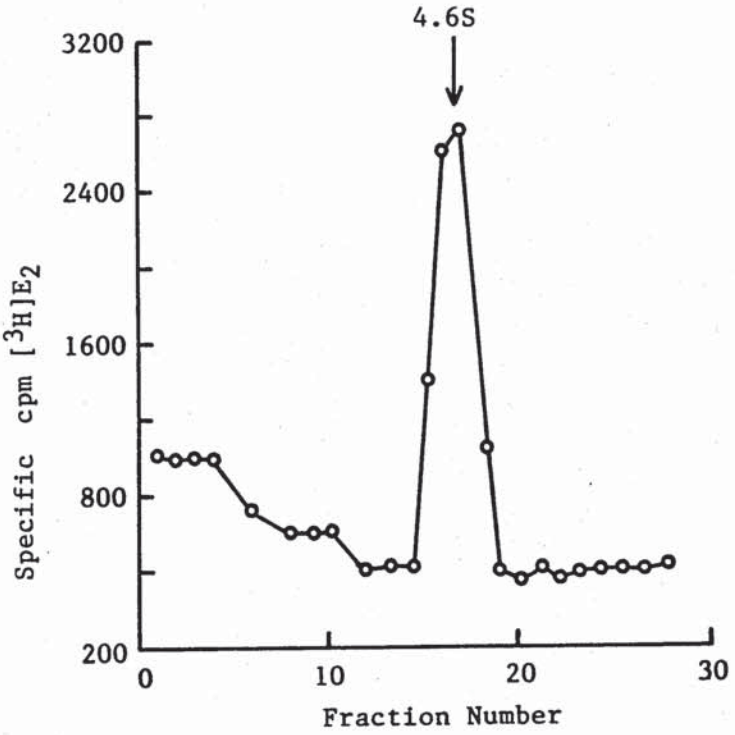
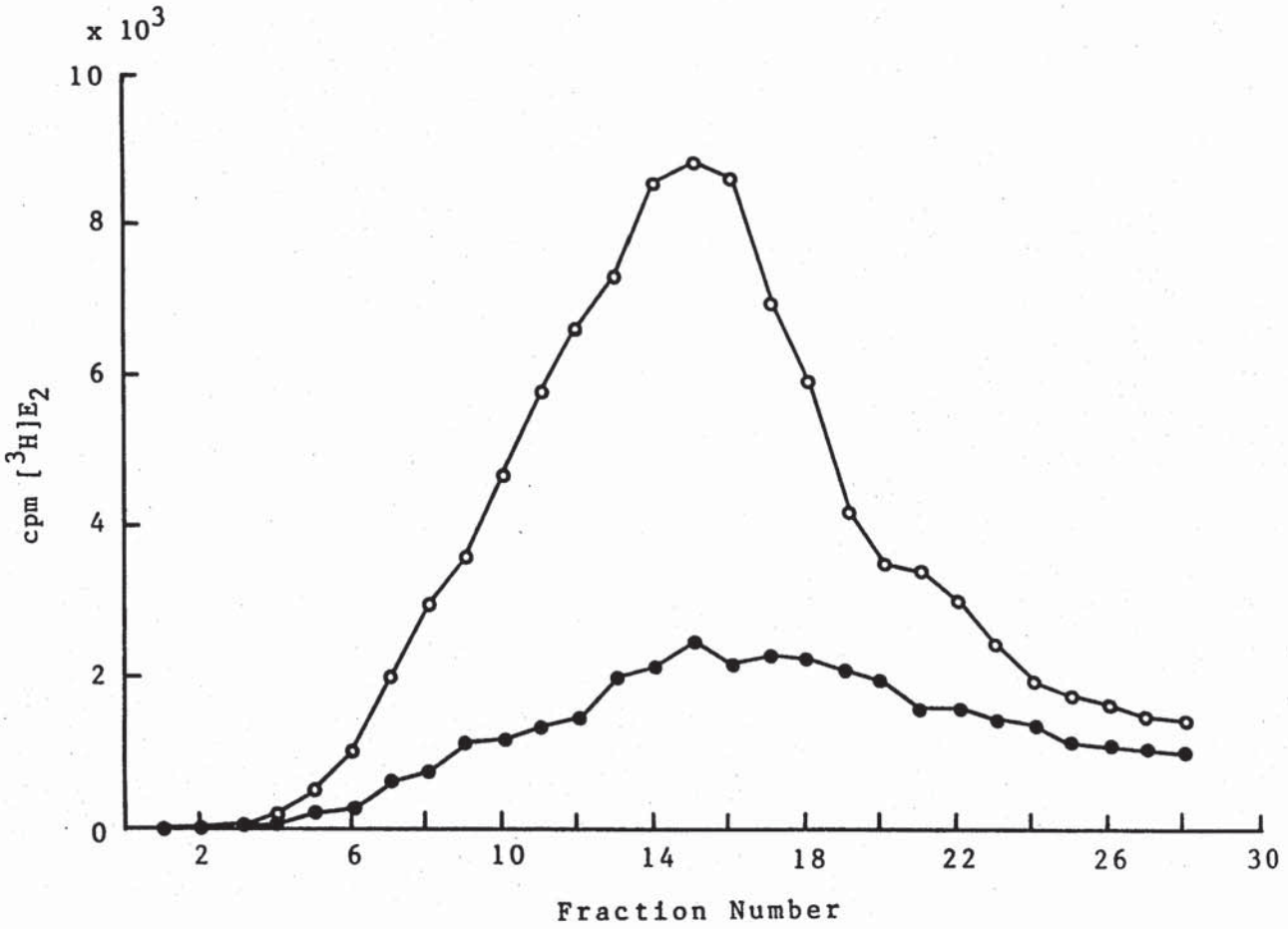

4.6S
3200
2400
1600
800
200
Specific cpm [3H]E2
0
10
20
30
Fraction Number

Figure 20: Effect of heating at 65°C on E_2-binding activity of oestrogen receptor. The affinity chromatography eluate was heated for 5 min in a 65°C water bath. The samples were quickly cooled to 4°C and incubated with 10nM [^{3}H]E_2. Bound E_2 was separated from free by standard gel filteration on Sephadex G-25 at 4°C (●). The elution profile of unheated oestrogen receptor but incubated with 10nM is shown (o).


x 10^3
10
8
6
4
2
0
cpm [3H]E2
2
6
10
14
18
22
26
30
Fraction Number

The oestradiol-binding proteins eluted from the affinity column lose their oestradiol-binding activity if they are heated for 5min at 65°C. The affinity binding activity characteristic of oestrogen receptors is known to be destroyed by such treatment, whereas nonspecific E_2 binding is actually increased by such heating (Figure 20).

3.3 ANTISERA

3.3.1 Titre

Of three rabbits receiving a primary immunization of 20-30µg of purified oestrogen receptor and 6 consecutive booster injections each about 10µg per animal, only 1 animal showed a reasonable level of response to the antigen. Figure 21 shows that the antibody titre of rabbit number 13 reached a plateau between 4 to 8 months after the primary immunization. The first significant titre was observed 3 months after the primary immunization. The titres fluctuated with time and were quite low, which could be due to the small amount of antigen injected during each booster.

3.3.2 Crossreactivity of Antisera

The antisera obtained from the early bleeds were screened using ELIZA where a rough guide to the titre was obtained. The antigen used were crude cytosol, semipurified ammonium sulphate precipitation and purified oestrogen receptor from human myometrium. Several cytosolic preparations known to contain oestrogen receptor have been checked for crossreactivity with the antiserum (Table 5). The antisera showed crossreactivity with several preparations from different species including rat and mouse uterine cytosols, ZR-75-1 and ER+ human breast cancer cytosols, Tissues known to lack oestrogen receptor were used as control. These included cytosol of skeletal muscle (4fmol/mg protein) and cytosol from ER- breast cancer biopsies.

Figure 21: Titres of antisera of 3 rabbits [(●) 002, (o) 028, (□) 13] after a primary immunization and subsequent booster injection. The method of titre determination is described in section 2.4.2.1. of materials and methods.

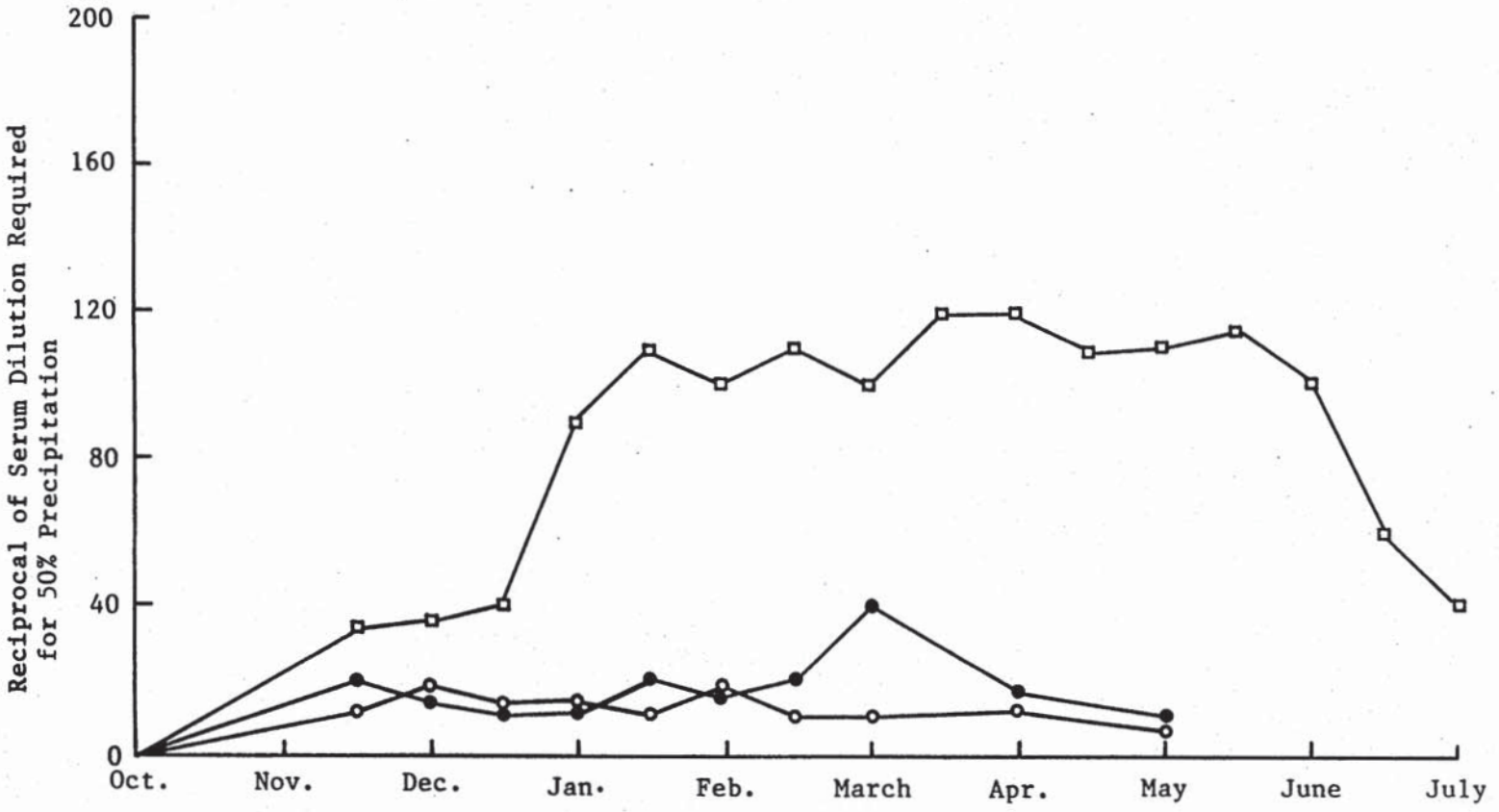

Reciprocal of Serum Dilution Required for 50% Precipitation
200
160
120
80
40
0
Oct.
Nov.
Dec.
Jan.
Feb.
March
Apr.
May
June
July

Table 5: Crossreactivity of the Antisera to Oestrogen Receptor from Various Species

Antibody	ZR-75-1	Human uterus	Human breast	Rat uterus	Mouse uterus
Rabbit antiserum	+	+	+	+	+

3.3.3 Purification of Antisera

The purification of the serum used an ion-exchanger from which the IgG was eluted by 40mM NaCl in phosphate buffer in a single asymmetric peak (Figure 22) and impurities are bound and retarded by the exchanger. The early fractions were the purest and so pool I was aliquoted into small tubes and stored at -20°C. Pool II was discarded. The protein obtained was calculated using absorbance at 280nm and was found to be 5mg/ml serum.

3.4 SPECIFICITY OF ANTISERUM

3.4.1 Double Immunoprecipitation Assay

The antisera were screened by an ELISA solid phase assay for a rough guide to the titre. However a more specific titre was determined using the double immunoprecipitation assay (Figure 23). The precipitation of the immuno-oestrogen receptor comolex could indicate the specificity of the antisera toward oestrogen receptor. The specificity of the antisera was further analysed using SDG and Western blotting techniques.

3.4.2 Sucrose Density Gradients

Sedimentation analysis of the oestrogen binding protein showed a single radioactive peak sedimenting at about 4S [mean value±SE (n=4) = 4.25±0.5] for high salt and at about 8S [7.9±0.4S] for low salt. The radioactivity was completely displaced by a 100-fold excess of unlabelled DES.

The antigen-antibody reaction was seen through the shift of sedimentation peak which was measured according to the internal markers [^{14}C] human-γ-globulin (7.1S) and [^{14}C] BSA. Figure 24 shows the clear separation of 4.6S and 7.1S markers which sediment in approximately a linear fashion. The sedimentation value of oestrogen receptor in low salt was affected by the antibody. In the presence of antibody the sedimentation value shifted from 8S to about 10S (Figure 25). In high salt the oestrogen receptor

Figure 22: Isolation of IgG from rabbit serum on DEAE ion exchanger. The precipitated protein (40% ammonium sulphate precipitation) was resuspended in 15ml water, dialysed in 10mM phosphate buffer, applied onto the column and eluted with 100ml of 10mM phosphate buffer containing 40mM NaCl. The early fractions under the peak (I) are the purest and the fraction in the tail region (II) was discarded. The protein was measured by the absorbance at 280nm.

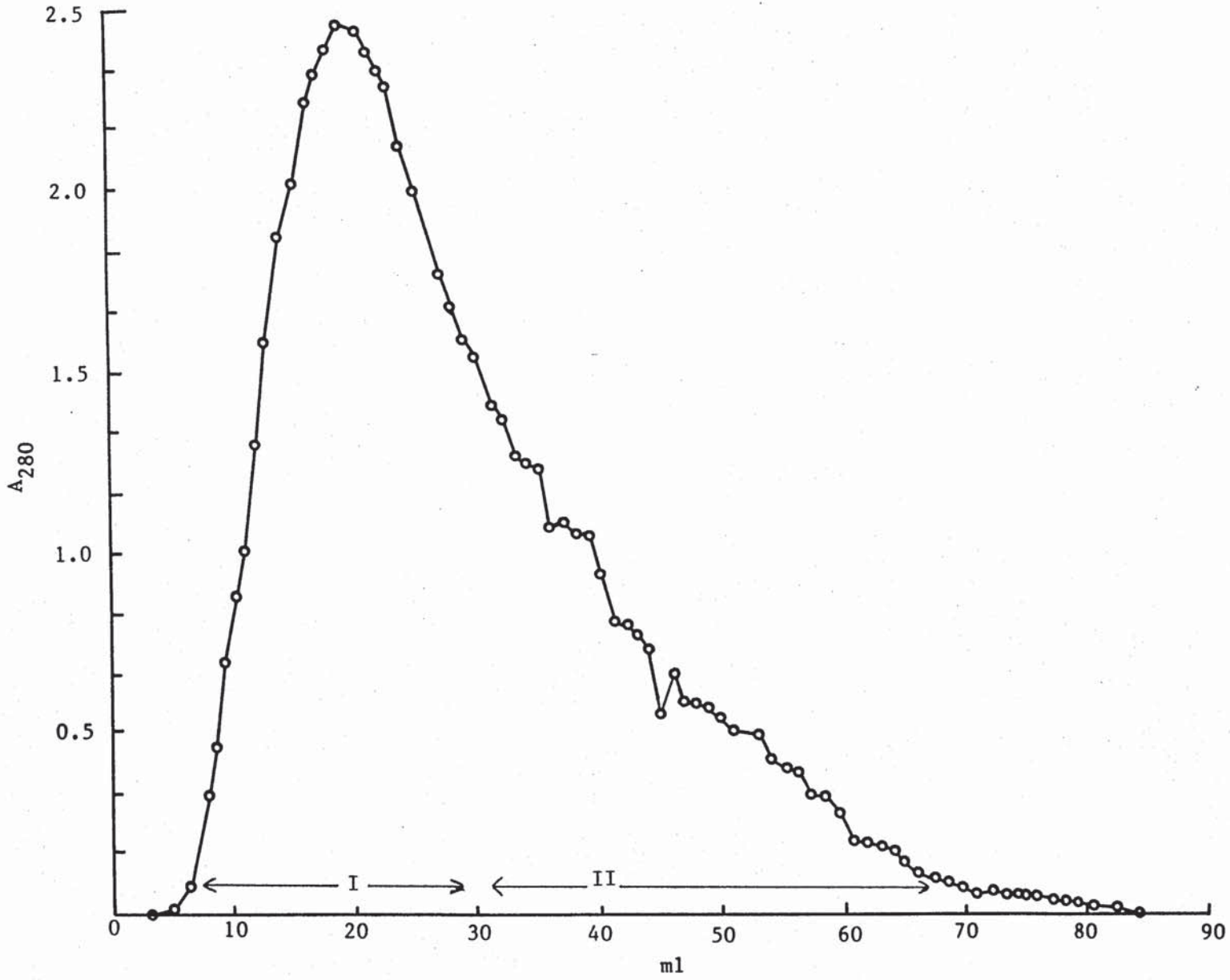

2.5
2.0
1.5
1.0
0.5
A_{280}
I
II
0
10
20
30
40
50
60
70
80
90
ml

Figure 23: The titre of March antiserum from rabbit number 13 using an immunoprecipitation method. The procedure was carried out as described in section 2.4.2.1. Specific binding was plotted on the y-axis using the following calculation

$$\frac{(B - N)}{(T - N)} \times 100$$

where:

N = non-specifically bound [^{3}H]ER in the normal rabbit IgG-N.

B = bound [^{3}H]ER and

T = Total count without IgG-N and IgG-ER.

1/dilution = dilution of IgG-ER.

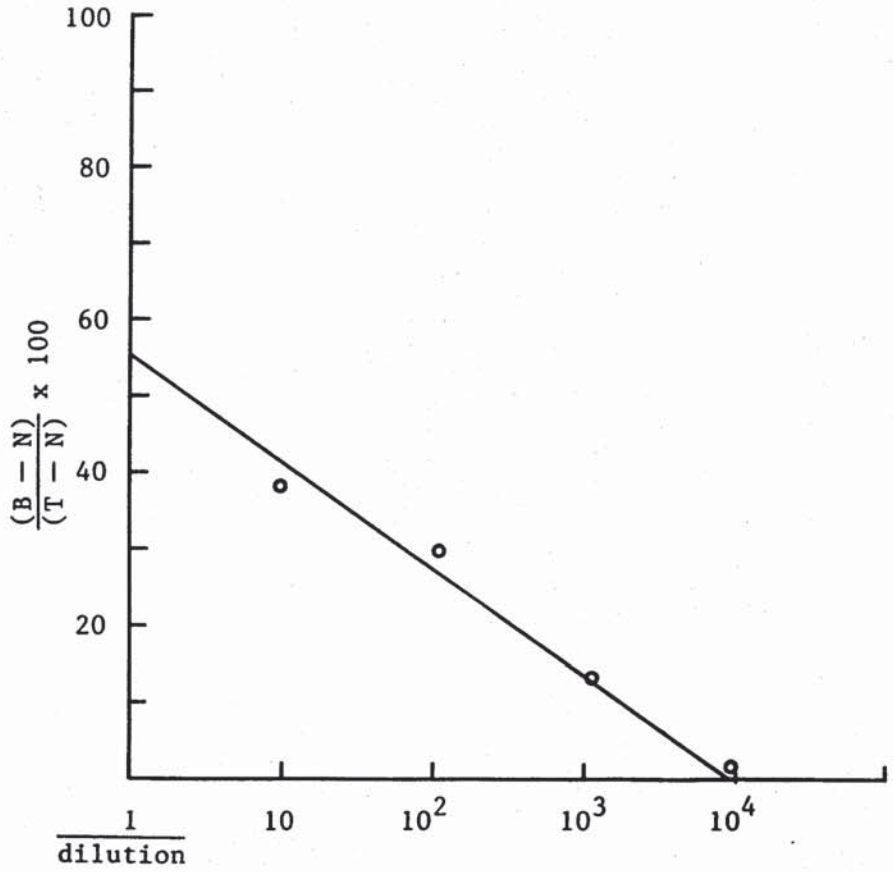

100
80
60
40
20
$\frac{(B-N)}{(T-N)} \times 100$
$\frac{1}{\text{dilution}}$
10
10^2
10^3
10^4

Figure 24: Separation of ^{14}C-labelled marker proteins on sucrose density gradient

[^{14}C]-BSA (4.6) and human-γ-globulin (7.1S) were mixed with 200µl aliquot of cytosol prepared from rat uterus (protein concentration 4mg/ml). Cytosol was then layered on the top of a 5-20% (w/w) sucrose density gradient prepared in TED and centrifuged at 230,000xg for 16h at 4°C. The rest of the procedure is described in section 2.4.3.

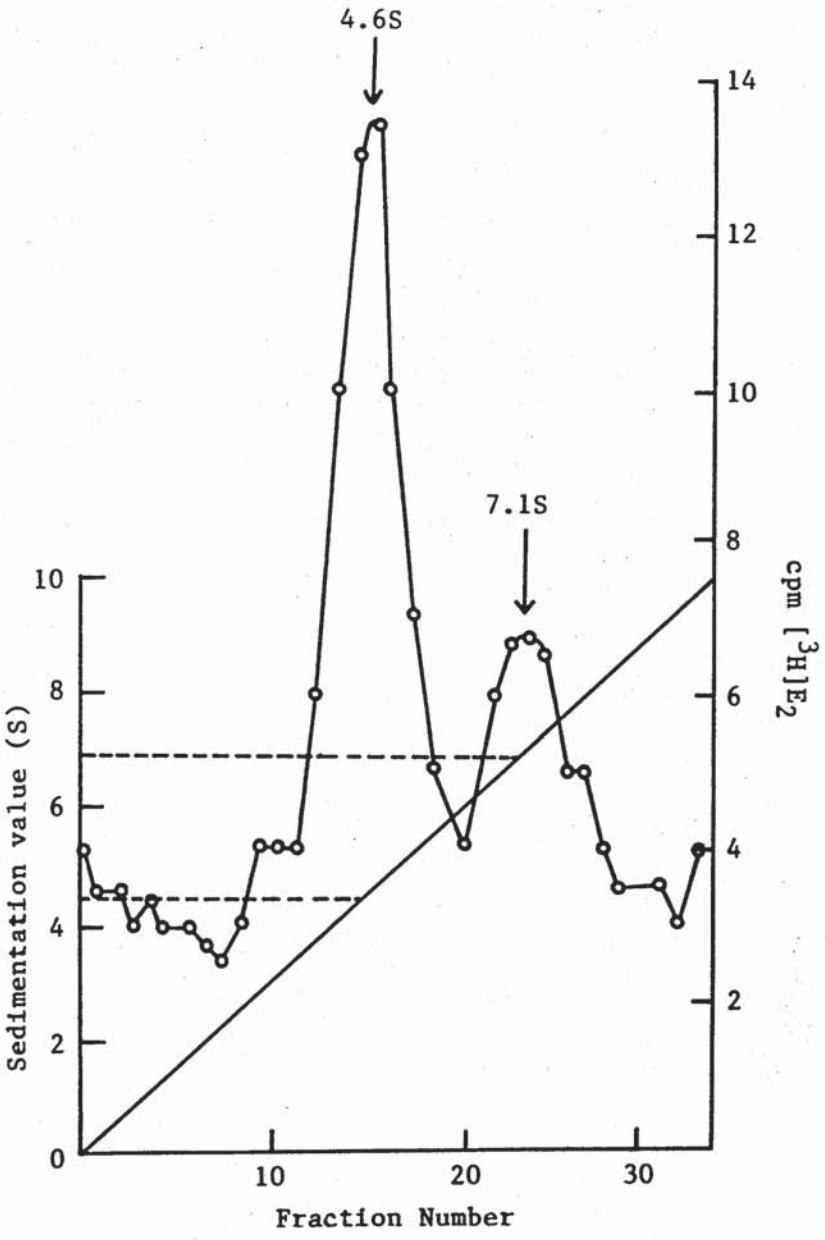
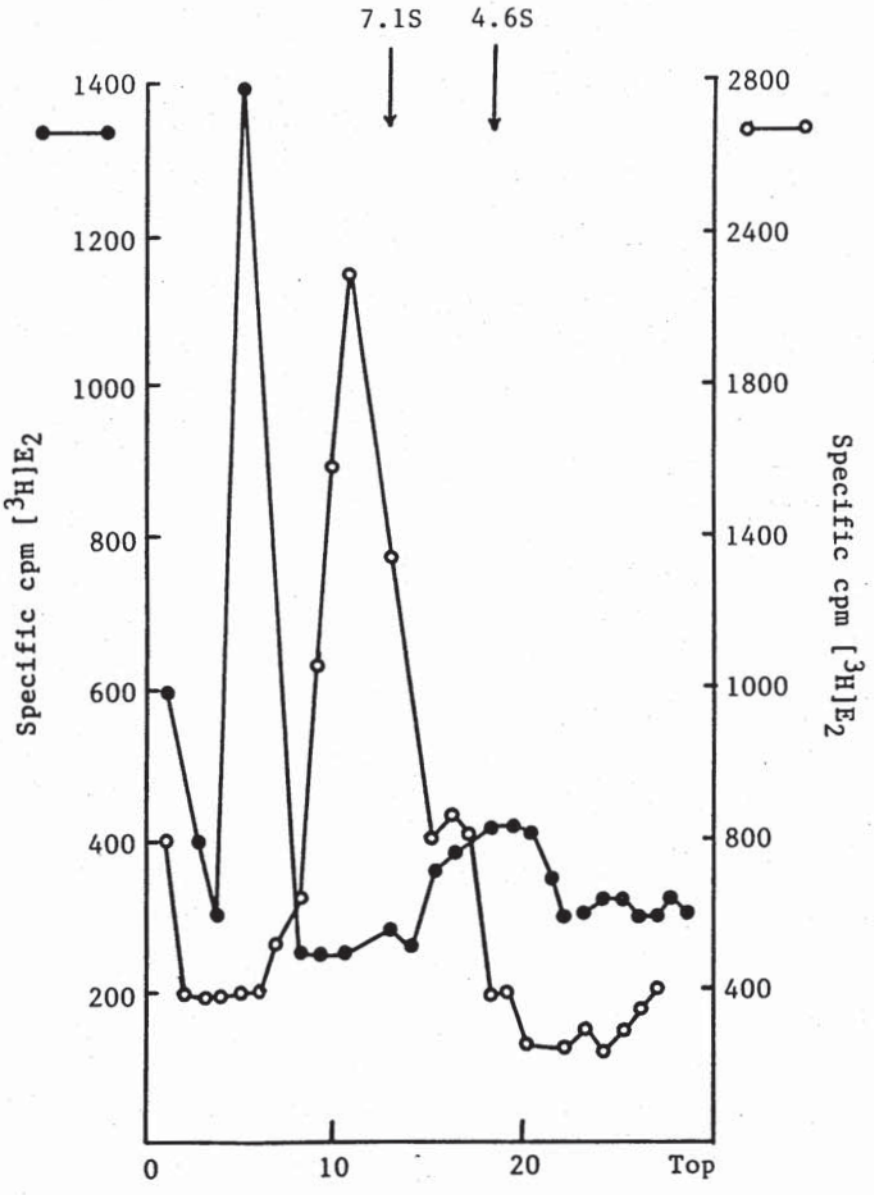

4.6S
7.1S
Sedimentation value (S)
cpm [3H]E2
Fraction Number
0
2
4
6
8
10
12
14
10
20
30

Figure 25: Effect of IgG-ER on the Sedimentation Values of Oestrogen Receptor (low salt).

Aliquots of [^{3}H]E_2-receptor complex were incubated with normal rabbit serum (o) or with antiserum (•) in a final volume of 250µl. After 4h at 0°C, 200µl of the samples were centrifuged for 16h at 230,000xg on a sucrose gradient in TEDK 10mM buffer. Arrows indicate the migration of standards (1) 4.6S BSA (2) 7.1S human-γ-globulin. Similar preparation in the presence antibody cause the sedimentation value to shift from 8S to about 10S.


7.1S
4.6S
1400
1200
1000
800
600
400
200
2800
2400
1800
1400
1000
800
400
0
10
20
Top
Specific cpm [3H]E2
Specific cpm [3H]E2

sedimentation value was shifted from 4S to about 8S in the presence of antibody (Figure 26). Rat ventral prostate cytosol labelled with [^{3}H] dihydrotestosterone and [^{3}H]ORG 2058 labelled rat uterine cytosol also were also run on SDG but there was no change in the peak of sedimentation value observed (Figure 27) in the presence of the antiserum.

3.4.3 Immunoadsorption Chromatography

Specificity was also checked using immunoadsorption chromatography. IgG-ER was immobilized onto sepharose-4B resin and incubated with cytosol from various preparations. The resin was washed to remove non-specifically bound protein. The retained proteins were then eluted with DES containing 0.5M sodium thiocyanate and 10% DMF. The eluate was analysed on native gels (Figure 28). A single band on the gel of each uterine sample (human, rat and rabbit) had a molecular weight in the region of 240,000. The dark region on the surface of the origin was due to oestrogen receptor aggregation which had failed to gain entry into the 4% gel in the continuous Tris-borate-EDTA buffer system. Sica and Bresciani (1979) added heparin in their buffer system to facilitate entry of protein into the gel while Skipper et al. (1985) used agar in place of PAGE for the stacking gel to allow entry of aggregating oestrogen receptor. The appearance of a single band indicated that the antiserum immobilized on the resin was retaining protein in a highly selective manner.

3.4.4 Time Course Study of IgG-ER Interaction with Oestrogen Receptor

The time course of the preincubation of the [^{3}H]E_2 cytosol with antibody is shown in Figure 29. As expected for specific protein-protein interaction, with the antibody present in limiting amounts, the time course indicates a single saturation curve.

3.4.5 Blotting

Transferring the electrophorised protein from the 5% SDS-PAGE onto the nitrocellulose paper overnight seems to be successful in

Figure 26: Effect of IgG-ER on the Sedimentation Values of Oestrogen Receptor (high salt).

Aliquots of [^{3}H]E_2-receptor complex were incubated with normal rabbit serum (o) or with antiserum (•) in a final volume of 250µl. After 4h at 0°C, the samples (200µl) were centrifuged for 16h at 230,000xg on a sucrose gradient in $TEDK_{0.4}$. Arrows indicate migration of standards (1) 4.6S BSA, (2) 7.1S human-γ-globulin. In the absence of antibody, the major radioactive component (receptor) sedimented in the region of 4S. Incubation of labelled cytosol with antibody resulted in a shift from 4S to about 8S.

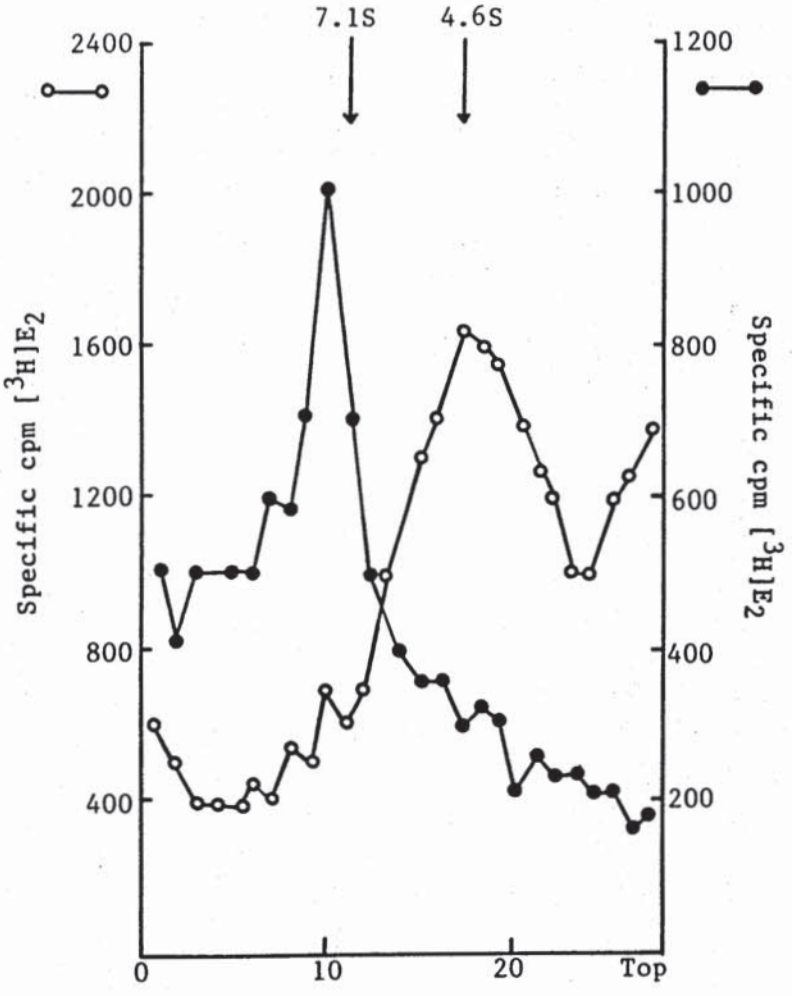

7.1S
4.6S
2400
2000
1600
1200
800
400
1200
1000
800
600
400
200
Specific cpm [3H]E2
Specific cpm [3H]E2
0
10
20
Top

Figure 27 Sedimentation pattern of progesterone and DHT receptors in the presence of antibody to oestrogen receptor.

Progesterone-receptor complex was prepared according to (Logeat et al., 1981) by incubating 1nM [^{3}H]ORG 2058 with rat uterine cytosol for 4h at 4°C. [^{3}H]DHT-receptor complex was prepared by incubating 2nM [^{3}H]DHT with rat prostatic cytosol (Greene et al.,1977) for 4h at 0°C. Aliquot from each receptor complex preparation was incubated with antiserum (50µg) in a final volume of 250µl. After 4h at 0°C, 200µl of the samples were centrifuged for 16h at 230,000g on a sucrose gradient in $TEDK_{0.4}$. No difference in the sedimentation value was observed in the absence and the presence of antibody. The radioactive receptors were found in the 4S region.

(•) [^{3}H]DHT receptor

(o) progesterone receptor

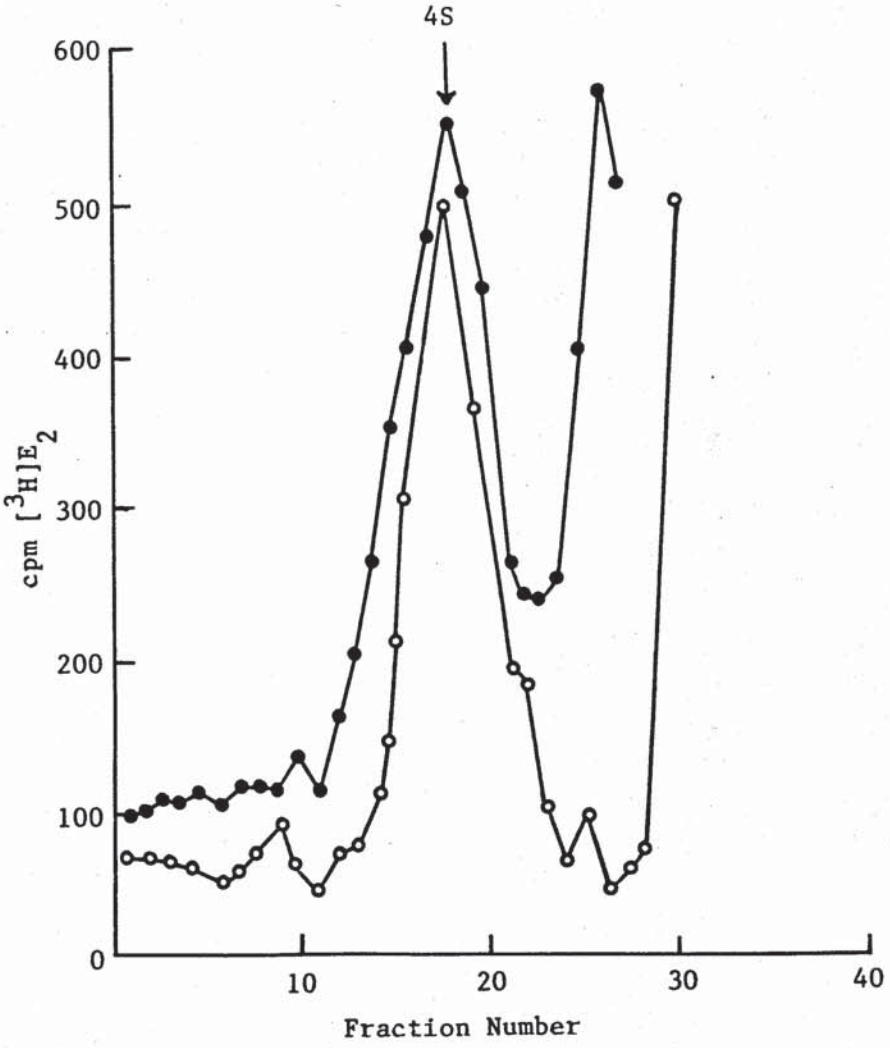

4S
600
500
400
300
200
100
0
cpm [^{3}H]E_2
10
20
30
40
Fraction Number

Figure 28: Characterization of Protein Eluted from Immobilized IgG (immunoadsorbent).
Cytosols containing oestrogen receptor from uterine tissues of different species (human, rat and rabbit) were prepared as in section 2.2.2.1. The cytosols were then incubated with antibody immobilized onto Sepharose 4B and the rest of the procedure was described in section 2.4.5.1. The eluates from the immunoadsorbent were run on PAGE in Tris-borate-EDTA continuous system.

lane 1. Standard molecular weight markers
lane 2. human myometrium
lane 3. rat uterus
lane 4. rabbit uterus

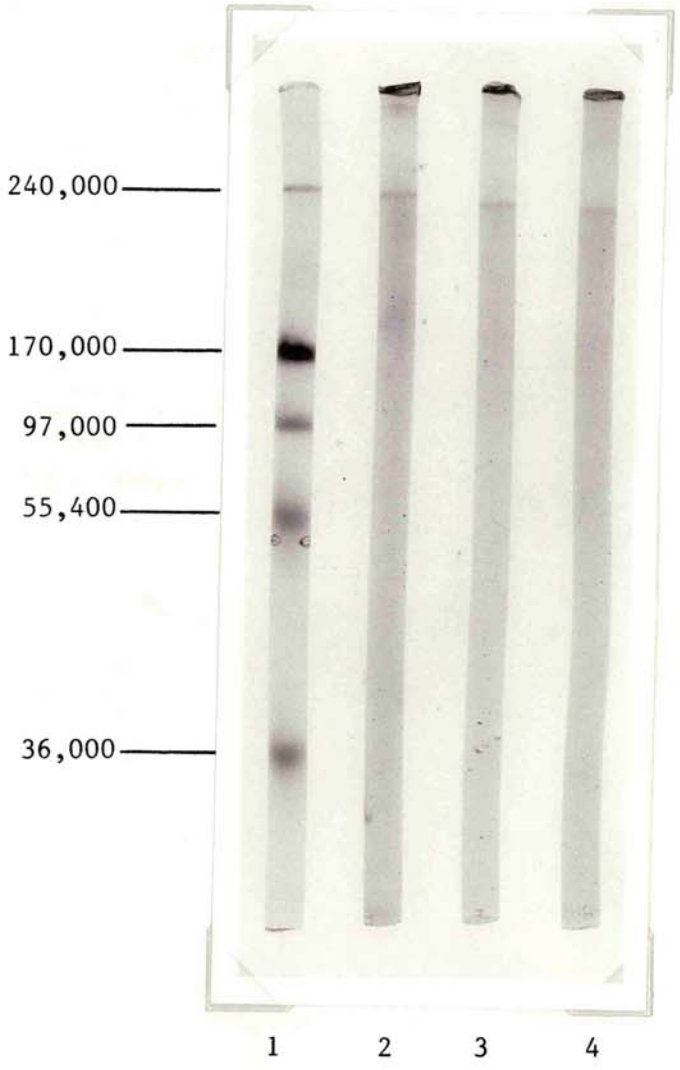
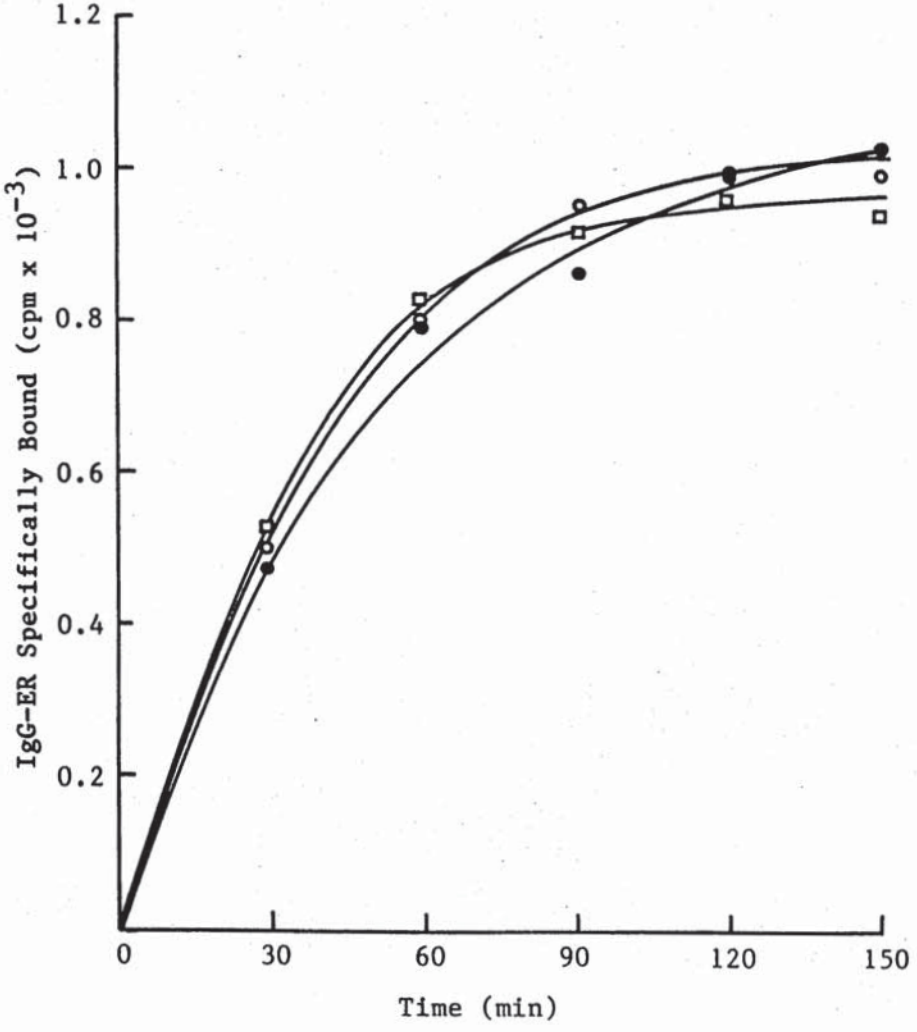

240,000
170,000
97,000
55,400
36,000
1
2
3
4

Figure 29: Time course of the interaction of IgG-ER with oestrogen receptor at 4°C. Aliquots of human uterine cytosol containing 0.1pmol of oestrogen receptor were preincubated for 1h at 0°C with 10nM [^{3}H]E_2. The complex were further incubated with immobilized IgG at 0°C for time periods indicated in materials and methods (section 2.4.5.2). The reaction was terminated by the addition of buffer. The immunoadsorbent was washed 3x2ml TED. Radioactivity bound to the adsorbent was measured. Each curve represents one independent experiment.


1.2
1.0
0.8
0.6
0.4
0.2
IgG-ER Specifically Bound (cpm x 10^{-3})
0
30
60
90
120
150
Time (min)

demonstrating the presence of antibody specific to the oestrogen receptor. Three preparations: crude cytosol, ammonium sulphate precipitate-treated oestrogen receptor and purified oestrogen receptor were run on the gel for 2h before transfer to the nitrocellulose paper. In each of the three samples, a band in the region of molecular weight 65,000 was evident (Figure 30). The appearance of these bands demonstrated that oestrogen receptor immobilized on nitrocellulose paper can be used to detect its antibody and secondly the specificity of the antisera can be deduced.

3.5 OESTROGEN RECEPTOR DISTRIBUTION AND TRANSLOCATION

3.5.1 Oestrogen Receptor Distribution in Oestrogen Responsive Tissues

The antiserum was produced against oestrogen receptor from human myometrium; therefore, it was tested for its capacity to react with the receptor from human myometrium and endometrium. Frozen sections of human uterus obtained from a patient of perimenopausal age were utilized for this purpose. Reactivity studies of the anti-receptor (anti-ER) antiserum with the human myometrium and endometrium revealed two types of staining. In the frozen sections of the endometrium processed for immunohistochemical demonstration of oestrogen receptor, cytoplasmic as well as nuclear fluorescence of the glandular cells lining the ducts were seen. The stromal cells also exhibited a weaker fluorescence. The nuclei were free of fluorescence in these cells (Figure 31a). This uneven distribution of oestrogen receptor in human endometrium had been reported by Tsibris *et al*. (1981). In the frozen sections of the myometrium, most of the cells (about 90%) exhibited cytoplasmic staining, with a few cells (about 10%) showing nuclear staining (Figure 31b). A section of the human myometrium was stained using haematoxylin/eosin (H&E) (Figure 31d).

In the immature rat endometrium, immunochemical staining was observed in both the cytoplasm and the nucleus of the epithelial cells of the endometrial glands. However, the staining of the

Figure 30: Electrophoretic blotting of oestrogen receptor from 5% SDS-PAGE.

Three preparations containing oestrogen receptor were run on 5% SDS-PAGE, transferred to nitrocellulose paper overnight and incubated with antibody to oestrogen receptor. Iodine labelled protein-A was used to detect the antigen-antibody reactivity. The nitrocellulose paper was then subjected to autoradiography.

lane 1. Crude cytosol from human myometrium
lane 2. Ammonium sulphate treated cytosol
lane 3. Affinity chromatography eluate

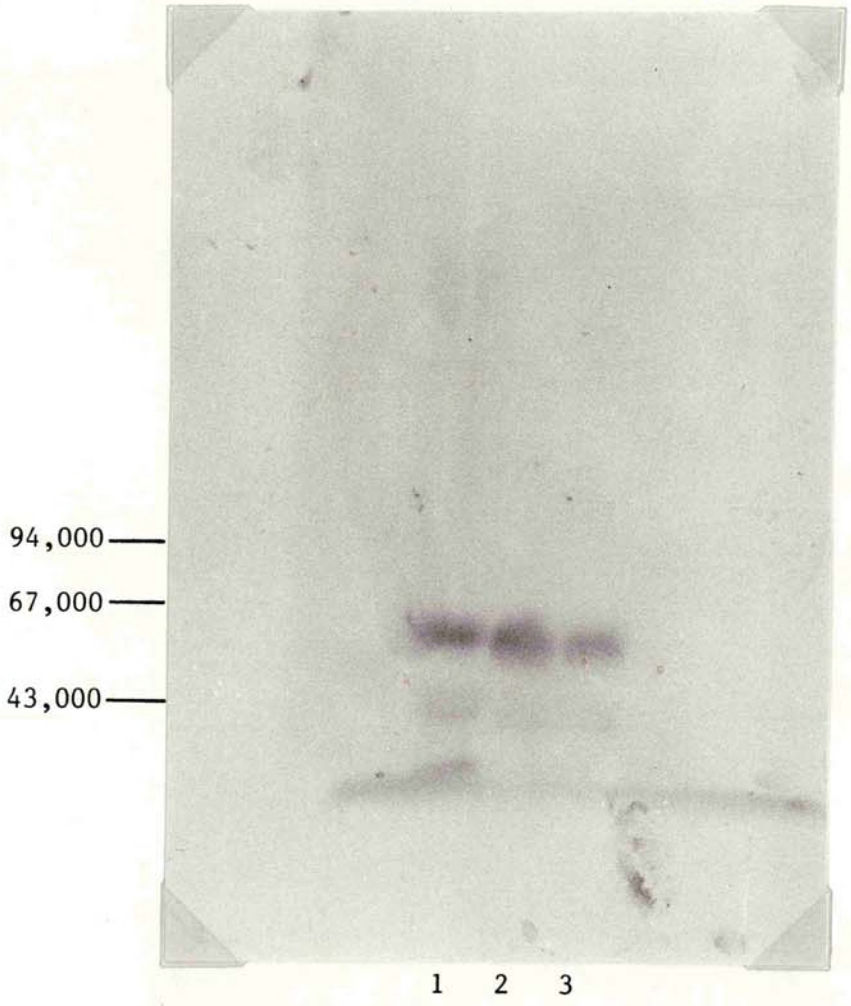
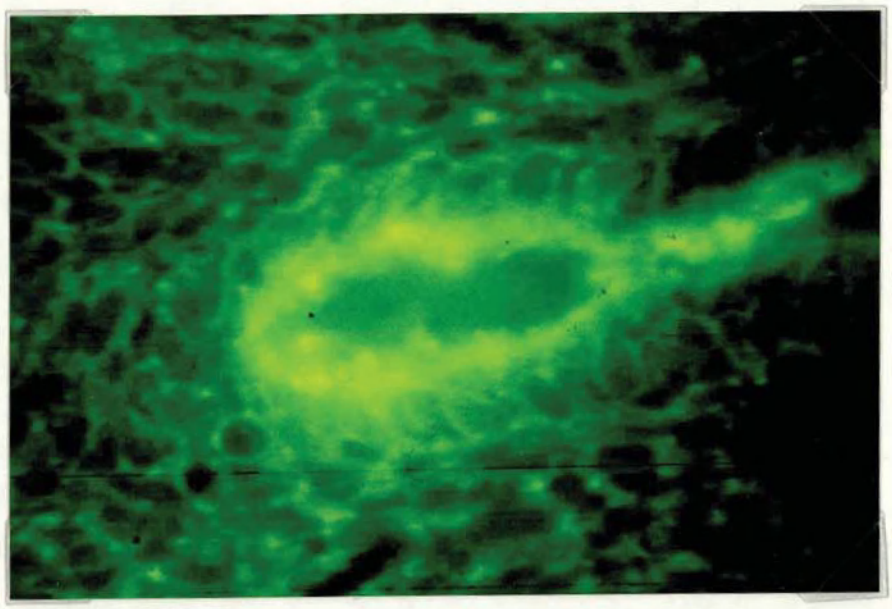

94,000
67,000
43,000
1
2
3

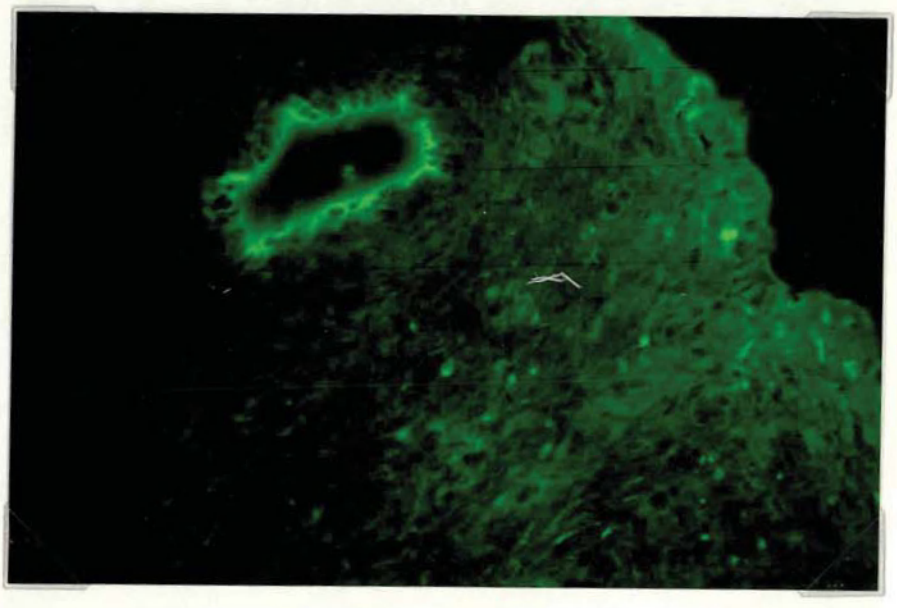
Figure 31(a-d): Oestrogen responsive tissues displayed the presence of oestrogen receptor by the IF technique.

(a) Frozen section of human endometrium was incubated with anti-ER antiserum followed by FITC goat anti-rabbit IgG. The tissue was observed using an oil immersion lens. Bright positive staining in the cytoplasm and nucleus of the cells lining the duct was observed. Stromal cells are weakly stained, mostly in the cytoplasm (x1000).

(b) Frozen section of human myometrium. IF was localized in the cytoplasm of most cells. Only a few cells showed nuclear staining (x400).



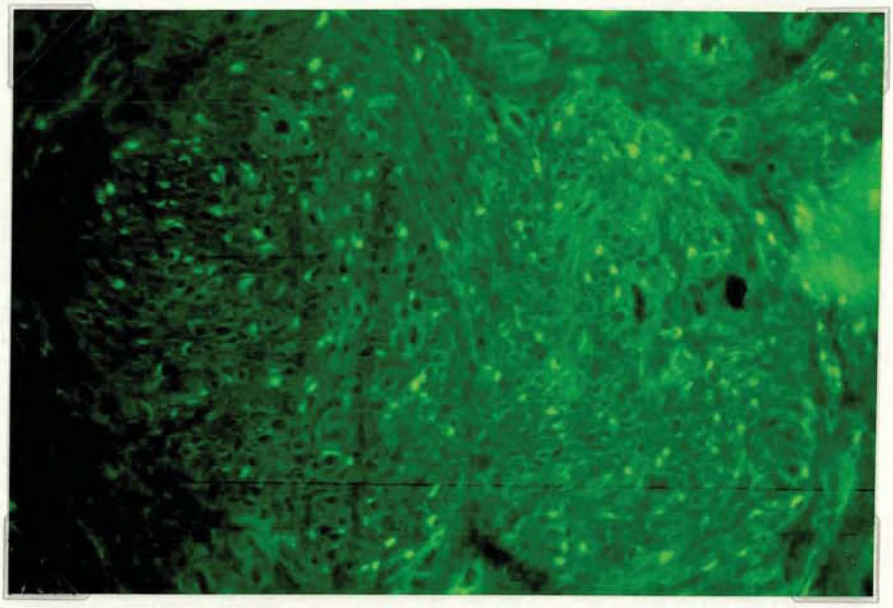
(a)



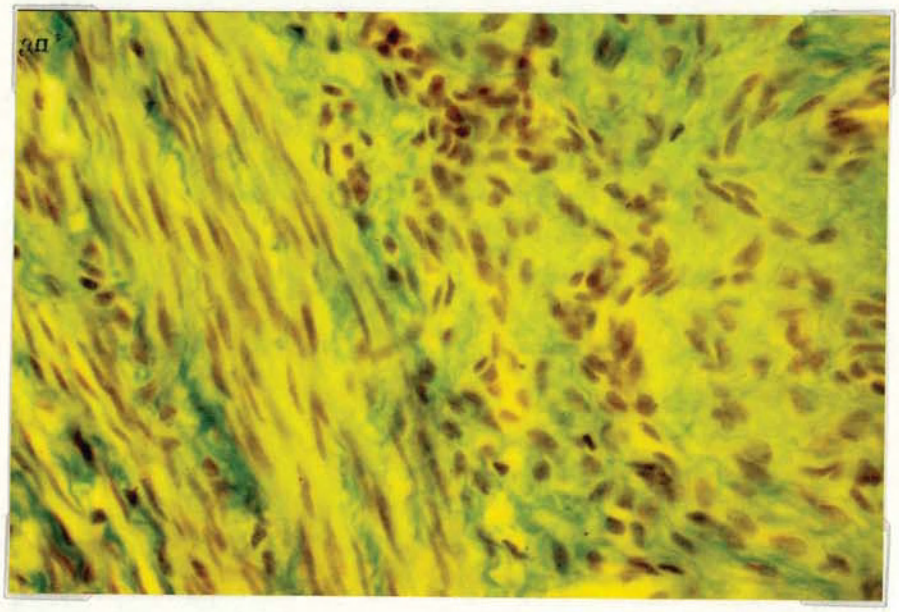
(b)

Figure 31(c): Demonstration of oestrogen receptor translocation in human myometrium. Adjacent sections from the same uterus were taken and incubated with 1nM E_2 at 37°C for 1h. A different staining pattern from the above (b) was observed. Most of the staining was seen in the nuclei with few cells having cytoplasmic staining. The increase in the number of nuclei stained showed that translocation had taken place (x400).

(d) Haematoxylin/eosin-stained frozen section of human myometrium showed the distribution of cells.



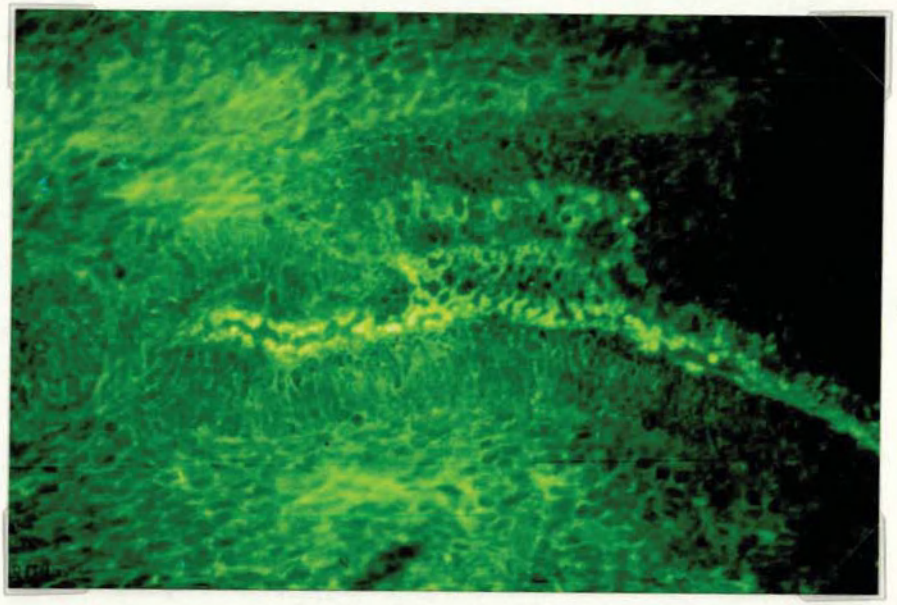
(c)



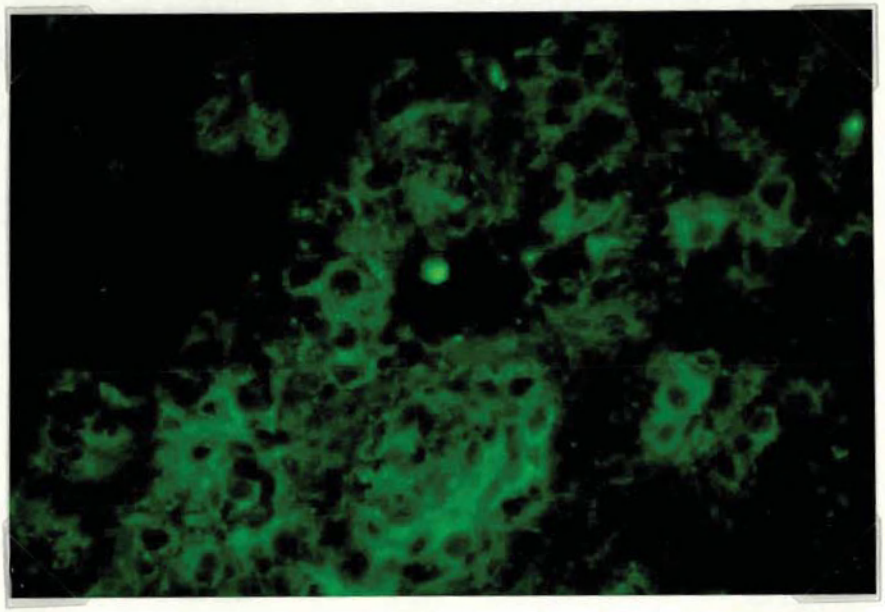
(d)

Figure 31(e): Frozen section of rat uterus. The tissue displayed a marked degree of IF staining. The fluorescence was seen localized in the cytoplasmic as well as nuclear compartments of the glandular cells, a similar pattern of staining to that seen in the human uterus. The stroma exhibited weaker staining, mostly cytoplasmic (x400).

(f) A breast carcinoma which was ER+ by biochemical assay (112fmol/mg cytosol). There was strong cytoplasmic fluorescence in some of the tumour cells. Faint IF+ was also observed in some cells (x400).



(e)



(f)

stroma cells was exclusively cytoplasmic (Figure 31e).

Frozen sections of ER+ breast carcinoma (112fmol/mg cytosol protein) showed positive cytoplasmic staining. The intensity of cytoplasmic staining varied in some areas (Figure 31f). The nuclear staining was not observed. The staining intensity varied from one cell subpopulation to another and some cells were negative. Similar results were obtained by Garancis et al. (1983) using monoclonal antibodies to oestrogen receptor. However this group used paraffin embedded tissues and the PAP technique as the method of receptor detection. Their results, using frozen instead of paraffin embedded sections were disappointing. Greene and Jensen (1982) who reported cytoplasmic staining using monoclonal antibodies on frozen sections of the breast, later reported nuclear localization of oestrogen receptor.

3.5.2 Oestrogen Receptor Distribution in ZR-75-1 Cells

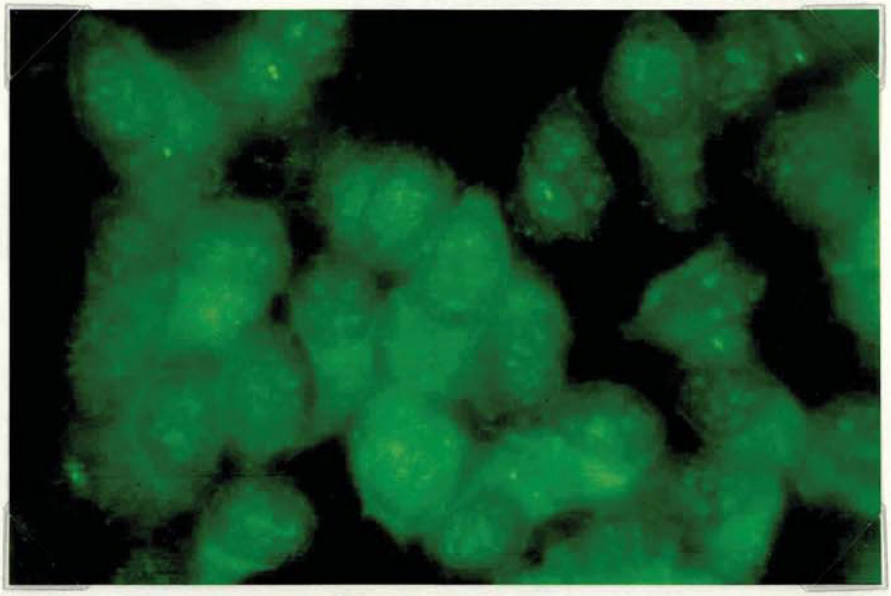
The experiments described were carried out on the ZR-75-1 cell line. Being derived from the metastatic pleural effusion of a patient with breast cancer, these cells are generally accepted as an appropriate model for hormone-dependent breast tumours. Biochemical assays showed that the cell line contains 30-50fmol/mg protein. In the cytoplasm of ZR-75-1 cells, the immunochemical staining was seen localized in granules. The staining was diffused, and it appeared as a fine granular precipitate. The cytoplasmic staining was present in the form of fine, yellowish-green intracellular particles which varied from a few to many (Figure 32a). The nuclear staining appeared in the form of fine, green (yellowish green) intranuclear particles which were often so numerous that the nuclei appeared to be almost entirely filled with the stain (Figure 32b). The same observation was reported in the pituitary cells by Morel et al. (1981) using goat antibody to oestrogen receptor. In the nucleus, they were distributed all over the chromatin. Detailed observations by Morel et al. (1981), using electron microscopy, revealed the distribution of the oestrogen receptor in the euchromatin and the neighbouring dense heterochromatin of the nucleus.

Figure 32 (a-g): In Vitro Translocation Experiments on ZR-75-1 cells.

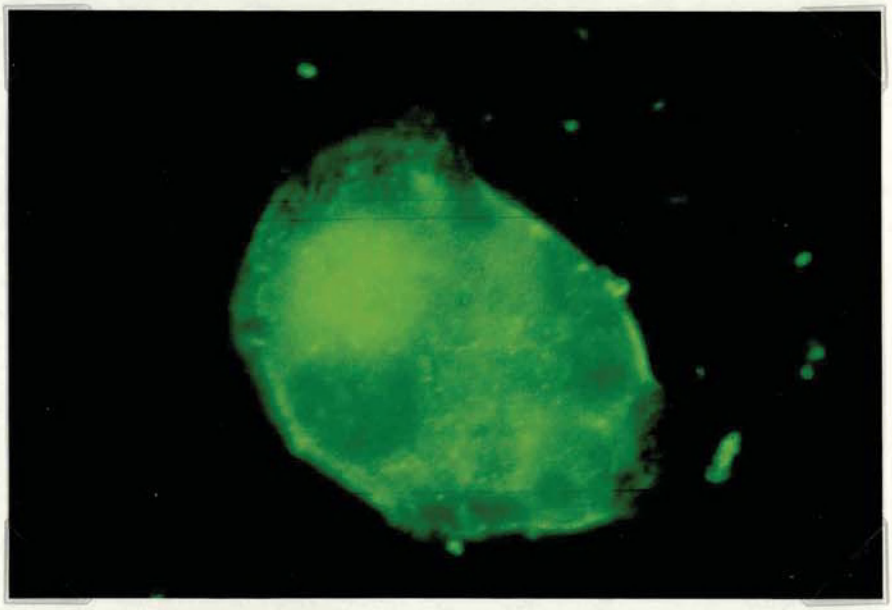
The ZR-75-1 cells were transferred to fresh medium containing 1nM E_2 or DES incubated for 1h at:

(a) 4°C prior to localization of oestrogen receptor using the IF technique. All the cells retained their IF in both nuclei and cytoplasm in the form of localized granules (x400).

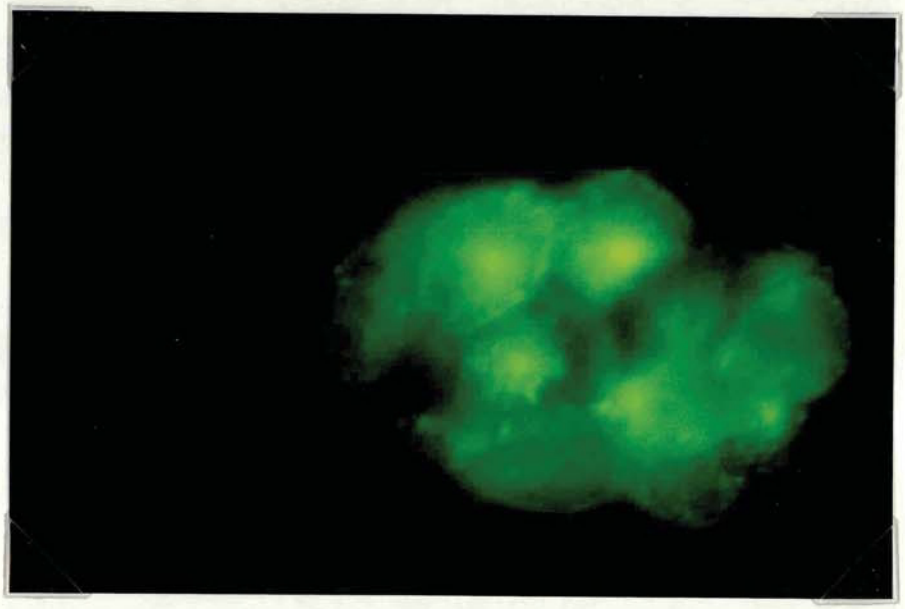
(b & c) 22°C prior to localization of oestrogen receptor. Besides the cytoplasmic staining, the cell also exhibited a dense, distinctive, nuclear staining which showed that translocation was beginning to occur (b) x1000, (c) x400.



(a)

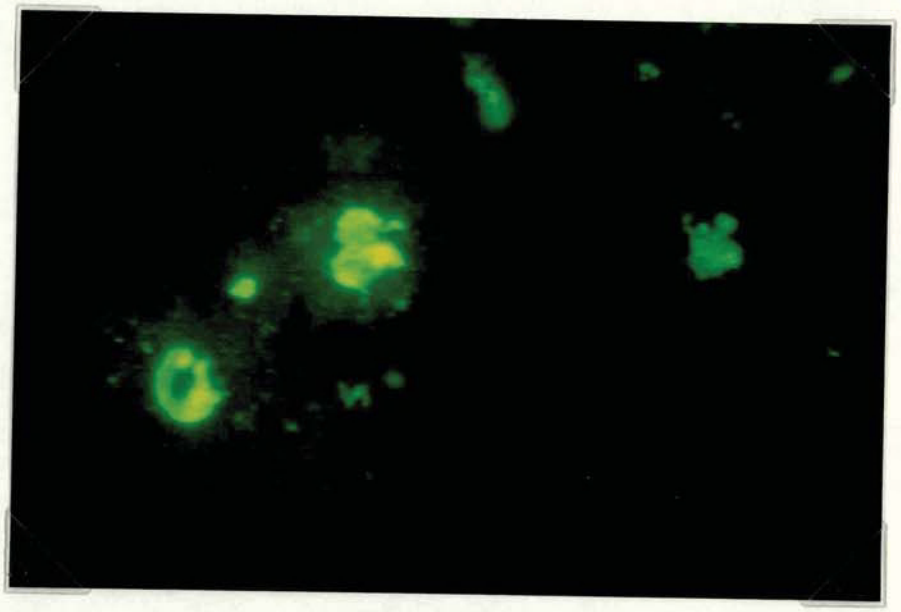


(b)

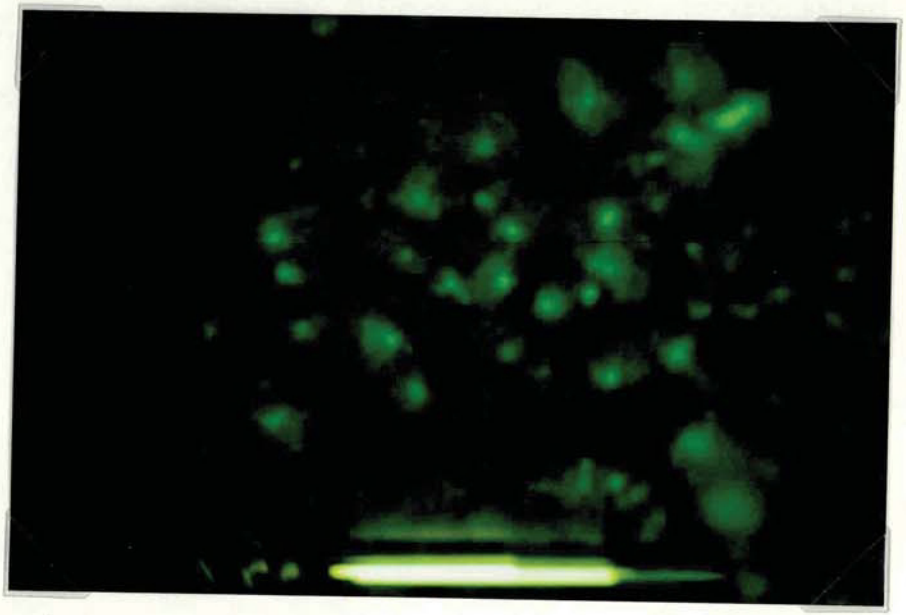


(c)

Figure 32(d & e): The ZR-75-1 cells were transferred to fresh medium containing 1nM E_2 or DES incubated for 1h at 37°C prior to oestrogen receptor localization. Only nuclear staining was evident. Fluorescence was not seen in the cytoplasm of the cell, making it difficult to see the outline of the entire cell (d) x1000, (e) x250.



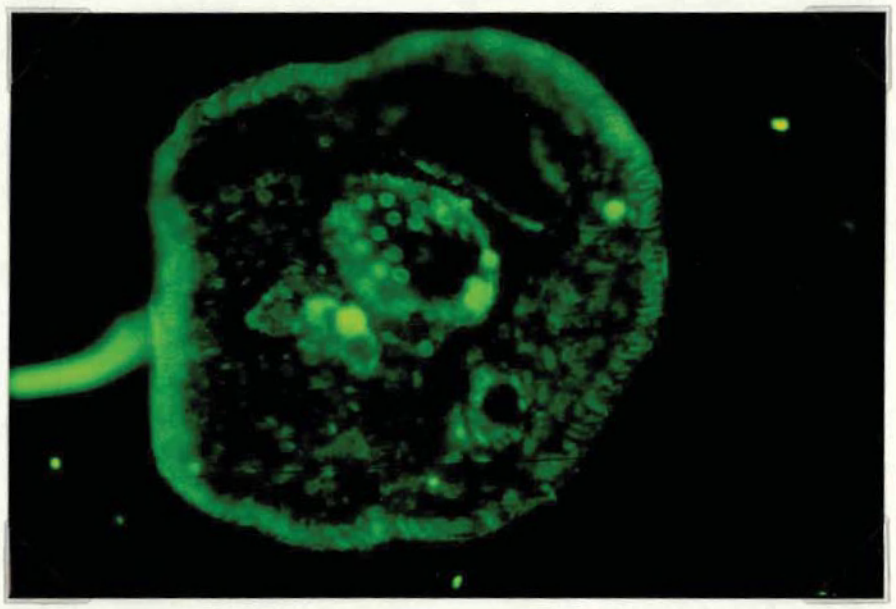
(d)



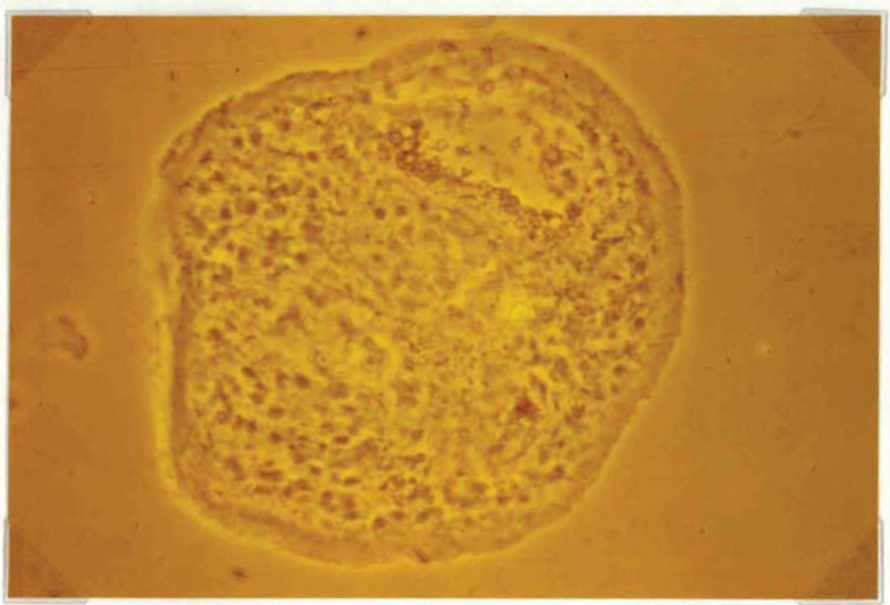
(e)

Figure 32(f & g): The cells were incubated with E_2 for 2h. Nucleolar fluorescent and a membrane fluorescence were demonstrated in this cell. The nucleolar staining appeared in spot like fluorescent in this highly magnified cell (x1000).

(g) The phase contrast photograph of (f) showing the detail structure of the cell (x1000).

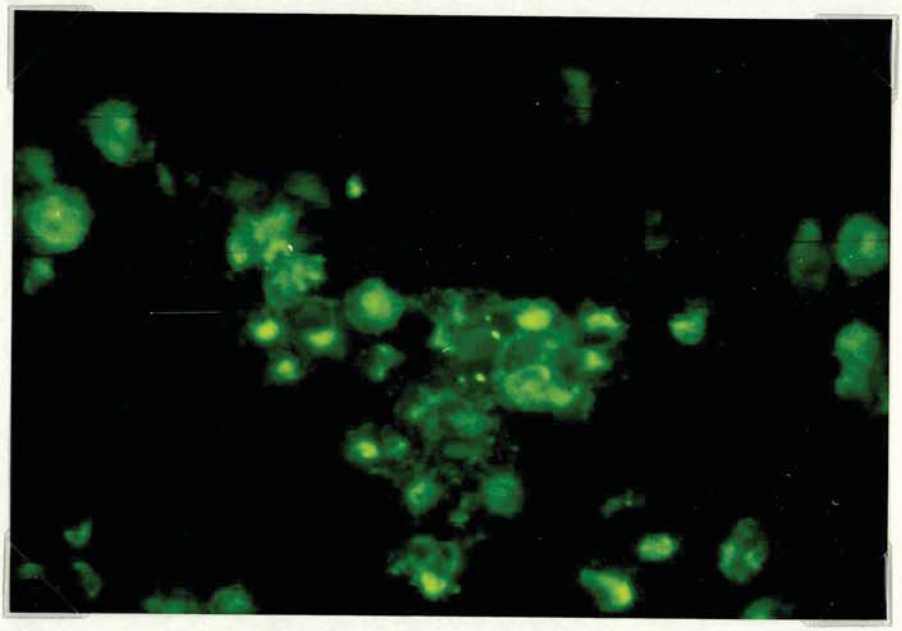


(f)



(g)

Figure 32 (h): Rat endometrial cells in culture. The cells were grown in the presence of E_2 at 37°C and fixed with methanol:acetone prior to oestrogen receptor localization. The IF was found to be distributed mostly in the nucleus of the cells. (x400)



(h)

3.5.3 Control Experiments

3.5.3.1 Reagent Controls

When the antiserum was preincubated with a saturating concentration of oestrogen receptor cytosol from positive human breast carcinoma (>500 fmol/mg protein), no such staining was observed i.e. no granules indicating immunoreactivity were present (Figure 33a). Another set of experiments was carried out on translocated receptor in ZR-75-1 cells with antiserum preadsorbed with purified oestrogen receptor and similar results were seen to occur. Preabsorbing the anti-ER antiserum with ER- cytosol of breast tumour was ineffective in eliminating the antibody-specific immunofluorescence (Figure 33b). Also preabsorbing the antiserum with E_2 (Figure 33c) showed similar staining distribution.

3.5.3.2 Tissue Controls

Frozen sections of ER- breast tissue showed neither cytoplasmic nor nuclear staining (Figure 33d and 33e). A section of ER- breast tissue was stained with H&E as shown in Figure 33f. Another negative control was obtained with spleen (Figure 33g).

3.5.4 Translocation of Hormone-receptor Complexes

Additional confirmation for the specificity of the antiserum to oestrogen receptor could be derived from the in vitro experiments on translocation.

3.5.4.1 Oestrogen Receptor Localization in Cells Grown in the Absence of E_2

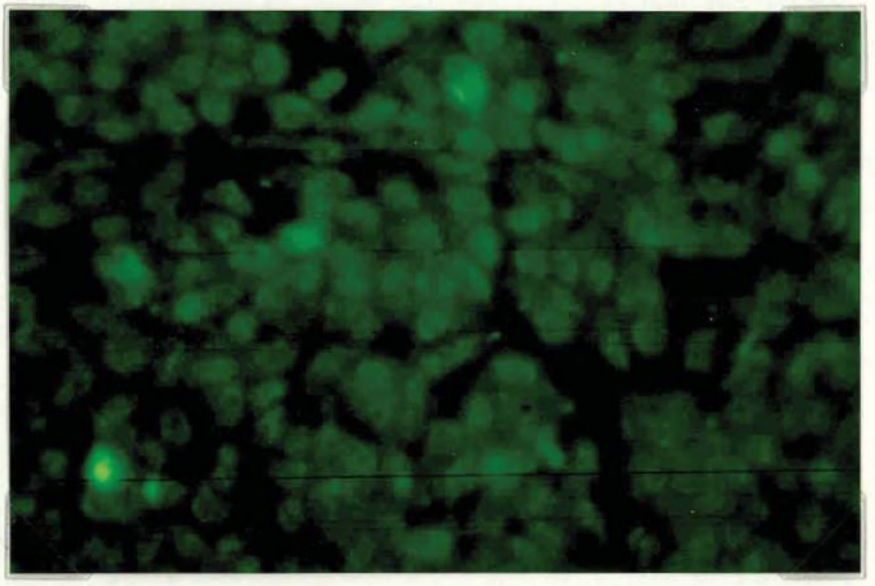
In all the cells grown for 1h in the absence of E_2, cytoplasmic as well as nuclear immunofluorescence was evident. If the cells were maintained in an oestrogen-free medium for 24 or 48h and then each left at 4, 22 or 37°C for 1h, the fluorescence was distributed both within the nuclei and in the cytoplasm. Figure 34a and 34b show the

Figure 33(a-h): The Specificity of the Antiserum by IF and Preabsorption Technique.

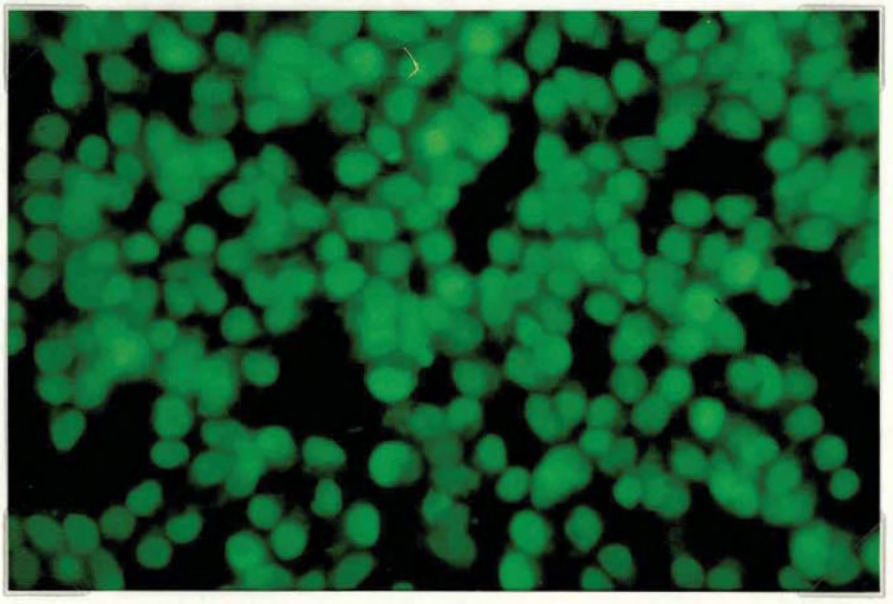
The specificity of the antiserum towards oestrogen receptor was checked by using preadsorbed antiserum in place of primary antiserum with ZR-75-1 cells. The anti-ER antiserum, was preadsorbed with various reagents:

(a) cytosol from ER+ breast cancer biopsies, >500fmol/mg protein (determined biochemically). The fluorescence in the ZR-75-1 cells was reduced from the normal level (x250).

(b) cytosol from ER- breast cancer. The intensity of the staining was the same as in cells incubated with unabsorbed anti-ER antiserum (x250).

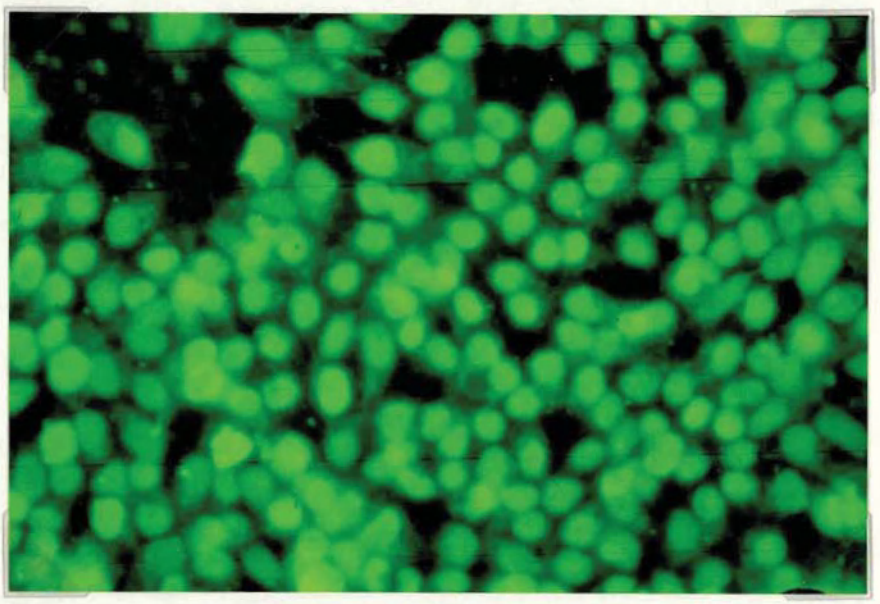


(a)



(b)

Figure 33(c): The antiserum was preabsorbed with E_2. This absorption does not seem to affect the fluorescence level in the cells (x250).



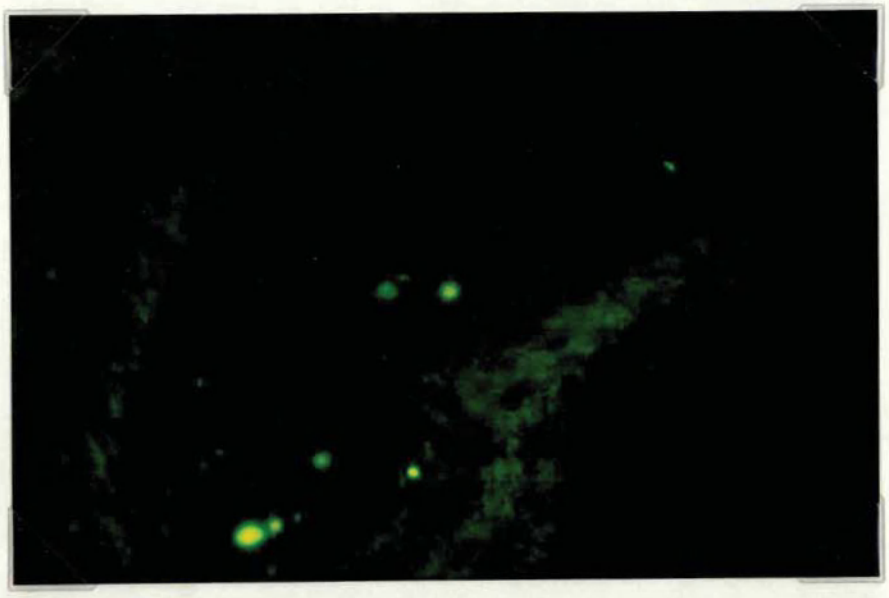
(c)

Figure 33(d-h): Various tissue controls were used to determine the specificity of the anti-serum.

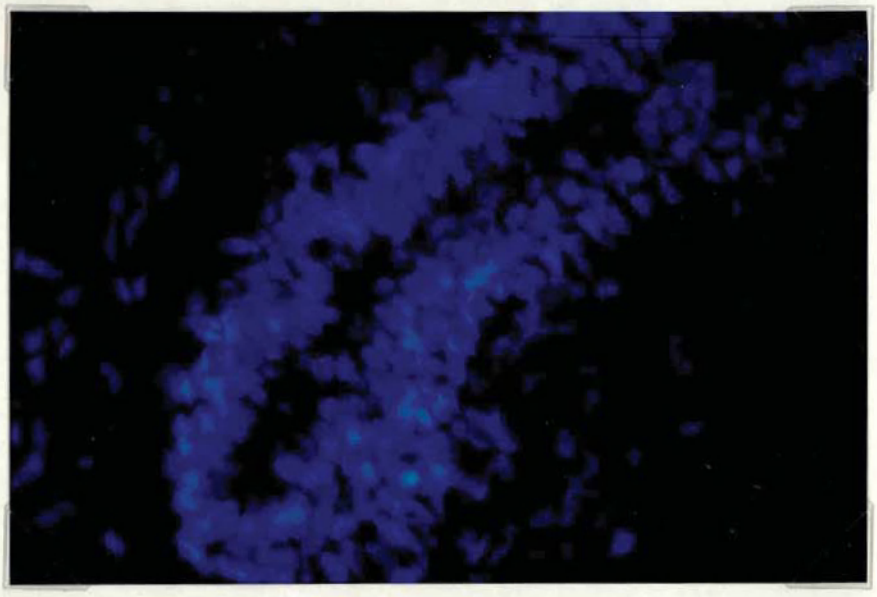
(d) A breast fibroadenoma which was ER- (biochemically determined) was taken as a negative control for human tissue. Negative cytoplasmic and nuclear staining indicated the absence of oestrogen receptor in this tumour (x400).

(e) The presence of the epithelial cells was shown by the staining of the DNA with Hoechst 33258 (x400).

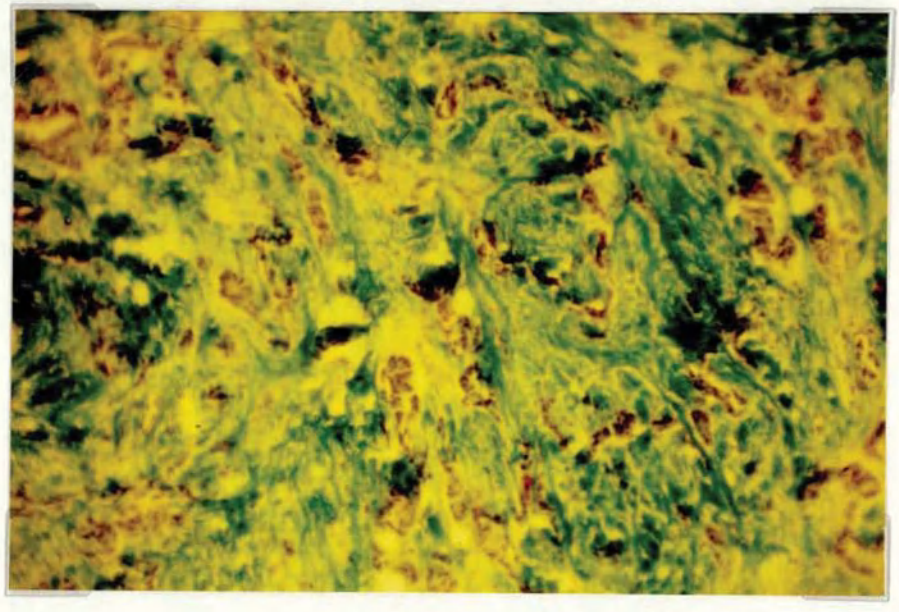
(f) A section of human breast stained with H & E (x100)



(d)



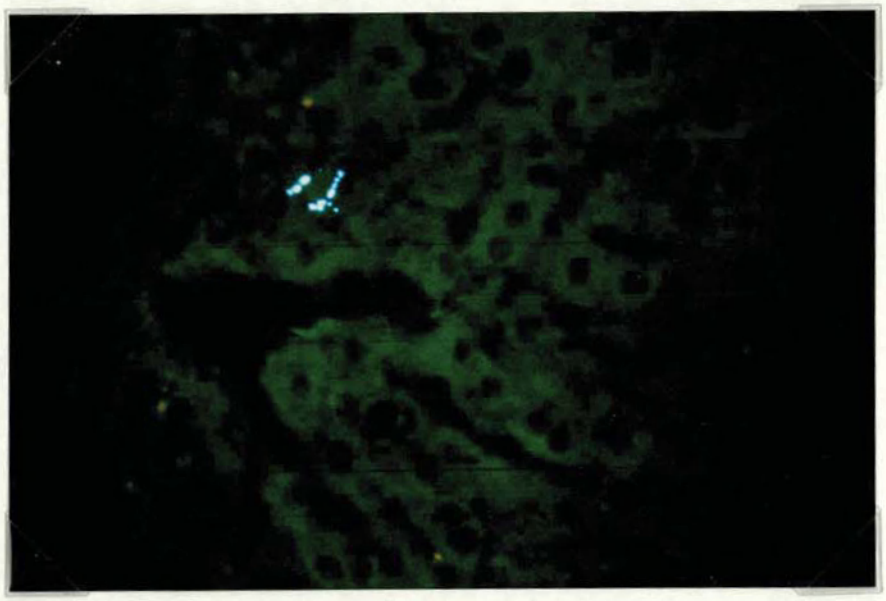
(e)



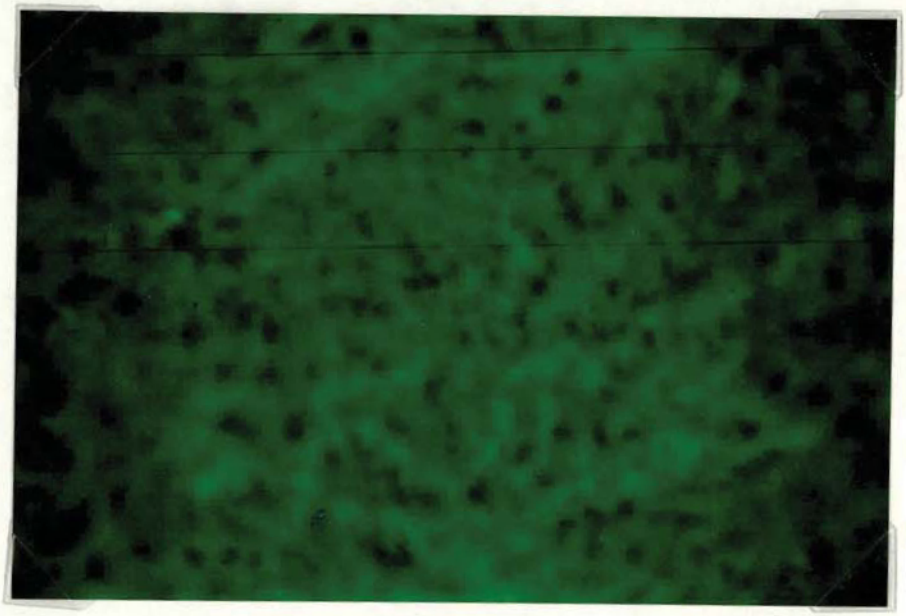
(f)

Figure 33(g): Rat spleen was taken as negative control for rat tissue. The frozen section was incubated with primary antiserum followed by IF localization ofthe oestrogen receptor. Only very faint non-specific fluorescence is seen in this negative tissue (x1000).

(h) A frozen section of rat liver processed for oestrogen receptor localization by the IF technique. Staining was seen in the cytoplasm though the intensity was much less than the uterine tissue of the same species (x1000).



(g)

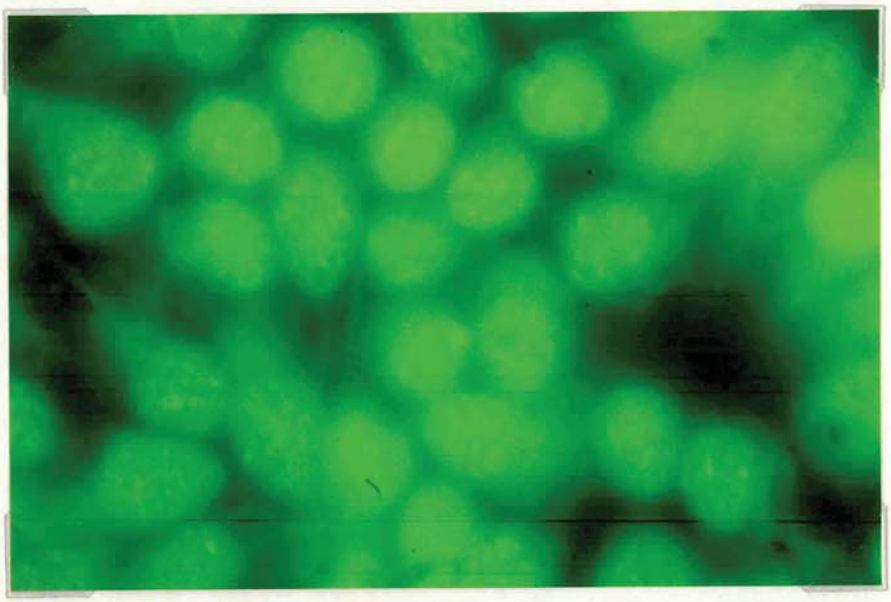


(h)

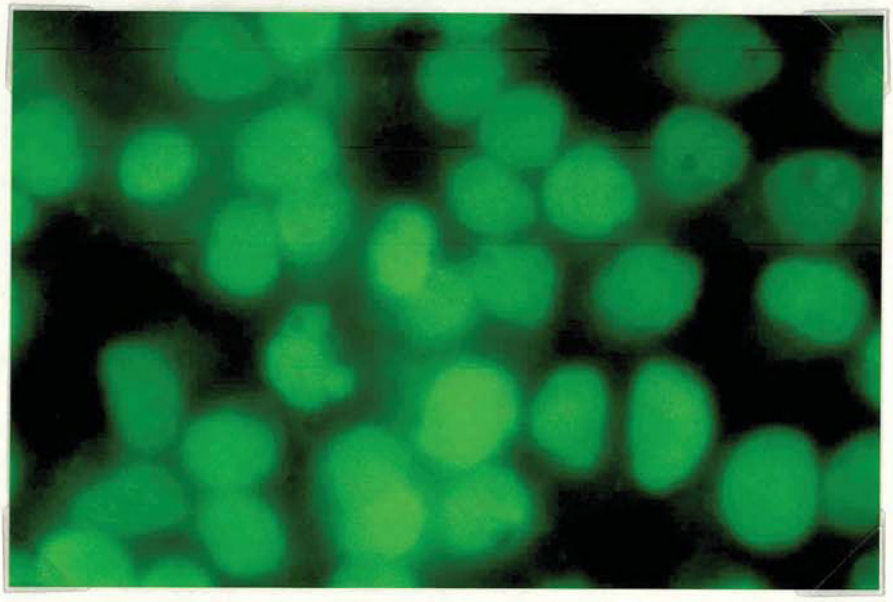
Figure 34: The ZR-75-1 cells were grown in the absence oestrogen and processed for oestrogen receptor using IF technique.

(a) ZR-75-1 cells unexposed to ligands at 4°C and processed for IF localization of oestrogen receptor. The IF localized in both cytoplasmic and nuclear compartment in all the cells.

(b) ZR-75-1 unexposed to ligand at 37°C and processed for IF localization. No change was seen in distribution pattern of the IF compared to (a).
(a) x400
(b) x400



(a)



(b)

IF distribution in cells maintained at 4 and 37°C respectively. Exposure of these cells for >48h in the medium without ligands at 22 and 37°C did not alter the cellular distribution of immunofluorescence.

3.5.4.2 Effect of Exposure to E_2 and DES

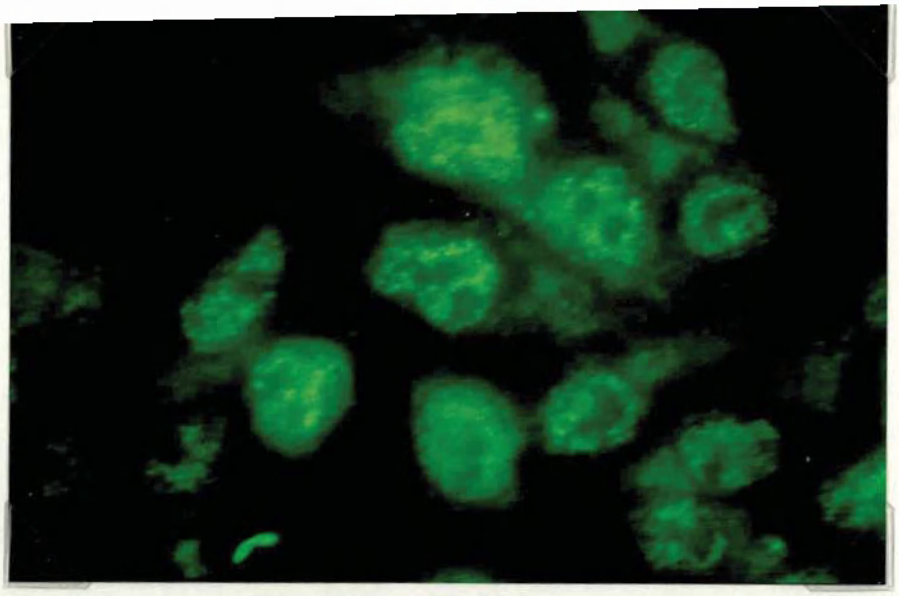
ZR-75-1 cells, grown in the continued presence of E_2 (10^{-9}M) for 24 and 48h, were incubated with ligand for 1h at 4, 22 and 37°C. The fluorescence, seen in the cells exposed at 4°C to E_2 or DES, appeared to be homogenously distributed over the cytoplasm as well as the nucleus, while those incubated with ligand at 22°C showed denser staining within the nuclear membranes (Figure 32b,c). Incubation at 37°C for 1h showed nuclear but no cytoplasmic staining (Figure 32d,e). When the incubation time was increased to 2h nucleolar-like spots of fluorescent with no detectable fluorescence in the chromatin network or the nuclear matrix ('nucleolar' fluorescence) (Figure 32f) were seen in cell. Raam et al. (1983), working on an endometrial cell culture and Tamura et al. (1984) on ER+ breast cancer frozen sections, incubated with different concentrations of E_2, DES, or monohydroxytamoxifen (2.5 to 250nM) for 2h at 37°C found that immunofluorescence was localized only in the nuclei. However, Lope-Pihie et al. (1985) found that 1h incubation was sufficient for complete translocation to take place which was found insufficient by the above mentioned groups. They found that when the incubation time was reduced to one hour, although the fluorescence was localized predominantly in the nuclei, in all the cells the cytoplasm also retained fluorescence. This was weaker compared to the controls, but when reexamined the fluorescence had become intranuclear and the cytoplasm was free of fluorescence. In other studies in vivo translocation of oestrogen receptor and progesterone receptor (PgR) has been demonstrated by Morel et al. (1981, 1984) on pituitary cells using goat antibody to oestrogen receptor and the PAP technique. They observed a reduction in cytoplasmic staining and an increase in nuclear staining after an injection of E_2 into an immature rat. In the present study, quantitative measurement of the staining had been carried out. Figure 36a, b and c are histograms of the ratio of staining in the nucleus (N) to staining in the extranucleus (E). A value more than 1 denoted translocation had taken place.

Figure 35(a-c): In vitro translocation study in ZR-75-1 cells in the presence of progesterone.

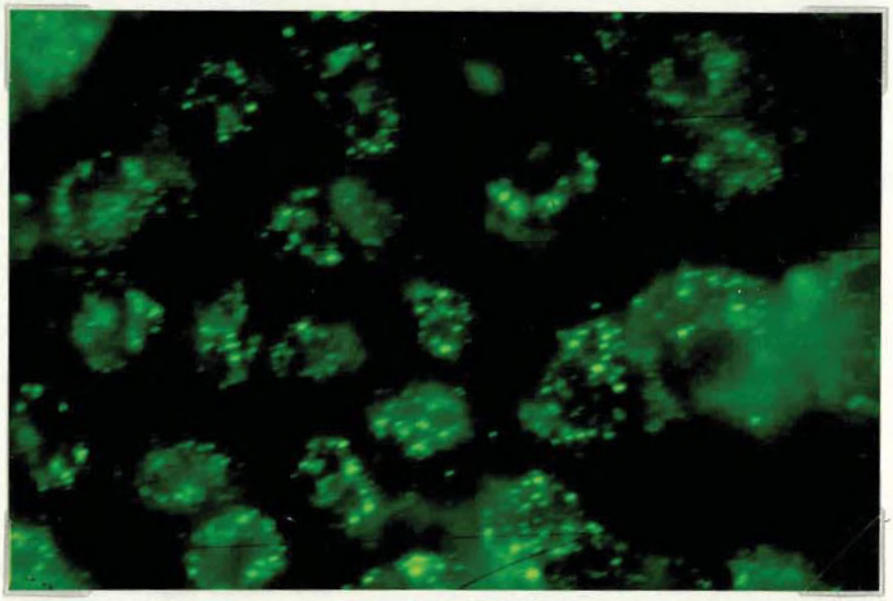
(a) The cells were transferred to fresh medium containing 1nM progesterone and incubated for 1h at 4°C. The cells were processed for IF localization of oestrogen receptor. The cells retained the IF in the cytoplasm only. The cytoplasm is vacuolated in most of the cells shown in this area (x400).

(b) When the cells were exposed to progesterone longer (24 and 48h), the staining pattern remained in the cytoplasm but the IF intensity was much reduced compared to cells grown in medium without exogenous progesterone (x400).

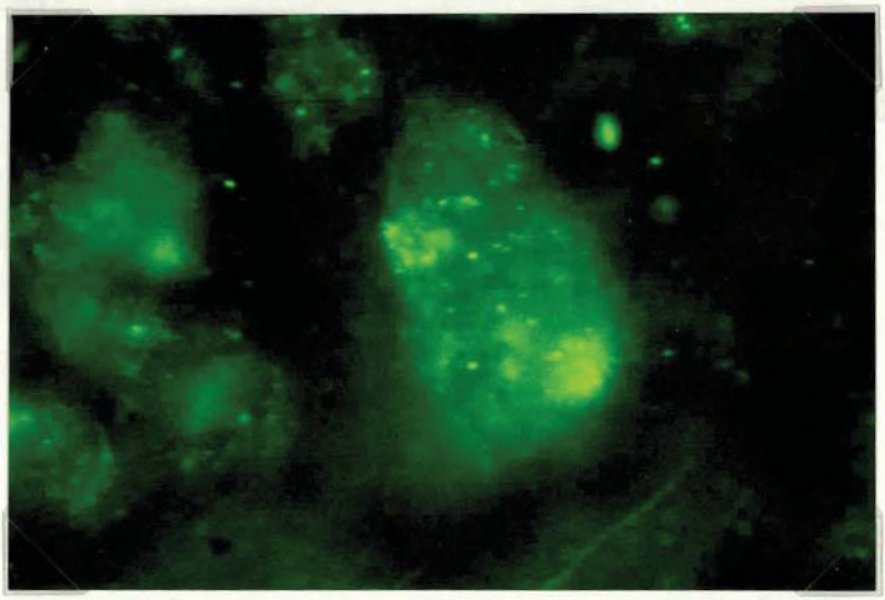
(c) Cells were transferred into progesterone containing medium and incubated for 1h at 37°C. They exhibited slightly stronger staining in the nucleus indicating that some translocation had taken place, though not completely as shown by the presence of cytoplasmic staining (x1000)



(a)



(b)



(c)

3.5.4.3 Incubation with 1nM Progesterone Alone

The cells grown in the presence of progesterone alone in E_2 free medium for 1, 24 and 48h showed a different pattern of staining from above. In the presence of progesterone (exposure for 1h at 4°C) the cells exhibited only cytoplasmic fluorescence (Figure 35a). The same was observed when the cells were grown for a longer period (24 and 48h) in progesterone. However the quantity of staining was obviously reduced when compared to cells grown in E_2 (Figure 35b) which is consistent with the finding that progesterone inhibits the synthesis of oestrogen receptor (Soutter et. al., 1979) in human endometrium (at late follicular phase). When cells were incubated at higher temperatures (22 or 37°C) for 1h, they exhibited nuclear fluorescence along with some cytoplasmic fluorescence (Figure 35c). Quantitatively, the extra-nuclear/nuclear ratio (E/N) of staining was <1 after incubating at 4°C in all the three cases but increased to >1 when incubated at higher temperatures (Figure 36c). In the presence of progesterone translocation seems to occur, even though quantitatively there was less than in the presence of E_2. Similar translocational behaviour was observed by Raam et al. (1983) and Tamura et al. (1984) on ER+ and PgR+ tumours. They found that translocation only occurred in ER+ tumours in the presence of progesterone. In ER+ PgR- tumours only cytoplasmic staining was found. Using goat anti-ER and anti-PgR antibodies, Morel et al. (1981, 1984) demonstrated the presence of both receptors in the cytoplasm of the same cells (gonadotropic, lactotropic and somatotropic cells) of the pituitary.

Biochemically, it has been seen that cytosol oestrogen receptor can be translocated to the nucleus in vitro by a process which depends on temperature, time and dose of E_2 (Sumida and Pasqualini, 1979; Sumida et al., 1982; Holt et al., 1983; Giambiagi et al., 1984).

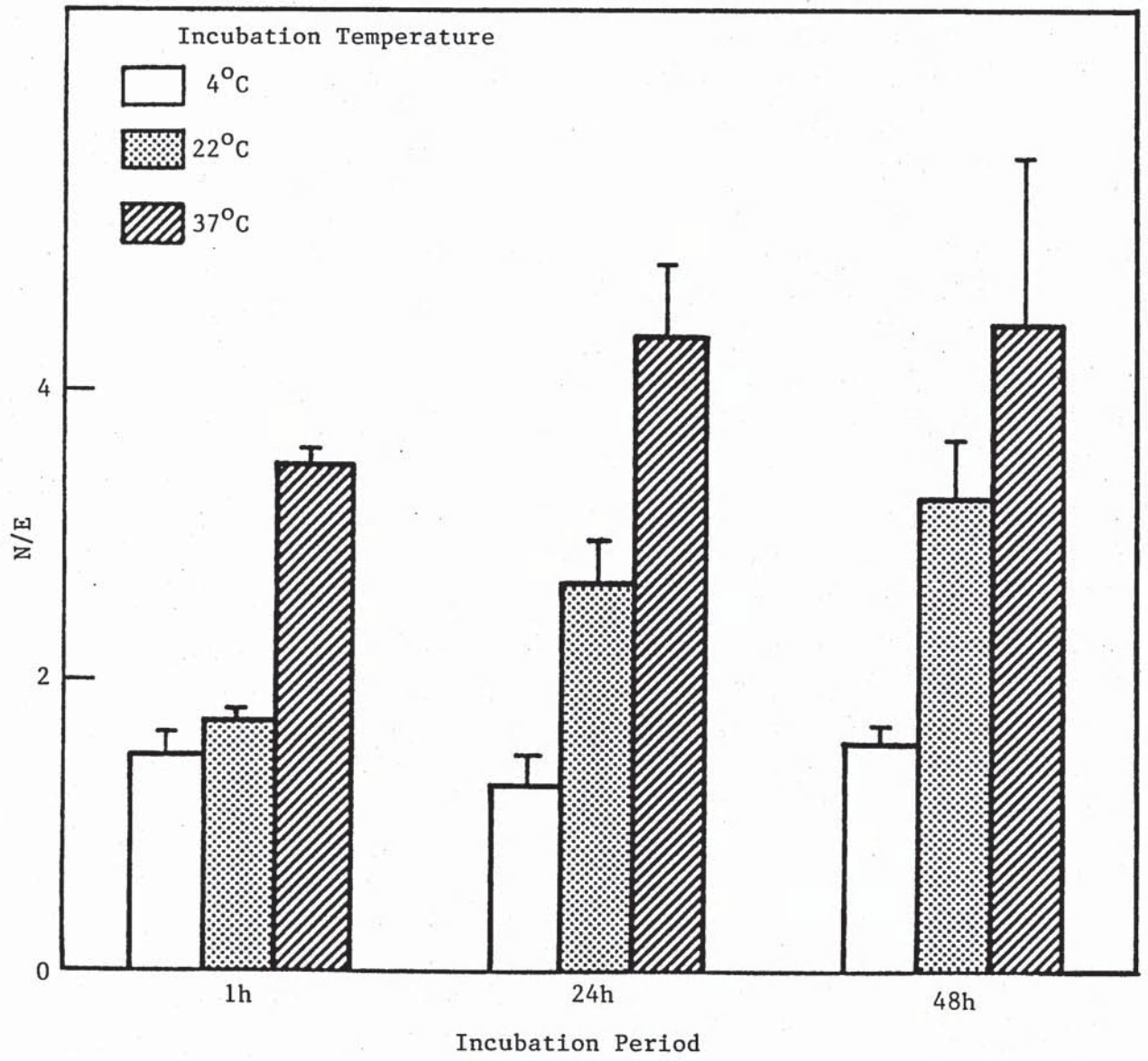
3.5.4.4 Myometrial Frozen Section

Incubating with 1nM E_2 for 1h at 37°C showed immunofluorescence

Figure 36: Quantitative values of intensity of IF staining. Comparison of the intensity of staining in the extranuclear spaces to that of nuclear spaces measured in an adjustable small window, available in the MPV compact photometry linked to fluorescence microscope. The ratio of nuclear intensity value to extranuclear was taken as translocation index of oestrogen receptor. One hundred and fifty cells were measured for each treatment in this experiment. Translocation experiments were carried out in:

(a) the presence of E_2
(b) the presence of DES
(c) the presence of progesterone
(d) Control experiments. Cells were incubated in medium without any steroid.

Figure 36(a): Fluorescence intensity ratio of nuclear to extra-nuclear space (N/E), measured in cells incubated with E_2. The results were the average intensity ratio ± SD. Each bar represents an average intensity ratio of N/E at different temperatures as indicated. All incubations were carried out for 1h.


Incubation Temperature
4°C
22°C
37°C
N/E
0
2
4
1h
24h
48h
Incubation Period

(a)

Figure 36(b): N/E from cells incubated with DES for 1h. There is no difference observed in the pattern of staining as well as the degree of translocation compared to Figure 36(a).

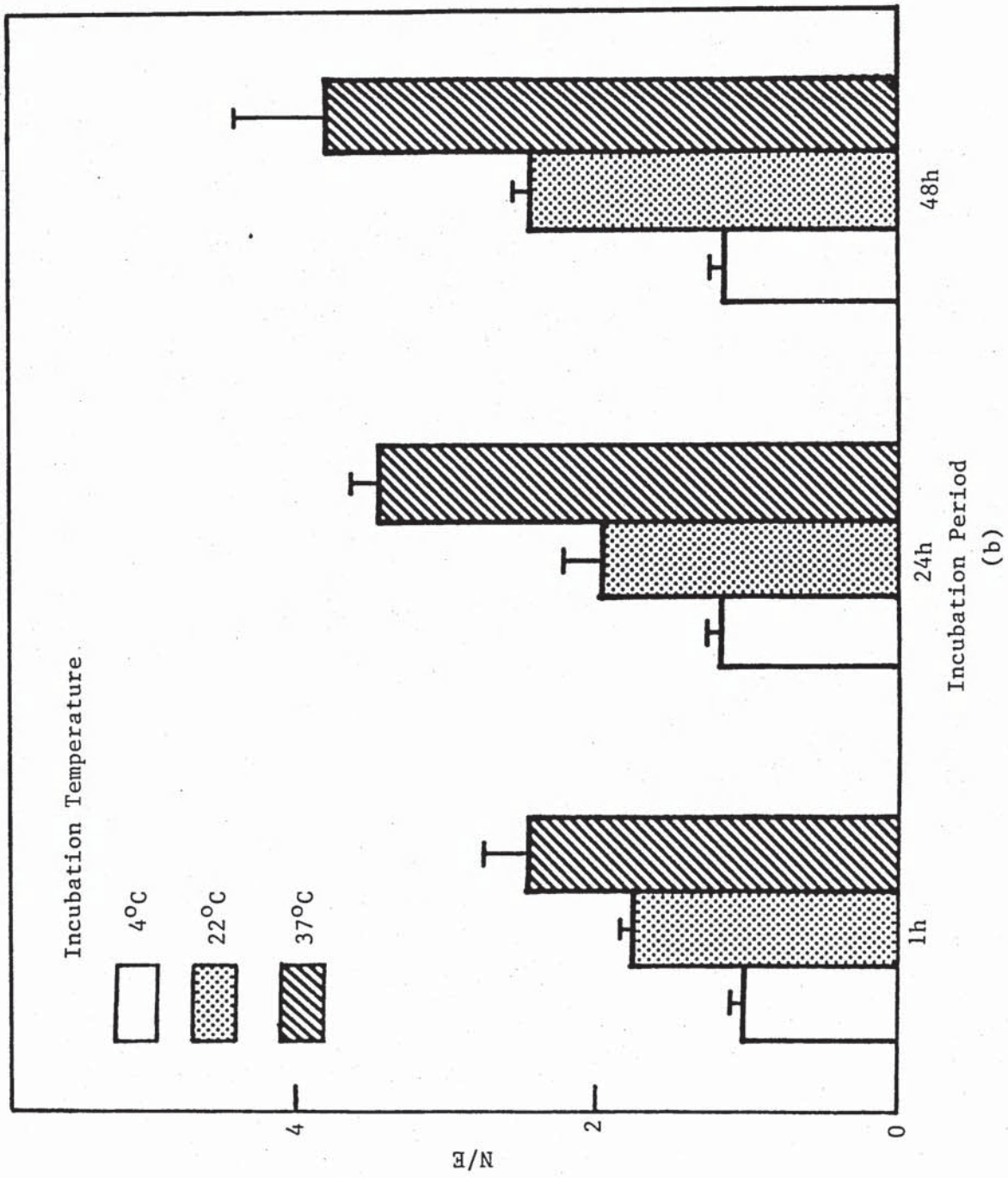
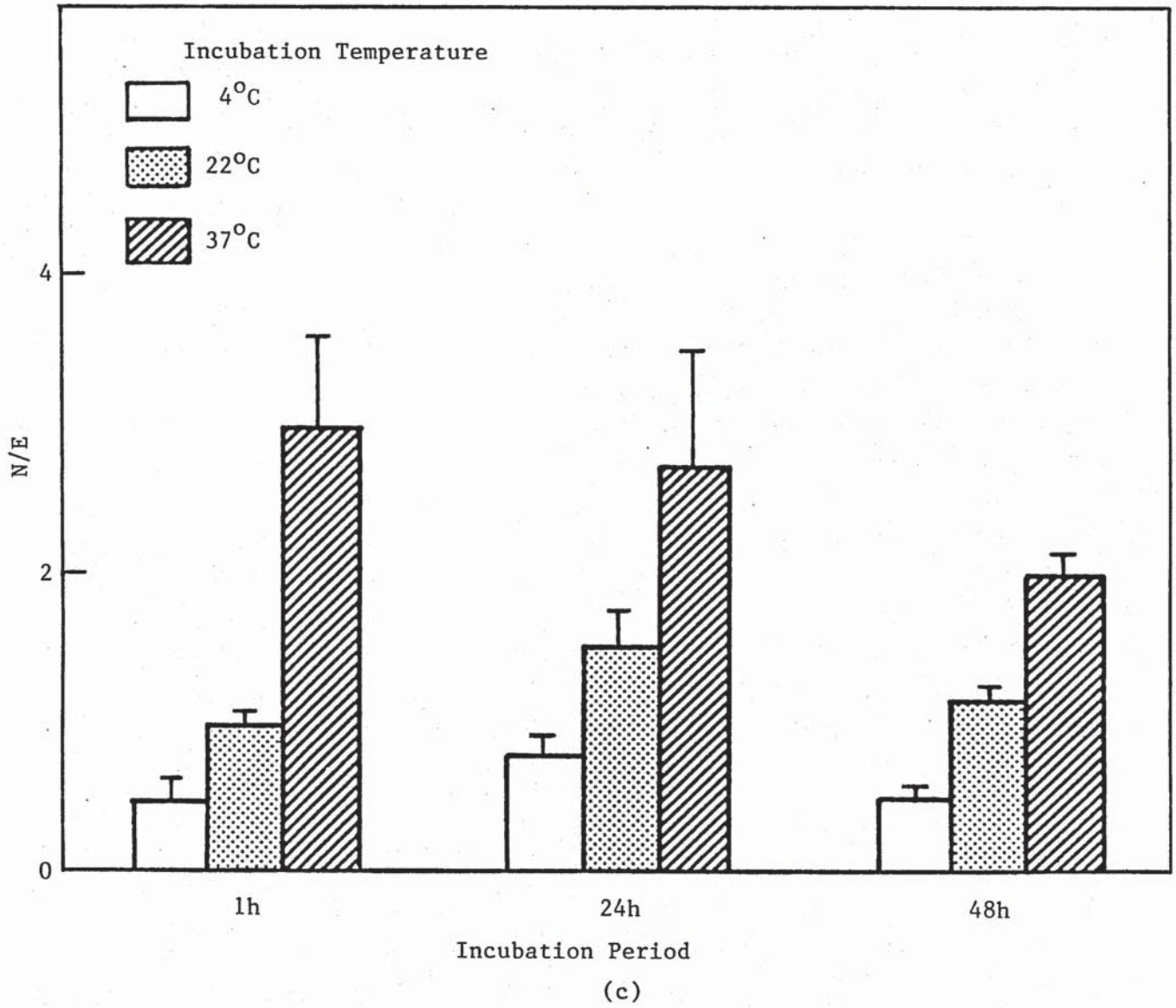
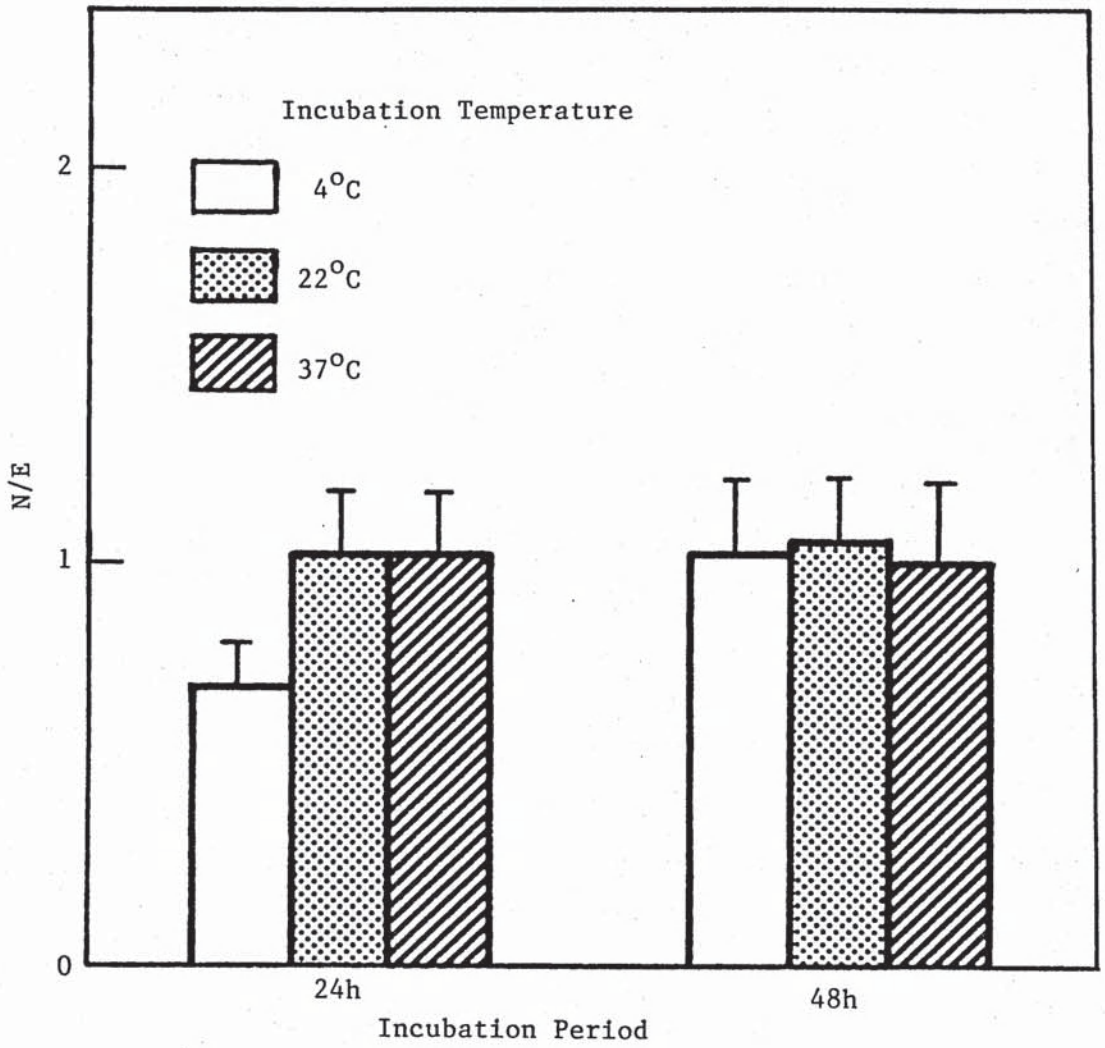

Incubation Temperature
4°C
22°C
37°C
N/E
0
2
4
1h
24h
48h
Incubation Period
(b)

Figure 36(c): N/E from cells incubated for 1h in progesterone. The value is usually <1 due to the absence of staining in the nucleus. Note the reduction in the degree of translocation (incomplete translocation) took place in these cells in the presence of progesterone after a long exposure.


Incubation Temperature
4°C
22°C
37°C
N/E
0
2
4
1h
24h
48h
Incubation Period

(c)

Figure 36(d): N/E measured from cells incubated in the absence of E_2. Note the absence of translocation as shown by the N/E value of about 1.


Incubation Temperature
4°C
22°C
37°C
N/E
2
1
0
24h
48h
Incubation Period

(d)

localized in the nuclei of most cells and in the cytoplasm in some cells (Figure 31c).

3.5.5 Tissue Cultures of Immature Rat Endometrial Cells in E_2 Containing Medium

Endometrial cells, cultured for this experiment by Dr. R. Field (Glasgow University), contained a population of glandular epithelial cells (Figure 32h). Some of the cells demonstrated cytoplasmic as well as nuclear immunofluorescence while others showed denser nuclear staining. The cells were grown in E_2 and immediately fixed (acetone : methanol) after removal from the growth medium.

3.5.6 Oestrogen Receptor in the Liver

Additional experiments were performed on frozen sections of immature rat liver. Immunoreactivity of oestrogen receptor was detected in the cytoplasm though the staining was less intense after incubation of the ultra-thin slices with the antibody diluted to 50-fold (Figure 33k). However, contrary to the result obtained in the human and rat uteri, there was very little immunoreactivity observed with more diluted antiserum (100-fold). This immunohistological observation was of interest, since the report of the presence of oestrogen receptor (Mester and Baulieu, 1972) and E_2 action (Greengard et al., 1965) in the liver.

3.6 QUANTITATIVE APPLICATION OF IMMUNOCYTOCHEMISTRY

3.6.1 Immunofluorescent Studies on FNAs of Breast Cancer

In 50 breast cancer cases, FNA samples were collected at the out patient breast clinic at the Western Infirmary and R.A.I., Paisley. Whenever possible a parallel experiment was conducted using the biochemical assay on the biopsy sample obtained at surgery and the results compared with those of the anti-ER antibody assay.

Immunofluorescence (IF) technique for detection of oestrogen receptor in FNA is very similar to that for frozen tissue and ZR-75-1 cells. Immediately after acetone:methanol or acetone fixation, the cells were incubated with anti-ER antiserum at 100-fold dilution for 45-60min, rinsed, then incubated with secondary antibody (which is goat anti-rabbit conjugated with FITC) and the receptor quantified using a MPV compact linked to a fluorescence microscope. For quantitation, the IF in the area occupied by the cell was measured. Some samples exhibited heterogeneity in the IF. The intensity values of the IF were divided into three categories viz. A = <30, B = between 30-60 and C = >60 (Figure 37). Each value was corrected by subtracting the IF from sample treated with normal rabbit serum in place of primary serum. If more than 90% cells fell into category A, the sample was considered negative. The number of cells counted was 100 to 150 cells. If the cell numbers in the FNA was less than this then the sample was reported as having insufficient cells.

Figures 38a,b and c show samples, positive by both anti-ER antibody and biochemical assays. The staining was clearly demonstrated in both the nucleus and the cytoplasm. Heterogeneity of the staining was observed and found to vary according to the oestrogen receptor status (Figure 39a,b,c). The antibody was selective, as indicated by the lack of any specific staining in cells taken from a tumour that was biochemically ER- (Figure 41a,b).

3.6.2 A Study of Heterogeneity of Tumour Cell Population in Relation to Oestrogen Receptor Distribution

3.6.2.1 ER+ Tumours.

The results of 21 ER+ tumours, by biochemical assay, are given in Table 6. Tumours which had an oestrogen receptor content of >400 fmol/mg cytosol protein were characterized by a high proportion of tumour cells and clearly a high degree of homogeneity with 5/21 cases composed exclusively of IF(+) cells (Figure 38a,c). Tumours with an oestrogen receptor content between 150 to 399 fmol differed

Figure 37: Quantitative values of intensity of IF staining

(a) Comparison of the intensity of staining with the oestrogen receptor concentration (cytosol and nucleus) obtained from the biochemical assay. Corrected intensity values were obtained by subtracting the staining with the normal rabbit serum from the staining of the cells with antiserum.

(b) Distribution of IF staining in the cell smears showing heterogeneity in cell population.

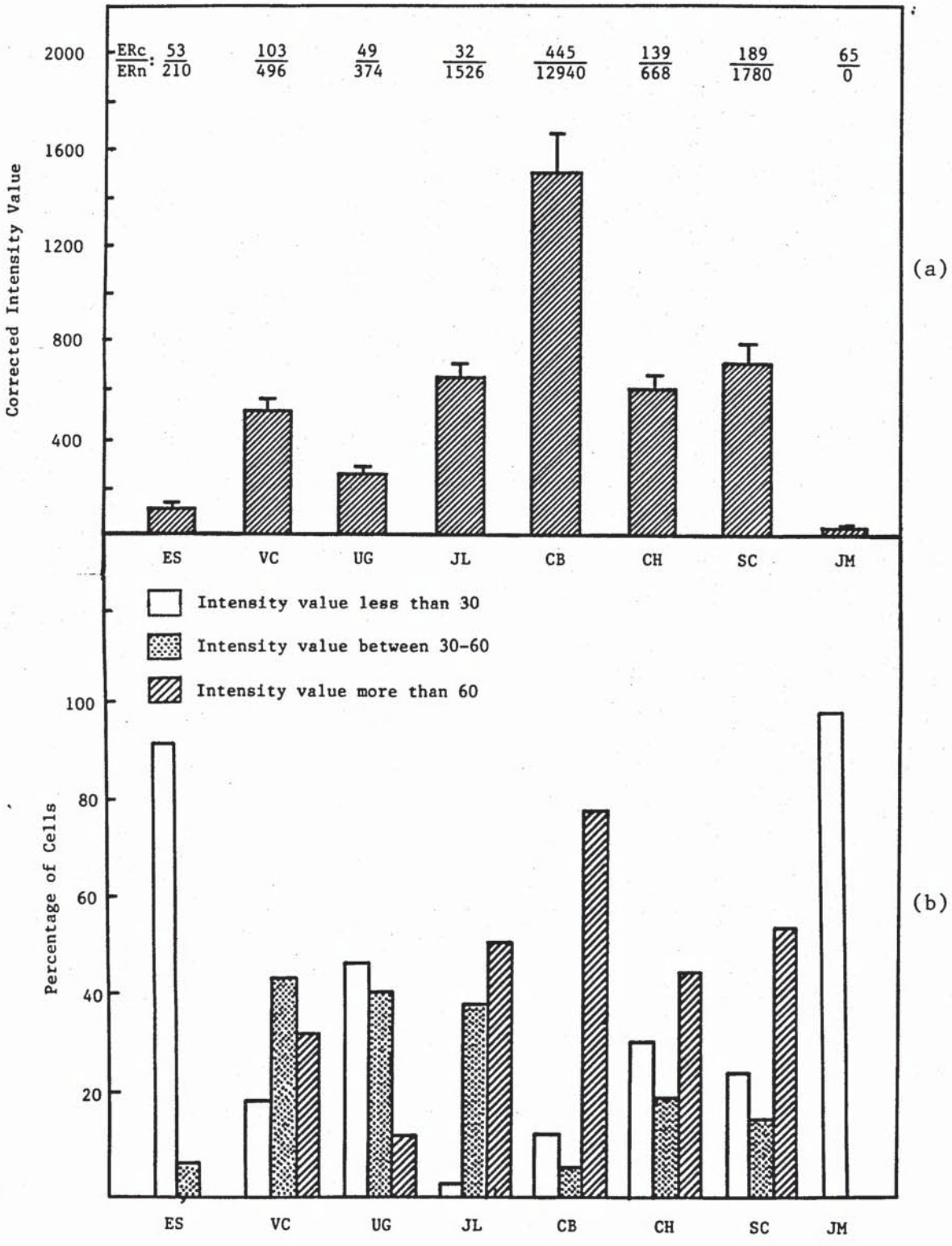

ERc/ERn: 53/210 103/496 49/374 32/1526 445/12940 139/668 189/1780 65/0
Corrected Intensity Value
2000
1600
1200
800
400
(a)
ES VC UG JL CB CH SC JM
Intensity value less than 30
Intensity value between 30-60
Intensity value more than 60
Percentage of Cells
100
80
60
40
20
(b)
ES VC UG JL CB CH SC JM

Table 6: Comparision of oestrogen receptor results by the biochemical method and anti-ER antibody assays.

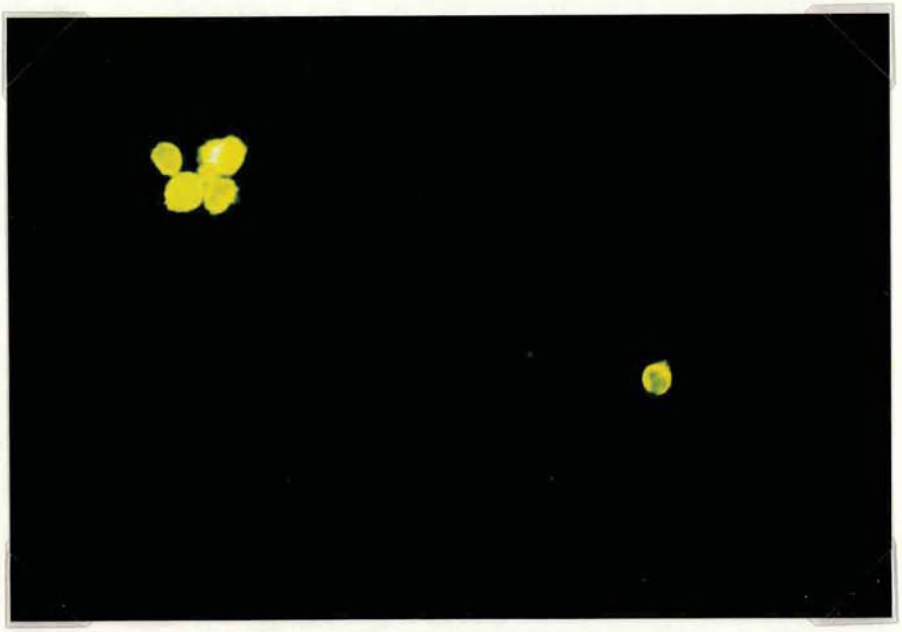
Oestrogen Receptor Status by Standard Biochemical Assay		Pre-operative FNA Anti-ER Antibody Assay	
		+	-
ERc+/ERn+	21	20	1
ERc-/ERn-	26	2	24
ERc+/ERn-	3	0	3
ERc-/ERn+	0	0	0
Total	50	22	28

Figure 38(a-c): The FNA samples were analysed using the anti-ER antibody assay.

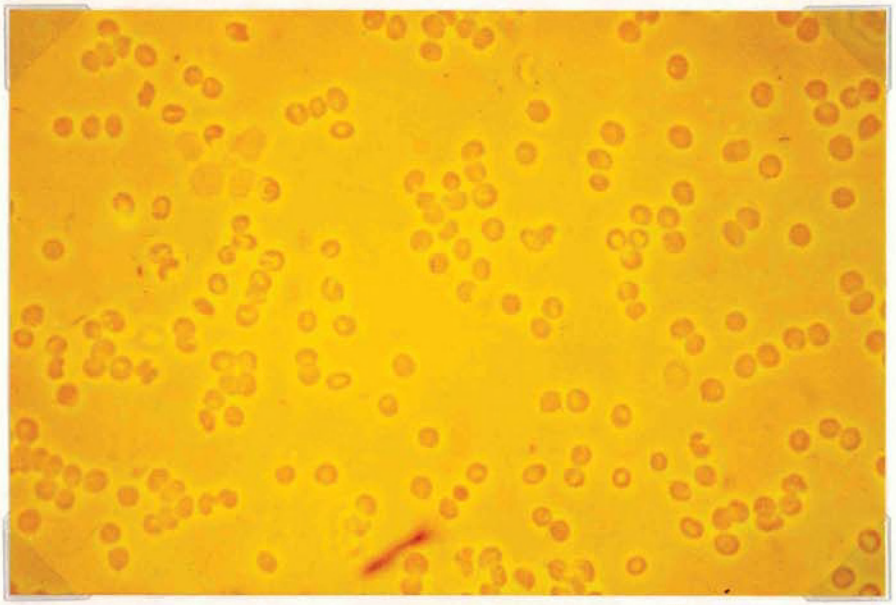
(a) Intense cytoplasmic as well as nuclear staining indicated that the anti-ER antibody reacted with most cells in this sample which fell into the group with >400fmol/mg protein (Patient C.B., Figure 37) (x250).

(b) The phase contrast showed the tumour cells among the red blood cells (x250).

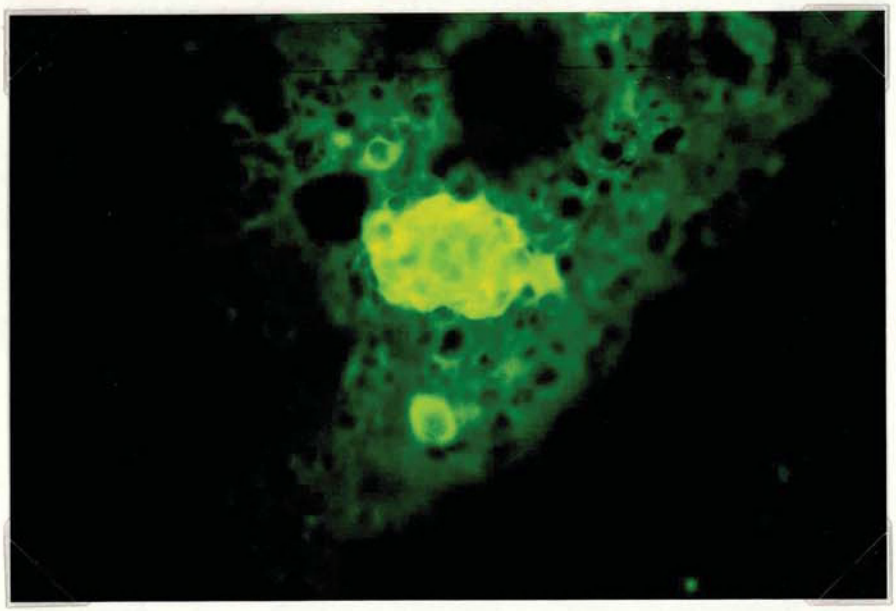
(c) FNA sample from another patient in the same group. Similar pattern of staining were seen (x250).



(a)



(b)



(c)

from the first category of tumours in exhibiting a degree of heterogeneity containing a mixed population of IF(+) and IF(-) cells (Figure 39a,b). The next category of ER+ tumours (20-149 fmol/mg) showed extensive tumour heterogeneity (Figure 39c). Tumour with no ERn (ERc+/ERn-) had >95% IF(-) cells (40a,b).

3.6.2.2 ER- Tumours

Of 26 biochemically ER- tumours, 24 were exclusively IF(-) and the remaining 2 contained a mixture of IF(+) and IF(-) cells of which the latter was in the majority. One sample showed a group of tumour cells which had nuclear staining (Figure 41c).

3.6.3 Anti-ER antibody assay Versus Standard Biochemical Assay

A good correlation of results was obtained with the oestrogen receptor status of the tumours for 47 out of 50 cases of FNA samples in which the biochemical data on oestrogen receptor values were available (Table 6). Twenty out of 21 cases were ER+ by both antibody and biochemical assays. In the one exception, it was not possible to estimate the proportion of tumour: mesenchyme cells in the biopsy, but in the FNA sample the number of IF(-) cells outnumbered the IF(+) cells.

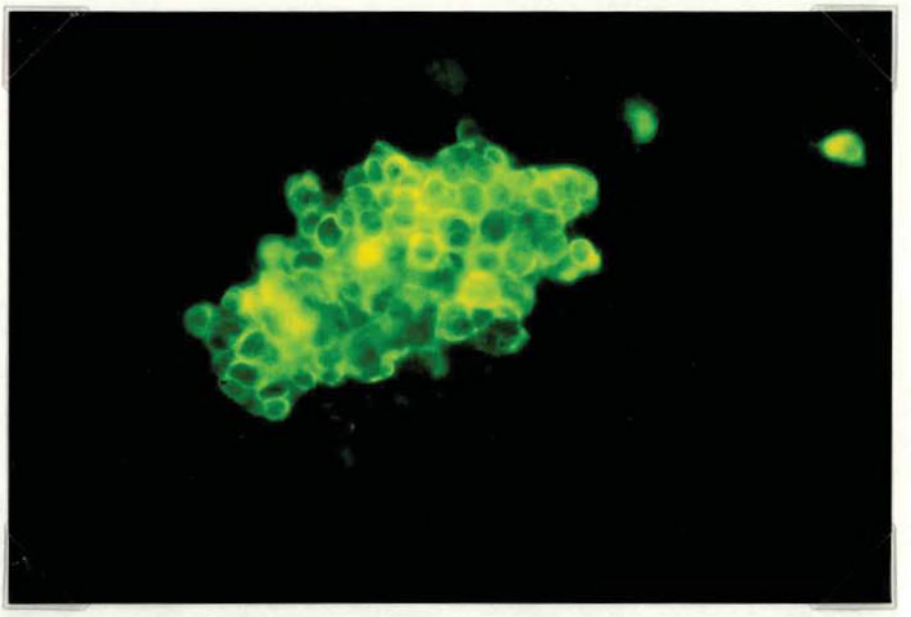
Since the smears of cells were spread over a wide area, it was impossible to count all the cells. Therefore 100 to 150 cells picked at random were quantitated and the number of IF(+) and IF(-) cells were tabulated based on the intensity values (Figure 37). No direct correlation was found between the intensity of IF and quantity of oestrogen receptor (Figure 37). However the cell morphology was very well preserved. Therefore, for cases in which the tissue material was limited, a study of cytological smears by anti-ER antibody procedure may prove to be the best alternative for determining the oestrogen receptor status of the tumour.

Figure 39(a-c): Photographs (a) and (b) were from FNA samples from tumours with 150-399fmol/mg protein (biochemically determined).

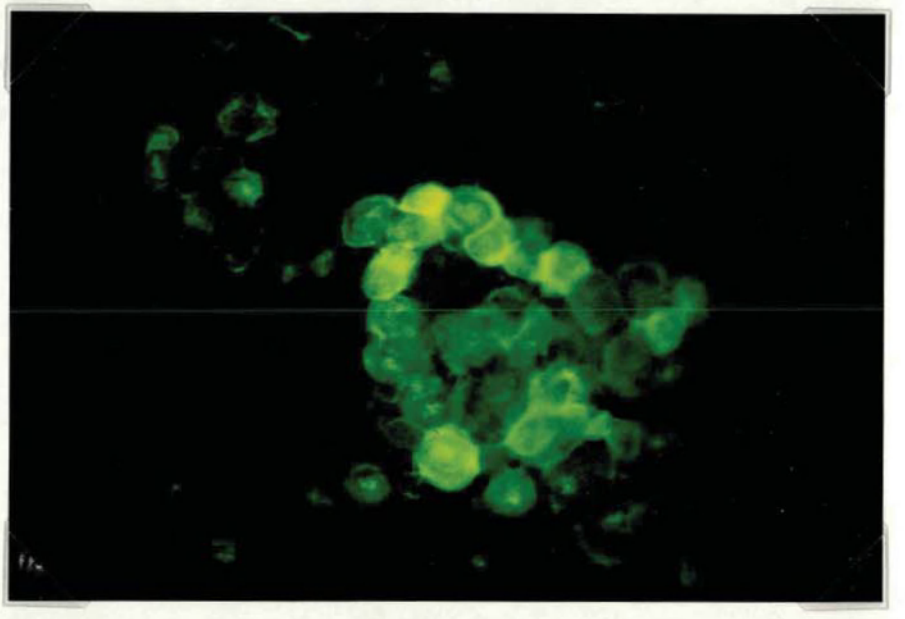
(a) Patient S.C., Figure 37. (x250)

(b) Patient C.H., Figure 37.
Heterogeneity existed among the IF+ tumour cells from these patients. The IF- cells appeared to be dispersed and small in number (x400).

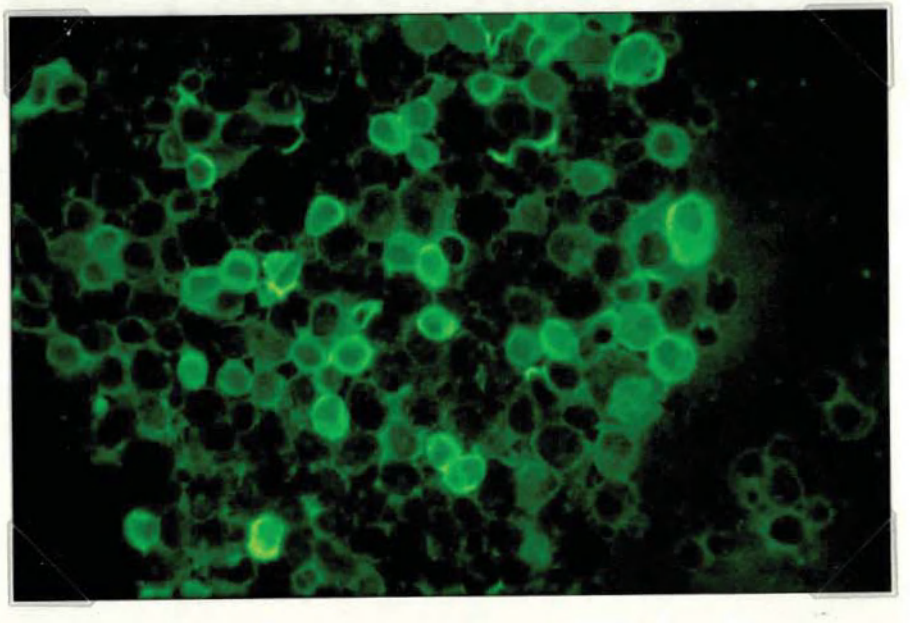
(c) This sample was from a group of tumours with 20-149fmol/mg protein (Patient U.G., Figure 37). Heterogeneity of IF+ and IF- cells are shown in this photograph (x400).



(a)



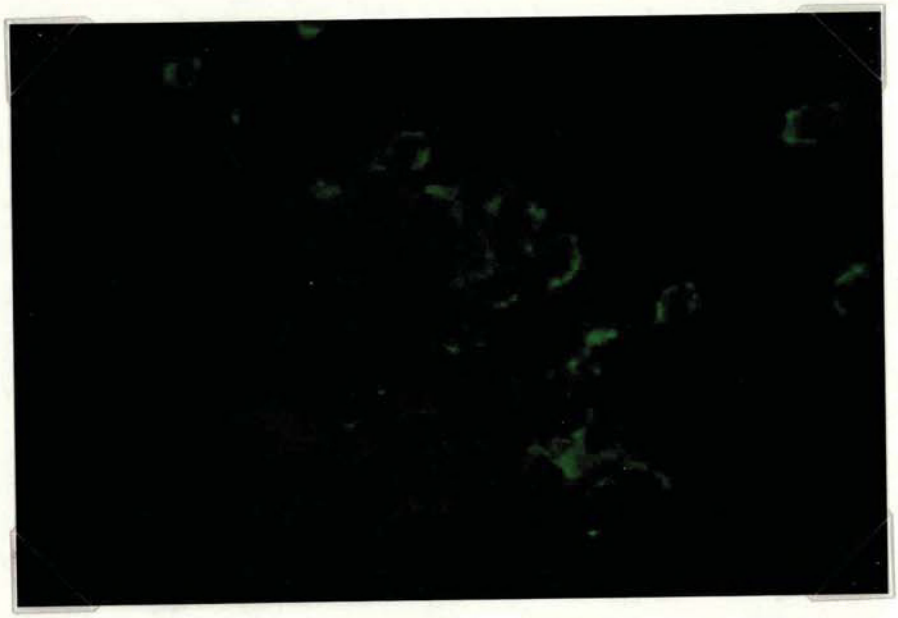
(b)



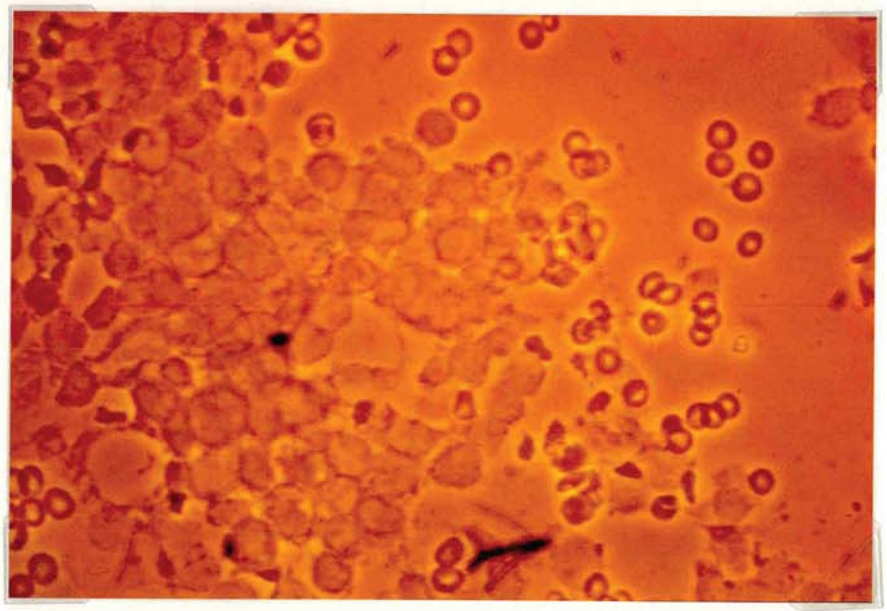
(c)

Figure 40(a): This sample (Patient J.M., Figure 37), which contained cytoplasmic but no nuclear receptor (clinically receptor negative) displayed cells which were IF(-).

(b) A phase contrast photograph of (a) (x400).



(a)

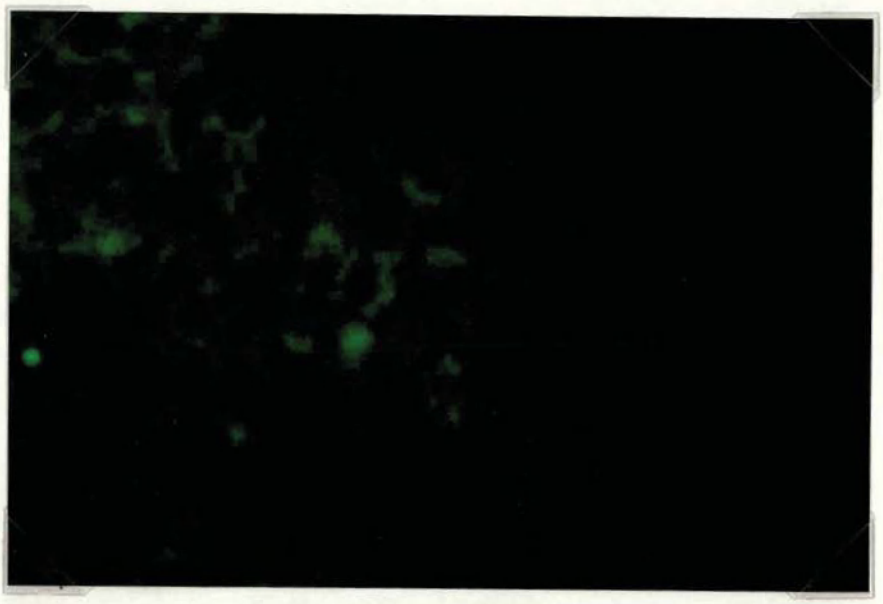


(b)

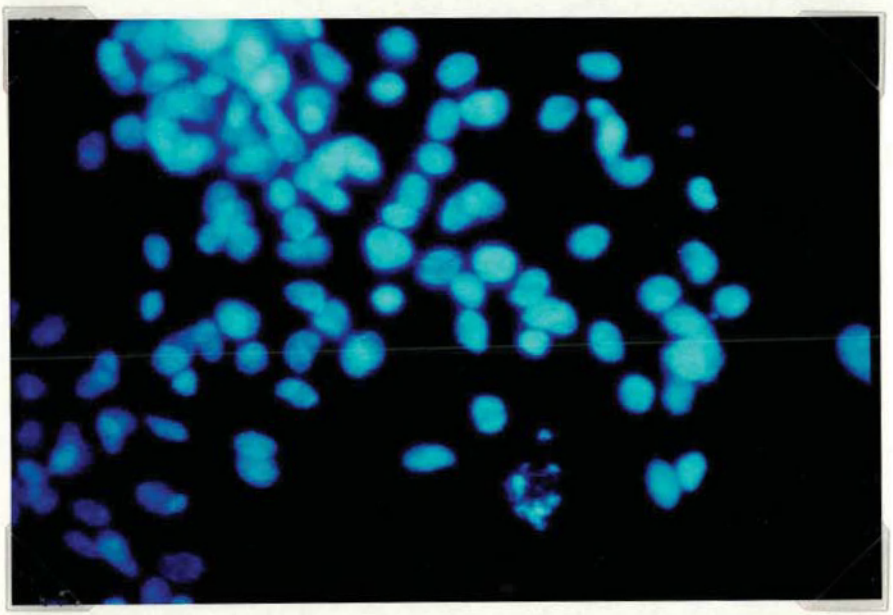
Figure 41(a): Antibody assay on ER- breast cancer. No cytoplasmic as well as nuclear fluorescence indicated the absence of any anti-ER antibody reactive cells in this tumour which is ER- by the biochemical assay (x400).

(b) The same sample counterstained with Hoechst. The presence of the DNA staining showed that tumour cells were present in this sample (x400).

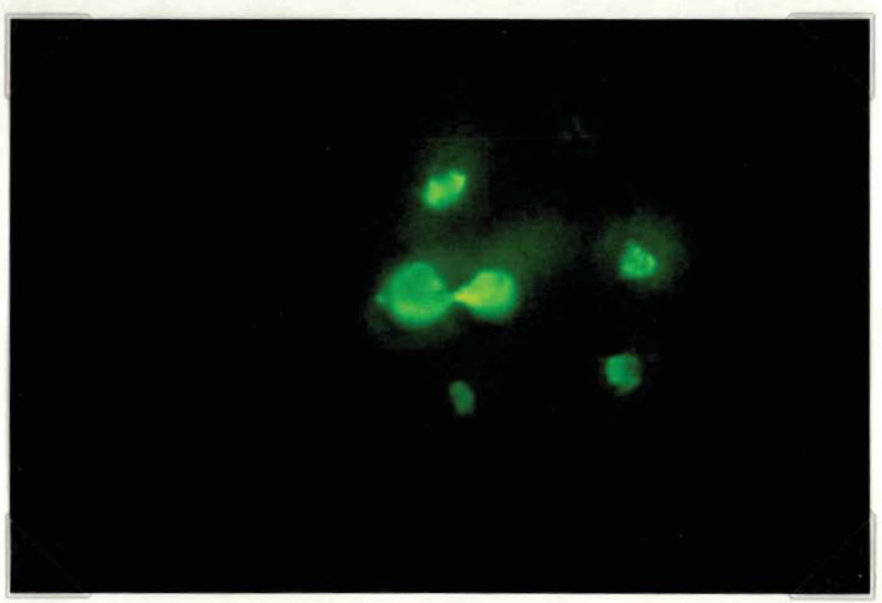
(c) Nuclear staining observed in a group of cells from one of the ER- breast cancer biopsies (determined biochemically) (x1000).



(a)



(b)



(c)

4.1 EARLY ATTEMPTS ON OESTROGEN RECEPTOR PURIFICATION

The initial efforts to purify the oestrogen receptor from human breast cancer tissue involved the use of protein separation by disc-PAGE, DNA-cellulose chromatography, DEAE Sepharose and Phenyl-Sepharose. Using these techniques the degree of purification obtained was less than 260-fold, primarily due to the loss of substantial amounts of binding activity.

Two peaks (peaks A and B) of protein binding were detected when the cytosol prepared from human uterus were run on SDS-PAGE. Peak A appeared at 240,000 molecular weight while peak B was about 65,000 molecular weight. The comparatively high levels of E_2 binding sites in the tracking dye peak of the uterine cytosol was probably due to vascularity of this tissue and hence high levels of $[^3H]E_2$ dissociating from the serum albumin. Skipper et al. (1985) reported the appearance of two peaks from rat uterine cytosol designated as F-fast peak and S-slow peak. The F peak had been calculated as 65,000 molecular weight. To account for the effect of stacking on the oestrogen receptor, a hypothesis was postulated by Skipper et al. (1985) based on the events occurred during the stacking process and on the three assumptions concerning the structure and transformation of the native receptor. These assumptions are as follows: (a) the peak of 65,000 molecular weight is the native receptor; (b) looking at the number of peaks, at least some of its subunits are different proteins which are bound together by noncovalent forces; and (c) dissociation of these subunits causes transformation. During the stacking process, each protein in the sample is concentrated into individual ultrathin zones which are arranged in an order according to their individual surface charge (Ornstein, 1964).

The hypothesis is stated as follows: (a) the oligomeric subunits of the receptor are dissociated by the stacking forces of

disc-PAGE; (b) the subunits do not reassociate because they are different proteins which are separated into individual zones of the "stack"; (c) from the third assumption (dissociation = transformation), the preceding steps result in transformation of the oestrogen-binding subunits to a DNA-binding state which then binds tightly to some extraneous component (perhaps RNA) in the crude cytosol, thus forming aggregates which are too large to move into the polyacrylamide-separating gels. Ben-Or and Chrambach (1981) also concluded that the glucocorticoid receptor is converted to similar aggregates in disc-PAGE analysis. Applying this hyphothesis to the observations seen in this study, peak A is the peak that is equivalent to the aggregated form of oestrogen receptor that is too big to pass through a great distance in the disc-PAGE. It was this peak that was being cut, pooled and injected to the experimental animal. This peak of 240,000 molecular weight, a species which according to others (Puca et al., 1973; Sica et al., 1976; Bresciani et al., 1978; Sica and Bresciani, 1979) is the large molecular weight oestrogen receptor.

According to the calculation, the amount of oestrogen receptor complexes used for immunizing the experimental animals for antibody production was equivalent to 100fmol, and assuming a molecular weight of 240,000 and a stoichometric ratio of four E_2 per molecule of receptor, 24ng was injected. However no antibody was detected even though Raam et al. (1973; 1981) reported that polyacrylamide had an adjuvant effect. Overestimation of the total protein injected was a possibility that cannot be excluded. The presence of non-functional receptors incapable of binding E_2, was also another possible factor. If the non-functional phenotypes had an electrophoretic mobility identical to that of the $[^3H]E_2$ -receptor complexes, they will be included to the protein bulk.

Using chromatography techniques, the purification procedure described in this study was based on the ability of the steroid-receptor complex to interact with DNA following an activation step. The differential affinity of the nonactivated and activated forms of the steroid-receptor complexes for DNA-cellulose

had proved a useful tool in the characterization or the partial purification (or both) of the androgen receptor in rat prostate (Mainwaring and Irving, 1973), the subunit of the progestin receptor in chick oviduct (Kuhn et al., 1977), the glucocorticoid receptor in rat liver (Kalimi et al., 1975; Wrange and Gustafsson, 1978) and the oestrogen receptor in rat uterus (Yamamoto and Alberts, 1972; Yamamoto, 1974).

Several groups had reported the partial purification of steroid receptor by chromatography procedures on DNA cellulose and/or DEAE Sepharose (Soloff and Szego, 1969; Puca et al., 1973; Wrange et al., 1979). However, these previous studies, as well as this work, did not result in either a sufficiently pure receptor preparation or in sufficient amounts of the purified receptor to allow further characterization of the protein by, for example, SDS-gel electrophoresis. Available evidence suggested that variable degrees of denaturation of the receptor molecule were responsible for the wide distribution of binding activity in eluates from DEAE Sephadex. Denaturation was likewise believed to be the cause of loss in the receptor activity occurring during the purification (Soloff and Szego, 1969).

The use of phenyl-Sepharose had some advantages. Phenyl Sepharose had a capability of binding non-activated receptor. The use of this matrix as the last chromatographic step was to recover as much oestrogen receptor as possible from the previous preparation. In addition, the eluted steroid receptor complex seems to be more stable in TED:ethylene glycol:glycerol solution than in buffer containing high concentrations of salt that were required to elute the complex from DNA cellulose and DEAE sepharose. Furthermore, when eluted with TED:ethylene glycol:glycerol, the resulting receptor preparation can be further characterized on SDS-PAGE without undergoing dialysis as the ionic strength was low. However, disc-electrophoresis showed that this final purification step resulted in a relatively heterogeneous protein content. Due to this reason a better and more efficient method of purification was sought.

4.2 AFFINITY CHROMATOGRAPHY

Because of the minute amounts of oestrogen receptor in target tissue extracts (less than 5mg of oestrogen receptor is recovered per kg uterine tissue processed) it was convenient to employ a concentration step using ammonium sulphate precipitation prior to the subsequent purification procedure. Purification of soluble oestrogen receptor of uterus was also facilitated by the treatment of the uterine cytosol with calcium ions in the presence of salt to yield a 'stabilised' 4S binding unit which did not aggregate or revert to the native low-salt form (8S) when the salt was removed (Sica et al., 1973).

The purification of oestrogen receptor had been hindered by various factors such as endogenous proteases, heat liability, and the low levels of receptor in available tissues. These problems could be overcome by the use of affinity chromatography, by the addition of the appropriate protease inhibitors, and by working at lower temperatures (Puca et al., 1977; Sherman et al., 1983). The problem of stabilization of the binding activity of the oestrogen receptor long enough to load it on an affinity column has been solved primarily by: (1) working at 5°C; and (2) using compounds which can act as protease inhibitors, in particular, EDTA. EDTA is presumed to inhibit a Ca^{2+}-activated protease present in uterus (Puca et al., 1972; Sica et al., 1976; Puca et al., 1977). All these compounds have been found to stabilize the E_2 binding activity of crude cytosol for at least 2 days at 4°C in TRIS buffer. Although E_2 affinity columns had been available for the last 10 years, they had been of limited use because of stability and elution problems due to certain physical properties of both E_2 and the receptor. The receptor has a K_d of 10^{-10}-10^{-11}M with a dissociation rate half-life of over a day at 4°C (Clark and Peck, 1979). The limited solubility of E_2 (micromolar range) in aqueous buffers and the fact that the unbound receptor itself is unstable required special attention. Overall, rapid processing of large volumes of material with low levels of unstable receptor, elution in a nondenaturing solvent, and storage under stable conditions were essential in the purification of the receptor protein.

The stability of the affinity column is essential and they should also have the proper selective binding affinity. Two procedures of synthesis of affinity resin have been published. One involved E_2 linked to agarose through the 17β position via an ester bond (Sica et al., 1973; Sica and Bresciani, 1979; Puca et al., 1980) and the other involved E_2 linked to agarose via a 17β -thioester bond (Greene et al., 1980).

Affinity chromatography with adsorbents containing steroid molecules linked to agarose through the 17β position via an ester bond had been employed in this study of oestrogen receptor purification. This method of purification has also been applied to oestrogen receptor purification by Luden et al. (1972) and specific serum binding proteins for testosterone by Burstein (1969) but they were not successful in the elution procedure. Luden et al. (1972) had described in detail the general problem also reported by others (Cuatrecasas and Anfinsen, 1971; Cuatracasas, 1972). Rosner and Bradlow (1971) had described the purification of corticosteroid-binding proteins from human plasma by using agarose columns containing cortisol hemisuccinate and Sica et al. (1973), on oestrogen receptor purification, using agarose columns containing E_2-hemisuccinate.

Some problems that had been commonly met were; (1) the removal of the noncovalently adsorbed hormone in the preparation of E_2 affinity chromatography. This is important before the columns could be used (2) The tenacious nature of the oestradiol-gel interaction requires the gels to be extensively washed over prolonged periods of time with very large volumes of organic solvents or with protein solutions such as albumin or crude uterine supernatant. This is important as it could alter the partitioning properties of E_2 between the gel and the aqueous medium and thus markedly enhance the release of adsorbed hormone from adsorbents, (Cuatrecasas, 1972; Luden et al., 1972). Failure to wash the gel adequately can result in the release of large quantities of E_2 into the sample containing oestrogen receptor. Release of adsorbed E_2 during chromatography of the sample may lead to the erroneous conclusion that the column is effectively removing the receptor from the sample. Of course, the

subsequent recovery of oestrogen receptor by specific elution was conclusive proof that the affinity column had extracted the macromolecule.

The amount of E_2 release was found to be negligible in this study. Puca et al. (1973) also reported the same observation, but others (Atrache et al., 1985; Binder, 1985; Lubahn et al., 1985) had reported a significant release of the free hormone especially when kept at room temperature. Even if there is a release of some free E_2 from the gel this does not necessarily mean that the adsorbent will be ineffective in selectively extracting the receptor from the sample. If the affinity of the free hormone released from the gel is not very different from that of the gel-bound hormone, the small amount of free hormone will not compete effectively with the much greater amount of immobilized hormone and the receptor will thus preferentially bind to the solid support. If, however the free hormone had a much greater (e.g. 10^3 times) affinity, a very small free fraction (0.1% of the total) would interfere with selective adsorption.

The application of tissue extracts to affinity columns with an E_2 derivative in which the hormone was attached to agarose through position 3-O-ester bond was reported to result in the catalytic cleavage of matrix-bound hormone molecules as well as resulting in the facilitated release of hormone which is adsorbed to the gel by noncovalent forces (Sica et al., 1973). The E_2 derivative in which the hormone was attached to agarose through position 17β of the E_2 molecule (Figure 7) proved to be more stable from attack of esterase, even though Lubahn et al., (1985) reported its lack of stability compared to the attachment of the E_2 derivative to agarose via the thioester bond. These E_2 derivatives retain substantial affinity for the receptor and immobilization of these derivatives on the agarose gels does not cause a further serious decrease in the affinity for the receptor. The presence of macromolecular polymers attached to the agarose backbone at several points could greatly increase the stability of the subsequently attached ligands. This phenomenon could reduce the loss of E_2 into the solution. The other

factor that should be considered is the basic cyanogen bromide linkage in the agarose gel. Sica et al. (1973) found a certain instability, which must be taken into consideration in experiments of receptor purification. However the presence of multipoint attachment of a branch protein copolymer should drastically reduce the potential problems which could result from spontaneous cleavage of the basic cyanogen bromide linkage groups. For this reason a branched copolymer of albumin or denatured albumin is used. Such a derivative also possesses numerous functional groups (acid-amino groups) which would serve as focal points for further substitution reactions. The present studies show that, at 4°C, these bonds are sufficiently stable to permit experiments under conditions where the release of free hormone is negligible and does not interfere with effective adsorption of the receptors to the gel.

Another factor which governed the release of free E_2 was insufficient washing by organic solvent. The gel can be diluted with unsubstituted agarose and still effectively adsorb receptor. This is due to the presence of the macromolecular arms, as explained by Cuatrecasas (1972). This diluted gel can be washed relatively easily to remove noncovalently bound steroid, and it will give little nonspecific adsorption of non-receptor proteins during chromatography of the uterine extracts. The hormone molecules in these derivatives are separated from the backbone of the agarose by about 150A and chemically neutral extensions, features which are known to be very favourable for selective interactions in affinity chromatography (Cuatrecasas and Anfinsen, 1971; Cuatrecasas, 1972).

4.2.1 The Use of Fresh Tissue in Antigen Preparation

In this experiment, the freezing of human uteri prior to oestrogen receptor isolation had no apparent influence on receptor molecular size. Atrache et al. (1985) reported a similar observation with calf uterine tissue. However, Sherman et al. (1983) had demonstrated a decrease in the size of glucocorticoid receptor complexes isolated from frozen rat liver as compared to fresh

tissue. The results indicated that the activity of particular enzymes responsible for receptor degradation may be lower in cytosol prepared from frozen uterus than in extracts derived from frozen liver. Other explanations for these observations include the differences in the levels of endogenous inhibitors and/or activators of proteolysis in different tissue systems.

The experiment did not use molybdate treated cytosol, since it was found that it did not change the sedimentation coefficient or molecular weight of the affinity purified receptor. Atrache et al. (1985) reported the same observation. They found difficulty in the isolation of molybdate-stabilized 9-10S oestrogen receptor from human uterus. In contrast to the calf oestrogen receptor, they found that the receptor from human uterus was completely converted from the 9-10S species present in crude cytosol to a 4S receptor during their heparin-Sepharose chromatography. This might suggest the existance of a component, present in human uterine cytosol, which allows salt-induced dissociation of the 9S oestrogen receptor even in the presence of molybdate. Purification procedures using Ca^{2+} ions and high ionic strength together inhibit aggregation and result in 4S oestrogen receptor on a sucrose gradient (De Sombre et al., 1969; Puca et al., 1970). Puca et al. (1971) showed that Ca^{2+} ions activate a macromolecular receptor transforming factor (RTF) which operated the transformation of 8S into stable 4S oestrogen receptor.

4.2.2 Extraction of Receptor by E_2-Succ-Alb-Sepharose 4B

Despite many reports regarding the instability of the adsorbent, it is of interest that the E_2-Succ-Alb was effective in terms of its ability to extract receptor molecules from tissue extracts. Oestrogen receptors bind to oestradiol-hemisuccinate with an affinity of $K_d=1.0\times10^{-7}M$, a value which is lower than the affinity of receptor for E_2 but slightly higher than the affinity of receptor for oestrone carboxymethyloxime ($K_d=1.3\times10^{-6}M$) (Atrache et al., 1985). This is consistent with the report of Bresciani et

al. (1970) who found that the oestrogen receptor has an affinity towards oestrone one order of magnitude less than E_2. This oestrogen-hemisuccinate displayed sufficient selectivity to be a useful ligand in the preparation of an affinity resin. Sepharose 4B, with spacer arm activation, gave a number of affinity gels ranging in steroid content from 0.3 to 0.5µmol/ml packed gel. The more dilute gels, in which nonspecific ion-exchange effects were eliminated by complete steroid coupling of the spacer chain gave consistently purer receptor preparations.

Purification of the receptor on the affinity column requires a ligand-free binding site. Incubation of human uterine cytosol with Sepharose-bound conjugates resulted in a significant decrease of cytosolic oestrogen receptor content. This decrease in oestrogen receptor content suggests an in vitro interaction of conjugate with cytosolic oetrogen receptor. As E_2 release was sufficiently low, it had been possible to detect the non-specific binding proteins contaminating the preparation. These experiments required prebinding of the receptor with saturating levels of very low specific activity E_2 and then loading, washing and eluting the affinity column in the regular manner. When the oestradiol-saturated crude extract was applied to the column and then subjected to affinity column chromatography under the same conditions as the crude extract, but without added E_2, the 65,000 molecular weight was singularly absent (Figure 18). Washing the column with insufficient amount of $TEDK_{0.4}$ showed the presence of all the other endogenous proteins (Figure 18) suggesting that they are not binding to the oestrogen receptor but rather directly to the affinity column by a nonspecific interaction which was oestrogen independent. Also after treatment of cytosol with control columns (resin without E_2-succ-Alb), very little oestrogen receptor was bound [not exceeding 10% according to this study, (Table 2)] which futher supports the low nonspecific binding of oestrogen receptor to the control materials. Thus this small percentage of binding was not due to conjugate-receptor interaction. This derivative, however, had been found difficult to handle in the elution steps thus making the yield very small but nevertheless very pure (Table 5).

Elution of the oestrogen receptor is achieved by DES (Coffer and King, 1980, 1981) at either 5° or 25-30°C (Sica and Bresciani, 1979; Greene et al., 1980; Puca et al., 1980). Warming to 30°C has been found to reduce the time necessary for E_2 to reach equilibrium binding with receptor, without significantly altering the binding affinity (Sica et al., 1973, 1980; Greene et al., 1980; Sica et al., 1981; Lubahn et al., 1985). Eluting the oestrogen receptor using E_2 had the disadvantage of eluting other proteins which have low affinity for E_2 but not for DES. This could be avoided by adequate washing with $TEDK_{0.4}$. Bresciani et al. (1971) reported that, with respect to E_2, the 8.6S and 4.5S oestrogen receptor both have an affinity 1.5 to 2.9 times higher for DES. Attempts to increase receptor yield by the use of buffers containing sodium thiocyanate were not very successful but adding 10% dimethylformamide into the mixture helped to increase the yield significantly (Table 3).

4.2.3 SDS-PAGE of the Affinity Eluate

The major endogenous band seen in the aliquot from the E_2 eluate pool was presumed to represent the oestrogen receptor as verified below. The presumptive oestrogen receptor band first becomes evident when E_2 binding affinity is initially detected in the eluate. A molecular weight estimation based on the migration of protein standards versus their molecular masses provided a value for this band of approximately 65,000. This was just smaller than bovine serum albumin at 67,000.

Purification to homogeneity is indicated by the presence of a single band on SDS-denatured gel electrophoresis at the same position as reported by others (Sica et al., 1973; Sica and Bresciani, 1979; Sakai and Gorski, 1984b) for classic mammalian oestrogen receptors in which the major species found were 65,000-70,000. A higher molecular weight species of 89,000, molybdate stabilized oestrogen receptor of calf uterus, had also been reported recently by Atrache et al. (1985). These authors did not rule out the fact that a non-steroid binding protein molecule

might be present. A similarity in size of cytosolic and nuclear uterine receptors has also been indicated by others (Erdos and Fries, 1974; Erdos and Fries, 1979; Katzenellenbogen et al., 1983). Calf, pig, mouse and rat uterine receptors all had been reported by Lubahn et al. (1985) to have similar binding affinities and, along with the human receptors, had the same maximum molecular weight on SDS-PAGE of 70,000. In smaller amounts in all cases, they found a "50,000" band was visible and, even more faintly, a "30,000" band was also seen. The relationship of the smaller bands to the 70,000 molecular weight band was not clear but probably represents common endogenous proteolytic activity occurring prior to the preparation of cytosol. This size heterogeneity was also reported by others (Katzenellenbogen et al., 1983; Van Oobree et al., 1983). Rabbit uterine oestrogen receptor, purified on a DES-agarose affinity column by Van Oosbree et al. possessed a 70,000 and a 50,000 molecular weight protein (Van Oosbree et al., 1983). Katzenellenbogen et al. (1983), by an affinity-labelling technique using tamoxifen aziridine, have demonstrated two similar sized bands in cytosol from rat uterus. Studies using gel exclusion chromatography in 6M guanidine-HCl yielded molecular weight estimates of 53,000-55,000 (Erdos and Fries, 1974; Erdos and Fries, 1979). Although 0.1M of 2β-mercaptoethanol was included in the elution buffer, the concentration might not be sufficient to bring a complete cleavage of the disulphide bonds.

The 50,000 and 30,000 molecular weight species of oestrogen receptor could represent active forms of the receptor either antigenically similar or smaller forms that are formed by cleavage from a larger precursor. The 30,000 molecular weight is the smallest size of receptor that is large enough to retain sufficient E_2 binding capacity so that it can be efficiently retained by the affinity column throughout the vigorous washing conditions (Lubahn et al., 1985). This hypothesis is supported by other reports where oestrogen receptors that have deliberately cleaved with endogenous proteases and examined by sedimentation and gel filtration analysis (Sica et al., 1976; Miller et al., 1981). However no such smaller

binding species were evident in this study, due to either the inability of such forms to bind to the affinity column, the stability of the receptor which occurred in the presence of Ca^{2+} thus avoiding proteolysis or, too small a quantity to be detected on the gel. However, the reason for the existence of these receptor species reported by others is not clear. It could be: 1) The presence of subunits of more than one receptor of similar size; (2) microheterogeneity of a single subunit; or (3) postsynthetic modification such as phosphorylation. Reports in the literature suggest that the receptor is phosphorylated (Migliaccio et al., 1982) and that dephosphorylation may cause some loss in E_2 binding (Auricchio et al., 1982; Auricchio et al., 1984).

Although there is growing support for the proposal that untransformed receptors are oligomeric proteins in vitro, questions concerning identity or dissimilarity of the polypeptide chains remains unresolved. Nonsteroid-binding components of molecular weight 58,000 and 13,000 have been proposed by Murayama et al. (1980) for the 8S oestrogen receptor. Separate reports indicate that, in untransformed 8S progesterone receptor complexes from chick oviduct, the A and B receptor subunits are associated with a 90,000 molecular weight component which does not bind hormone (Dougherty et al., 1984). A hetero-oligemeric structure composed of molecular weight 90,000, 41,000, 40,000 and 24,000 units has been suggested for the unactivated glucocorticoid receptor (Grandics et al., 1984).

The 8S oestrogen receptor has a molecular weight of 240,000 for a purified receptor under nondenaturing conditions, and the apparent molecular weight of 65,000 for the SDS-dissociated unit appears consistent with a tetrameric model proposed separately by Raaka and Samuels (1983) and Sherman et al. (1983). The isolation described in this thesis using SDS-PAGE also gave a 65,000 molecular weight protein again supporting the tetramer model.

4.2.3.1 Identification of the Band as Oestrogen Receptor

The 65,000 molecular weight protein band on SDS-polyacrylamide gels was identified as oestrogen receptor by two criteria. First, the 65,000 molecular weight protein becomes evident only when the receptor was detected during the elution of the affinity column. The other contaminant proteins present with the 65,000-dalton band were all observed earlier in the wash fractions and displayed no E_2 binding activity. Secondly, preloading of the receptor with E_2 prior to passing it through the affinity column prevented the receptor from binding. Thus the 65,000 molecular weight band was prevented from binding to the column and was not observed in the E_2 eluted fractions.

This 65,000 molecular weight protein of SDS denatured oestrogen receptor showed a single peak with a sedimentation coefficient of about 4S. Results from earlier experiments (section 3.1) proved the presence of nondenatured receptor of 240,000 molecular weight protein. While there is uncertainty concerning the molecular weight of the native cytosol receptor, these results are in good agreement with studies by other groups (Puca et al., 1971, 1972, Sica et al., 1973 and Sica and Bresciani, 1979) who calculated that nondenatured receptor has a molecular weight of 240,000 and sedimented at 8S while SDS denatured protein has a molecular weight of 64,500 with a sedimentation values of 4S. Thus the results indicate that the human uterus oestrogen receptor subunit is one and carries a single hormone binding site. It appears that the native state in cytosol, several subunits are associated to form a larger quaternary structure, but the number of interacting subunit cannot be specified which could be four as seen through the size of the nondenatured molecular weight protein.

4.3 RECOGNITION PATTERN OF ANTI-RECEPTOR ANTISERA

Using ELISA, the immunoglobulins from the sera of the rabbit immunized with human uterine receptor shown to cross react with

oestrogen receptor from all sources tested including cytosol receptor from ZR-75-1 cells, human, mouse and rat uterus and ER+ human breast cancer, suggesting the conservation of a common antigenic determinant in oestrogen receptor across a variety of species.

4.4 SPECIFICITY OF THE IgG-ER

4.4.1 Sucrose-density Gradient

Oestrogen receptor sediments at 8S in low ionic strength sucrose gradients (0.01M KCl) and at 4S in high ionic strength sucrose gradients (0.4M KCl) (Gorski et al., 1968). Anti-receptor antibody interaction was also studied using the $[^3H]E_2$ as a marker both for double-antibody precipitation and SDG.

The antibody raised against oestrogen receptor showed two interesting properties. First, it reacted with E_2-receptor complex without interfering with the ability of the receptor protein to bind or retain the hormone, so the labelled steroid can be employed as an indicator of antibody receptor interaction. Second, the antibody reacts with oestrogen receptor to produce non-precipitating immune complexes presumed to consist of one molecule of immunoglobulin associated with each receptor protein molecule. Thus, antibody-receptor interaction can be conveniently studied and quantified by the shift observed in the sedimentation peak of a $[^3H]E_2$-receptor complex when subjected to ultracentrifugation in a sucrose density gradient in the presence of immunoglobulin from an immunized animal, compared with that from a control animal (IgG-N). The shift in the sedimentation values had been employed as indicator for antibody-antigen reaction by many authors (Greene et al., 1979, Raam et al., 1981, Coffer and King, 1981; Holt et al., 1983; Giambiagi et al., 1984). The increase in size of the shift (4S to 8S in high salt, and 8S to 10S in low salt) which is equivalent to an increase of about 25,000 molecular weight is good confirmation that antibody interacts with oestrogen receptor at a 1:1 ratio. The same

observation was also reported by others (Moncharmont et al., 1982, Moncharmont and Parikh (1983), Moncharmont et al., 1984).

4.4.2 Reactivity of IgG-ER with Different Steroid-binding Proteins

Results of screening studies for cross-reaction of IgG-ER with other steroid-binding proteins indicated no reaction with rat uterine cytosol progesterone receptor and rat ventral prostate dihydrotestosterone receptor. The antisera also did not react with free $[^3H]E_2$ label. The IgG-ER antibodies cross-react with cytoplasmic oestrogen receptor derived from oestrogen-sensitive target tissue but not to progesterone receptor or androgen receptor. As IgG-ER did not react with rat progesterone receptor or dihydrotestosterone receptor from rat prostate, this confirms that different steroid hormone receptors lack immunochemical similarity (Greene et al., 1979, Coffer and King, 1981).

4.4.3 Immunoprecipitation: Double antibody method

The IgG from the serum of a rabbit immunized with purified receptor contained antibodies to the receptor protein was further characterized by double antibody precipitation. Comparing IgG-ER with IgG-N, a significant amount [about 60% (Figure 23)] of human myometrial cytosol $[^3H]E_2$-receptor complex was precipitated by the antiserum. Greene et al. (1977), using antiserum to oestrogen receptor, also raised in rabbit, reported similar observation but their method of antigen purification was from the nuclei of calf uterine tissue.

4.4.4 Immobilized IgG-ER on Sepharose

The ability of the immobilised IgG to bind to oestrogen receptor in a saturable manner revealed two facts. The binding of antibody to oestrogen receptor is concentration dependent and the oestrogen receptor that bound to the resin could be eluted and characterised. The eluted protein was analysed on native PAGE and

found to have 240,000 molecular weight. The molecular weight falls into the range that had been reported by Sica et al. (1976); Puca et al. (1972); Moncharmont et al. (1982, 1984). The same protein were run on SDS-PAGE gave a molecular weight of 65,000 (Sica and Bresciani, 1979; Moncharmont et al., 1984), suggesting that the aggregate seen on PAGE could be a homo-tetramer.

4.4.5 Detection of Antigen by Antibody Binding on Blots

The method allows detection of antibody specificity for its protein antigen by autoradiography and is simple and precise. The immobilized proteins were detectable by immunological procedures. All additional binding capacity on the nitrocellulose was blocked with excess protein; then a specific antibody was bound and finally, a second antibody directed against the first antibody was added. The second antibody was radioactively labelled with [^{125}I]. The specific protein was then detected by autoradiography. It has the advantage of detecting as little as 100 pg of protein. It has been found that oestrogen receptor transferred to nitrocellulose sheets remained there without being exchanged over several hours.

The electrophoretic blotting technique described here produces replica of proteins separated on PAGE. A quantitative transfer was obtained with proteins from gels containing oestrogen receptor. Still there remains the possibility that certain classes of protein do not bind to nitrocellulose. To check this, the gel left after transfer was silver nitrate stained and found to be clear.

4.5 IMMUNOCYTOCHEMISTRY

Before the immunocytochemical experiments were carried out careful measures had been taken to obtain optimal conditions.

4.5.1 ZR-75-1 Cells

The growth medium of the cell-line was changed to fresh medium

without E_2 6 days before each experiment. This long pretreatment of the cells with charcoal treated heat-inactivated calf serum and, hence E_2 free medium is required to completely remove E_2 from these cells. However, more than six days was not recommended, since the cells grow very slowly and might cease to grow altogether, as observed by Allegra and Lippman (1980). They also observed an obvious decrease in cell adhesiveness in oestrogen deprived cells and detached cells are easily seen in the medium.

4.5.2 Fixatives

ZR-75-1 cells were used. They were removed from the growth medium and immediately fixed in: (1) acetone (100%), (2) methanol:acetone (1:1) and (3) ethanol (100%). The best results were obtained with 100% acetone which gave a clear background and comparable intensity. Fixation with alcohol did give receptor localisation but with less intensity and slightly higher background. Allowing the tissue to air dry (30-60min) before fixation, had no appreciable effect on the intensity of specific staining. However, the length of time that tissue or cells were in fixative had an important effect on the ability of the antibodies to locate the oestrogen receptor. Prolonged fixation did affect the specific staining for oestrogen receptor. Fixation with methanol:acetone for longer than 30min led to a progressive loss of specific staining. The same was true with 100% acetone and 100% alchohol.

Unfixed tissues or cells was not suitable for oestrogen receptor localization as there was progressive loss of specific oestrogen receptor staining when the cells were incubated in PBS prior to application of immunostaining. Greene et al. (1984) reported that it was due to the escaping of the oestrogen receptor into the washing buffer. This could also be due to the oestrogen receptor not being fixed on to the slides as there was loss of cells together with the absence of staining. The cells should be adequately fixed e.g. using paraformaldehyde which causes perforations in the cell membrane (Ewijk et al., 1984) which allow

the antibodies to penetrate the cells. In addition, the unfixed tissue was observed to have more nonspecific background staining than fixed tissues even though some non-specific binding could be avoided by preabsorbing the antiserum with 1% BSA overnight at 4°C.

The stability of the receptor probably depends on storage temperature. Temperature seems to have no effect on the specific staining if the fixed cells, frozen sections or FNAs were kept at 0-30°C for a few hours and then subjected immediately to immunostaining. Also, if the fixed cells and tissues are kept at -20° or -70°C for more than 24h there was some preservation of the specific staining, but storage for 1 week or more gave noticeably weaker staining intensity and after 1 month of storage, specific staining was usually not detectable by this method.

4.5.3 Immunocytochemical Methods

A number of histologic methods for localizing oestrogen receptor in tissue sections have been reported. These methods depend on immunocytochemical localization of putative receptor-bound steroid (Kurzon and Sternberger, 1978; Pertschuk et al., 1978; Farley et al., 1982). None of these methods has yet been validated as capable of detecting specific receptor proteins (Castaneda and Liao, 1975; Chamness et al., 1980). In fact, a comparative study of patients with breast carcinoma showed the absence of any correlation between histochemical methods and biochemically determined steroid receptor levels or clinical response (McCarty et al., 1982). Besides, a valid histochemical technique for the localization of oestrogen-binding proteins would be desirable since it could offer the attractive possibility of studying steroid hormone-receptor interactions at the individual cell level, and possibly the heterogeneity of the oestrogen receptor distribution between cells thus adding significantly to the elucidation of the basic mechanism of steroid hormone action.

The presence of cytoplasmic and nuclear oestrogen receptor in

oestrogen responsive tissues and human mammary carcinoma cell-lines could be detected by using the immunocytochemical technique. An effective and most economical way of studying the oestrogen receptor localization and translocation phenomenon would involve the use of histochemical methods. One such histochemical method has been described by Nenci et al. (1976, 1980). These authors studied E_2 kinetics in breast cancer cells by incubating the cells at different temperatures in E_2 and detecting the intracellular localization of the hormone by using rabbit anti-oestradiol antibody and fluorescein-labelled goat anti-rabbit immunoglobulins. The authors' claimed that their experimental results demonstrated receptor kinetics rather than the steroid kinetics. This brought criticisms of the method published. The points of criticism have been summarized by Chamness et al. (1980) who emphasized that the hormone binding methods cannot distinguish steroid binding of Type I receptors from that of Type II receptors.

4.5.4 Specificity of Antibody

An important criterion is that the specificity of the antiserum be restricted to the hormone receptor under study and that there is no interaction of the antibodies with other proteins or hormones.

The aim of the experiments was to present immunochemical and cytological evidence for the presence of oestrogen receptor in the target cells using anti-receptor antibody. The presence of any nonsecific staining could be eliminated by preabsorption with BSA (0.1%) overnight at 4°C or 1h at 37°C. Application of these antibodies to immunocytochemistry required a clear demonstration that they recognised receptor in E_2 responsive cells and tissue sections. Competition studies were set up. Oestrogen receptor protein, found in cytosol of breast cancer tissue (500-1000fmol/mg protein) and used as the diluent, was found to be capable of preventing the antibodies from binding to their respective antigenic determinants in the E_2 target cells (Figure 33a). However, other

cytosolic components present in receptor-depleted cytosol were unable to compete effectively for the antibodies (Figure 33b). These competition studies provide strong support for the presumption that the antibodies were recognising tissue oestrogen receptor. The chances of nonspecificity was very small since the immunocytochemical reaction was obtained in this work after only a short exposure to 100-fold diluted antiserum. This suggests the presence of antibodies of high specificity for the corresponding antigens.

The specificity of the anti-receptor antiserum that was used in this study has been investigated by biochemical and immunochemical methods. Evidence had been presented that the antiserum does not react with E_2 (Figure 33c), or cellular proteins other than oestrogen receptor. The following results obtained in this study utilizing cells from an E_2 target organ such as myometrium or endometrium from both human and rat further substantiate the restricted specificity of the antiserum to oestrogen receptor.

(a) The antibodies recognise a protein which translocates in response to E_2 and DES (Figure 32a,b,c,d and 31c). Translocation of the protein from the cytoplasm to the nuclear compartment of cells incubated not only with E_2 but also with DES ruled out the possibility that the antiserum detected the hormone and not the receptor. A complete translocation of the protein from the cytoplasmic to the nuclear compartment testified that the antigenic sites recognized belonged to the Type I oestrogen receptor which, unlike Type II, is known to translocate under these conditions (Muldoon, 1980).

(b) The anti-receptor antiserum recognised cytoplasmic oestrogen receptor with unoccupied steroid binding sites in the human ZR-75-1 cells as evidenced by the presence of fluorescence in the cells exposed to 37°C in a E_2 free medium (Figure 34a,b). This is also further evidence that the antibody recognise oestrogen receptor, rather than E_2.

(c) The antibodies also react with oestrogen-occupied cytoplasmic receptors (cells which had been incubated with E_2 or DES). The antiserum interacts efficiently with oestrogen receptor saturated with E_2 or DES. No difference in the intensity of fluorescence was observed when compared to the cells from E_2 free medium.

(d) The antibodies cross-react with the nuclear form of receptor even when they are bound to the acceptor sites in the chromatin. In the ligand incubated cells, an absence of cytoplasmic fluorescence, unaccompanied by an emergence of nuclear fluorescence, would be expected if the antibodies failed to recognize nuclear forms of the receptors. This was not observed in this study. Fluorescence was clearly seen to be transferred from the cytoplasm to the nuclear compartment.

The antiserum raised in rabbit against the human uterine receptor preparation was apparently monospecific when applied to uterus of rat origin, but this cannot definitively rule out contaminant antibodies reacting with unknown proteins in very low concentration, as discussed previously.

The subcellular distribution of the receptor was of great interest. The presence of the receptor in the cytoplasm itself was an interesting result, since the biochemical methods deal in fact with 'cytosol', that is only an experimentally defined cytosoluble fraction of tissue extract. The shift in the receptor subcellular distribution in favour of the nucleus, after E_2 administration, agrees with the understanding of steroid hormone receptor translocation. The *in vitro* translocation studies revealed the specificity of the antiserum and the fact that oestrogen receptor present was of Type I, because of its ability to translocate to the nucleus. Frozen sections, either fixed prior to storage at -70°C or immediately fixed following sectioning, showed mostly cytoplasmic with occasional nuclear staining. The mechanism of oestrogen receptor action in target cells, as originally proposed (Gorski *et*

al., 1968; Jensen et al., 1968) proposed that cytoplasmic oestrogen receptor became activated following the binding of E_2, and was translocated to the nucleus. Oestrogen receptor present in the soluble fraction of a cell extract is assumed to be cytoplasmic (low-salt extracts).

The phenotype (8S, 4S and 5S) of the receptor could not be deduced from these experiments. However the 'nuclear type' receptor, through large numbers of studies, could be classified as either 5S or 4S. Raam et al. (1982) reported rabbit antibodies to oestrogen receptor that recognized the 4S rather than the 5S form in the nuclei of MCF-7 cells.

4.5.5 In Vitro Translocation Studies

In addition to the specificity characteristics of the anti-ER antiserum, several interesting facts related to the oestrogen receptor translocation emerged from this study:

(1) Binding of the hormone is perhaps an essential prerequisite for the transformation of cytoplasmic receptors into the nuclear form in normal uterine cells. This conclusion is drawn from the fact that the immunofluorescence was exclusively cytoplasmic in the cells grown in E_2 free medium while nuclear fluorescence was observed only in cells incubated with E_2 or DES. Such results were obtained in most of the cell population.

(2) Binding of the hormone perhaps increases the binding affinity of the oestrogen receptor to the acceptor sites in the nucleus. This was seen in the ZR-75-1 cells where immunofluorescence was in the nucleus only after incubating the cells with E_2 and DES.

(3) One nanomolar concentrations of ligand was effective in inducing transformation and allowed complete translocation of cytoplasmic oestrogen receptor to the nuclei to take place.

(4) One hour incubation with the hormone at 37°C appeared to be sufficient for complete translocation of oestrogen receptor into the nuclei for the normal myometrial cells and breast cancer cell-line. Incubating at 22°C resulted in incomplete translocation, with the cells retaining residual fluorescence in the cytoplasm at the end of the experiment. Since the translocation of these cells was complete at 37°C this indicates that the partial translocation initially observed was not due to abnormalities in oestrogen receptor nor to the antiserum detecting cytoplasmic proteins other than oestrogen receptor. The data obtained by Eckert and Katzenellenbogen (1982) for MCF-7 cells using radiolabelled E_2 also indicated completion of oestrogen receptor translocation to the nucleus within an hour.

(5) The pattern of distribution of oestrogen receptor obtained with anti-ER antisera, consisting of oestrogen receptor hormone complex formation in the cytoplasm, binding to the nuclear membranes and/or chromatin, and the eventual translocation and retention in the nucleoli-like spots in the chromatin are analogous to the observation of Raam et al. (1983) who studied the intracellular oestrogen receptor movement using anti-oestrogen receptor antibodies.

The in vitro translocation studies could also extend our knowledge of the specific acceptor sites where the nuclear binding occurred. Increasing the translocation time to 2h (Raam et al., 1983), tended to reveal distinct spots localised in the nuclear compartment rather than on the general surface of the nuclei. This finding could support the specific acceptor sites hypothesis (Raam et al., 1982) for ER-DNA interaction. The fact that cryostat sections can be used for such studies is very useful.

However these studies were not consistent with the findings of Greene's group (Greene et al. 1984; McClellan et al 1984; Press and Greene, 1984; Press et al., 1984; King et al., 1985) who developed

monoclonal antibodies to oestrogen receptor and found the location of the receptor was mostly in the nuclei. They suggested the presence of the cytoplasmic oestrogen receptor in the tissue extract was merely an artifact of extraction whereby the weakly bound nuclear receptor became soluble in low salt buffer during homogenization.

The findings from this study fit some of the information in the two-step model. When it was originally postulated there was no direct proof. The mechanism of action in target cells was hypothesized to consist of a number of steps including (a) diffusion of E_2 into a target cell, (b) high-affinity, specific binding of E_2 to cytoplasmic oestrogen receptor, (c) activation of the oestradiol-receptor complex, (d) translocation of the activated complex to the nucleus, (e) binding to nuclear chromatin and (f) induction of specific RNAs (Gorski et al., 1968; Jensen et al., 1968). This model would predict that the majority of the receptor should be cytoplasmic, under conditions in which E_2 levels are low, and nuclear when receptor is saturated by high levels of E_2 The obvious explanation for migration to the nucleus is that only the oestradiol-receptor complex can bind to DNA in a physiologically significant manner (Yamamoto and Alberts 1972). The results obtained in this study are consistent with the proposed model except that even in the absence of E_2 some oestrogen receptor could still be detected in the nucleus. Results obtained by Raam et al. (1982, 1983) and Morel et al. (1981) also working with rabbit antibodies against oestrogen receptor, and Morel et al., (1984) with antibodies against progesterone receptor, followed the model very closely, and as did those of Greene and Jensen (1982) using monoclonal antibodies to oestrogen receptor. Later, King and Greene (1984), also working with monoclonal to oestrogen receptor and Welshons et al. (1984) independently discovered purely nuclear localization of oestrogen receptor. However, King and Greene (1984), King et al. (1985), and McClellan et al. (1984) used fixatives such as glutaraldehyde and paraformaldehyde which are known to cause crosslinking of proteins by interaction with free amino groups (Hopwood, 1972; Collins and Goldsmith, 1981). This was adjusted for optimum preservation of

chromatin (McClellan et al.,(1984). While Morel et al. (1981), Raam et al. (1983), Tamura et al. (1984) and also the present study used fixatives which conserved cytoplasmic protein without any particular emphasis on the preservation of chromatin. Various possibilities stated below cannot be ruled out. The cytoplasmic oestrogen receptor detected by the rabbit antibody in this study could be related to oestrogen receptor newly synthesized, which is believed to occur in the cytoplasm. Another possibility is the presence of the degraded form of oestrogen receptor which is still capable of binding E_2 but such a molecule would be unlikely to translocate. They could be lower molecular weight proteolytic fragments (Thibodeau et al., 1983) which are generated during the purification of antigen. This is unlikely since the gel of affinity eluate demonstrated only one band at 65,000 molecular weight. Another important factor is the effect of fixatives. Fixation with acetone or alcohol might cause the lability of protein to take place since Brandtzaeg (1981) observed a loss of cytoplasmic proteins from cells fixed with ethanol. This could result in the distribution of 'loosely associated nuclear bound receptor' (King et al., 1985) in the cytoplasm in the absence of ligand. The presence of ligand might increase the binding affinity for its sites in the nucleus thus not detecting any cytoplasmic receptor. Another possibility could be the property of the oestrogen receptor itself which could be activated spontaneously even in the absence of other stimuli, such as elevated temperature (20°C), increased salt concentration (0.4M KCl), or added steroid, all of which have been necessary for the activation of other steroid receptors (Thibodeau et al., 1983).

In conclusion, with the help of biochemical techniques, nuclear translocation of cytoplasmic receptor has been demonstrated in oestrogen responsive cells. Like the biochemically measured receptors, the antigenic determinants, detected immunohistochemically with the rabbit antiserum, can also be fully translocated. Utilizing anti-ER antiserum, failure to detect any proteins other than oestrogen receptor in the target cells by immunofluorescent procedure was consistent with the antiserum containing only

antibodies directed against oestrogen receptor.

Intracellular movement of the receptor protein can be studied in vitro using antibodies directed against steroid receptors, especially if the antibody population contains a mixture of antibodies that recognize receptors in both hormone-occupied or unoccupied forms, and the receptors bound to the nuclear chromatin.

Polyclonal antibodies directed against cytoplasmic oestrogen receptor are excellent tools for investigating the action of oestrogens and antioestrogens in hormone-responsive cells and for understanding the abnormalities associated with the translocation process in malignancy. The methodology described can easily be adapted to study the effect of progestins on oestrogen receptor synthesis. In addition, the heterogeneity of oestrogen receptor distribution that cannot be examined by using radiolabelled hormones can also be investigated by using the immunohistochemical method described.

4.6 OESTROGEN RECEPTOR QUANTITATION OF FNA SAMPLES

The measurement of oestrogen receptor has been generally accepted as clinically valuable in selecting patients for hormone therapy. It has been known that the likelihood of a successful response to endocrine therapy is increased at least tenfold in ER+ breast cancer patients (McGuire et al., 1975b). Small quantity samples such as in the form of FNAs can be easily obtained from out-patient clinics. Another important aspect of FNAs was in providing information regarding both the prognosis and the likelihood of response to endocrine therapy before surgery. This pre-operative knowledge helps the surgeons in selecting both the extent of surgery and the type adjuvant therapy.

The biochemical assay has the disadvantage that a drug such as tamoxifen may interfere. Further the biochemical assay gives only an average value for receptor concentration and has no ability to

answer the essential question concerning the proportion of tumour cells relative to total cell content (fat, stroma etc.) and the proportion of ER+ and ER- cells in the biopsy.

Present methods for the determination of steroid hormone receptors depend on the radioactive steroid as a marker for the receptor protein and have several inherent disadvantages. Some of the receptors in the tumour may be bound to endogenous hormone and remain undetected unless some type of exchange assay is also carried out. The binding of steroid to receptor, though strong, is non-covalent, so the radioactive marker is subjected to displacement by dissociation, by exposure to heavy metal ions, or by degradation of the rather labile receptor protein during storage and processing of the tumour specimen. In fact, it is believed that deterioration of the receptor during sample manipulation may be one reason why clinical response to endocrine therapy is occasionally seen with apparently ER- breast cancers. The two analytical procedures most commonly used for separating the receptor-bound steroid from the excess unbound hormone (SDGA and DCC with Scatchard plot) are costly in terms of time and/or instrumentation and usually require a tumour specimen larger than is available with metastatic cancers. Due to these reasons many biochemical bioassays had been developed. Among these bioassays are iso-electric focussing or selective retention of oestrogen receptor on DE-81 cellulose paper (Barbonel et al., 1977) or small Sephadex columns (Barnes et al., 1977). Isoelectric focussing had been tried on FNAs and shown to correlate well with the biochemical technique (Silfversward et al., 1980) but the limitation still lies in its inability to fully analyse the given sample for heterogeneity.

Until recently only autoradiography, using [^{3}H] ligands with high specific activity, has been used to visualize steroid-binding cell subpopulations at physiological concentrations (10^{-9} to 10^{-10}M) of hormone. However, autoradiography requires the preparation of 4μm frozen sections and long exposure times at low temperatures. These drawbacks make this technique inappropriate for widespread clinical use. The use of steroid hormones conjugated with

fluorescent molecules has been reported to detect other types of receptor (Type II). This lower binding affinity could also be due to the size of the steroid ligand with the bulky fluorophore resulting in a large structural perturbation that reduces the affinity for the receptor drastically (Lee et al., 1977; McCarty et al., 1977; Chamness et al., 1980; Pertschuk et al., 1981). Attempts to use these agents at higher concentrations raises the possibility that sites other than the receptor are being detected (Chamness et al., 1980; McCarty et al., 1981; Pertschuk et al., 1981).

4.6.1 Tissue Heterogeneity

One of the limitations of current receptor assays that would not be overcome by an immunobead assay, DCC (titration assay) or SDGA (sedimentation analysis) is the inability to assess tissue heterogeneity. If a breast biopsy contains ER+ tumour, but the amount of tumour tissue is very small, then the DCC assay will yield low or negative values. There are ample examples of the lack of quantitative agreement among assays carried out on different portions of the same large breast cancer. A great deal of effort has been devoted to the development of methods for the visualization of ER+ cells within a heterogeneous cell population. Successful measurement of receptor at the cellular level requires at least 2 criteria: (a) the molecule used as a label must interact with the receptor with high affinity and high specificity; and (b) the method of detection must be extremely sensitive. In the latter case, the sensitivity of the detection device and the detectability of the tag (for instance, its specific activity for autoradiography or its fluorescence emission for fluorescence microscopy) are complementary factors. With the availability of the specific antibody prepared against human myometrium receptor, specific and also sensitive methods for immunocytochemical assay of receptor became feasible.

Recent studies by cytochemical and fluorescence methods have indicated that most breast tumours are composed of ER+ and ER- cells (Pearson et al., 1978). Cellular heterogeneity is a particularly important issue in the case of human breast tumours, where it has

been used to explain the varied clinical response to hormone therapy in ER+ cases (Silfversward et al., 1980). Furthermore, it is thought that a proper knowledge of the cellular heterogeneity of a tumour would be useful in selecting an appropriate combined therapy.

4.6.2 Biochemical Versus Anti-ER Antibody Assay (IF-antibody Assay)

It is very important to realise that the quantity of oestrogen receptor measured by biochemical assay can be directly related to the intensity of IF only if the tumours are homogenously positive with an equal amount of oestrogen receptor in all the tumour cells. Conversely, samples with both high and low ER+ cells may show little quantitative correlation between IF and biochemical values An antibody assay of 7 ER+ and 1 ER- (biochemical classification) tumours revealed complete qualitative agreement but little relationship between quantity of oestrogen receptor and IF intensity presumably due to a lack of homogeneity in oestrogen receptor content of the tumour cells. An analysis of IF data on the ER+ tumours revealed that 83% of tumours with >400fmol of oestrogen receptor were homogeneous i.e. contained only IF(+) cells in category B and C in the multiple areas of the slide examined. A distinct trend towards greater heterogeneity, not only in tumour cellularity, but also in antigen status of the tumour cells was observed in tumours containing from 50-399 fmol/mg of oestrogen receptor. The majority of these tumours contain a mixture of IF(+) and IF(-) cells. It is also evident that IF(-) cells made up more than 50% of the tumour when oestrogen receptor content was less than 59 fmol/mg protein.

Biochemical and IF antibody technique agreed qualitatively on 20 out of 21 ER+ tumour which had an adequate quantity of tumour cells. With the biochemically ER- tumours, 2 out of 26 patients were found positive by the antibody. Of these, one patient found positive by IF antibody technique had the biochemical assay done on a tru-cut sample i.e. much less tissue than normally required. The other

patient was premenopausal and her tumour was very heterogeneous in its staining pattern. Some cells exhibited extensive nuclear staining giving an overall significant IF value although many others were negative (Figure 41c). However, the data indicate a high correlation between the biochemical and immunohistochemical methods qualitatively.

Interestingly patients with breast tumour clinically ER- i.e. ERc positive and ERn negative was found to be negative by this assay. The distribution of IF(-) cells seem to be homogeneous in the smear inspected. One example from a patient having such tumour is shown in Figure 40a. This finding could offer an explanation as to why the group of patients does not show a response to endocrine therapy.

CONCLUSION

The procedure for purification of human myometrial oestrogen receptor involved the application of affinity chromatography employing E_2-Succ-Alb-Sepharose 4B, and elution with DES. For antibody raising, the amount of receptor injected is very small, only between 10-30μg compared to 300μg used by Greene et al. (1979) to immunize a single rabbit and 700μg of the purified protein to immunize a goat.

Experimental data have been gathered to check the specificity of the antibodies towards oestrogen receptor. The presence of anti-oestradiol antibodies has been excluded since there is no sedimenting radioactive immune complex seen when $[^3H]E_2$ is reacted with IgG-ER. Similarly when $[^3H]E_2$ is added to the Sepharose immobilized IgG-ER, the latter does not bind $[^3H]E_2$. IgG-ER induces a shift in the sedimentation coefficient of the oestrogen receptor from 4S to 8S (high salt); and 8S to 10S (low salt). The IgG-ER has no effect on the sedimentation rate of $[^3H]$ DHT receptor or $[^3H]$ progesterone receptor. The selective binding to E_2 target tissues together with the demonstration of translocation is added proof of anti-receptor activity. Lastly, the evidence shown by the immunoblot experiments clearly indicates that the antibodies recognise a protein of 65,000 molecular weight.

The antibodies have been used as probes for detecting and localising oestrogen receptor in the target cells. An immunofluorescent technique using anti-receptor antibody has been applied to FNAs from human breast cancer. This technique shows a good correlation with the results of the DCC assay. It offers the additional possibility of detecting receptor-positive cells in heterogenous tumours, which cannot be shown conclusively by the DCC technique. As more highly purified preparations of antibody become available, it should be possible to use them, in kit form, to identify oestrogen receptor in FNAs in routine breast cancer clinics.

A PROPOSED MODEL FOR STEROID HORMONE ACTION

The mechanism for oestrogen action is still debatable. Thus it is difficult to propose a model which is not subject to critism. Since part of the result obtained in this study fits the two-step mechanism model for oestrogen receptor, this classical model is still preserved. It is merely the case that the location of the receptor must be changed. In the classical model it was established that empty receptor had little or no affinity for the nucleus thus residing in the cytoplasm alone, while the most recent findings reported that oestrogen receptor resides in the nucleus alone. Interestingly the present study using immunofluorescence technique and antibody to oestrogen receptor, showed that both empty and filled receptors are residing in the cytoplasm as well as in the nucleus which includes nuclear membrane, chromatin and nuclear matrix. A model is then proposed.

Steroid diffuses into the cell down a concentration gradient. Once into the cell, the steroid comes into contact with the oestrogen receptor. The receptor-hormone complex is then formed. The binding of the steroid to the receptor causes activation which results in an increase in affinity towards the chromatin. The binding kinetics of the activated receptor to specific sites in the chromatin may be helped by non-histone chromosomal protein.

Another possibility is the fixatives artifact. However, artifacts brought about by fixation cannot be ruled out. The empty receptor might be residing loosely in the nuclear component. Thus with fixatives like alcohol or acetone, the empty receptor is easily detached and distributed in the cytoplasm.

How the receptor is released from the nucleus is not known. It could be through dephosphorylation or the presence of specific proteases which have a high Km for the receptor.

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