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CHARACTERISATION OF NUCLEAR BINDING SITES FOR OESTROGEN RECEPTOR COMPLEXES

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A thesis submitted in partial fulfilment for the degree of
Doctor of Philosophy in the Faculty of Medicine

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TO MY PARENTS AND GRANDPARENTS

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Abbreviations

The standard abbreviations, as recommended in the "Biochemical Journal, Policy of the Journal and Instructions to Authors, 1984", are used throughout this thesis with the following exceptions.

bp - Base pairs

BSA - Bovine Serum Albumin

cAMP - Cyclic Adenosine Monophosphate

CI 628M - α - [4-pyrrolidinoethoxy] phenyl-4-hydroxy- α' nitrostilbene

DCC - Dextran Coated Charcoal

DES - Diethylstilboestrol

5α DHT - 5α -Dihydrotestosterone

DFP - Diisopropyl fluorophosphate

DNase - Deoxyribonuclease

DTT - Dithiothreitol

E_2 - Oestradiol-17 β

GH3 - Rat Pituitary Tumour Cell Line

H - Cytosol labelled with Radioactive Ligand alone

H+C - Cytosol labelled with Radioactive Ligand in presence of 200 fold excess of cold ligand

HEPES - N-2-hydroxy-piperazine-N'-2 Ethane Sulphonic Acid

HSP - Heat Shock Protein

H 1285 - 4(N,N-diethylaminoethoxy)-4'-methoxy- α-(p-hydroxyphenyl)- α'-ethylstilbene

IgG - Immunoglobulin G

Kb - Kilobase

Kd - Dissociation Constant

MCF-7 - Human Breast Tumour Cell Line

POPOP - 1,4 Di-(2-(5-phenyloxazolyl))-benzene

PPO - 2,5-Diphenyloxazole

RNase - Ribonuclease

SDS PAGE - Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SHBG - Sex Hormone Binding Globulin

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Summary

1. The literature concerning the mechanism of action of steroid hormones, has been reviewed. In particular that relating to steroid receptor proteins and their interaction with the cell nucleus and nuclear material has been stressed.

2. Nuclei have been isolated from human myometrium and their purity assessed using standard criteria. The isolated nuclei have been extracted using either 2M NaCl or 0.1M H_2SO_4. The resultant extracts have been immobilised on sepharose 4B and assayed for the ability to specifically bind activated, labelled oestrogen receptor complexes.

3. Both nuclear fractions have shown significant ability to specifically bind activated, labelled oestrogen receptor complexes.

4. The relative abilities of oestrogen receptor from a variety of sources to bind to the 2M NaCl soluble nuclear extract/sepharose resin has been assessed. Receptor from immature rat uterus was found to bind in a reproducible, saturable manner to the myometrial nuclear extract and was used as the source of oestrogen receptor for all subsequent studies.

5. The effect of assay conditions on the binding activity at 4^oC has been assessed. The presence or absence of EDTA or DTT in the assay buffer had no significant effect. A KCl concentration in the range of 0.12-0.15M was necessary to observe the maximum amount of specific saturable binding. When oestrogen receptor was prepared in the presence of sodium molybdate, no decrease in binding activity was observed, indicating the presence of molybdate did not interfere with the interaction of the oestrogen receptor complex and the immobilised components.

6. The effect of digestive enzymes on the ability of both nuclear fraction/sepharose resins to bind activated, labelled oestrogen receptor has been assessed. Only proteolytic enzymes were observed to reduce the binding activity indicating that the immobilised nuclear component involved in binding activated, labelled oestrogen receptor in proteinaceous.

7. Saturation analysis of the binding of activated, labelled oestrogen receptor to both nuclear extract/sepharose resins revealed a single class of high affinity, saturable binding site (Kd $3\text{-}4 \times 10^{-11}$M) with other non-specific lower affinity binding sites being present.

8. Both nuclear extract/sepharose resins have been analysed for specific binding sites for androgen, glucocorticoid and progesterone receptor complexes. The nuclear material solubilised from human myometrium by mild acid treatment contained specific binding sites for all three classes of steroid-receptor complex. The nuclear material soluble in 2M NaCl only contained specific binding sites for androgen and glucocorticoid receptor complexes. In all cases the number of binding sites were lower than the number available to activated, labelled oestrogen receptor. Both nuclear extract/sepharose resins contained binding sites for tamoxifen-oestrogen receptor complexes. The number of sites available for the tamoxifen-oestrogen receptor complex was much less than those available to bind oestradiol-oestrogen receptor complex.

9. Competition between the various steroid-receptor complexes for binding sites available on both the nuclear extract/sepharose resins has been assessed. Competition between anti-oestrogen oestrogen receptor complexes and oestradiol-oestrogen receptor complexes has also been assessed. The data suggest there are both unique binding sites for each steroid-receptor complex and a population of common binding sites.

10. Nuclei have been isolated from immature female rat kidney, liver, spleen and uterus. The 2M NaCl soluble nuclear fraction was then isolated in each case and analysed for the ability to specifically bind activated, labelled oestrogen receptor complex. The ability of the various preparations to bind activated, labelled oestrogen receptor was uterus $>$, liver $>$, spleen $>$, kidney.

11. Protein blotting techniques have been used in an attempt to identify the nuclear protein(s) which are involved in the binding activity. Both oestrogen receptor labelled with ^{125}I-oestradiol and rabbit antiserum raised against human myometrial oestrogen receptor in conjunction with ^{125}I-protein A, have been used in a search for a discreet protein fraction(s) involved in binding oestrogen receptor. The use of the latter technique revealed a faint signal around M_r approximately 15,000 which is worthy of further investigation.

1. Introduction

1.1 Control and Regulatory Mechanisms in Higher Organisms

The human body has two major control systems to regulate both the way in which it perceives and reacts to its surrounding environment and to regulate its biochemical function. These are (1) the nervous system and (2) the endocrine system which involves the ductless or endocrine glands. There is interaction between both systems at various levels but in general the nervous system is involved in the rapid transmission of information within the body. This is achieved by electrical impulses which pass along the nerve fibres of the body. The endocrine system is concerned with the regulation of metabolism, including aspects of cellular metabolism, growth and differentiation. Communication in this system uses hormones. The effects of hormones can take place in seconds, (e.g. the catecholamines) or can extend over a much longer period of time (e.g. steroid hormones).

The endocrine glands are ductless tissues which secrete the hormones directly into the blood stream. Thus, hormonal responses are relatively slow compared with nervous transmissions.

A hormone is a chemical substance that is secreted into the blood stream at one location in the body and has its physiological effects at another, distant site. These distant sites have come to be known as target tissues. One hormone can effect more than one target tissue and it is possible for target tissues to be responsive to more than one hormone. The tissues which are affected by a hormone are defined by the presence of a chemical receptor (usually protein in nature) on or within the target cell. These receptor molecules enable a target tissue to perceive a hormonal signal and this is the initiation point for the further events which are induced by the interaction with the hormone. These events may include changes in enzyme activity (e.g. phosphorylation and/or dephosphorylation),

transport activity and longer term responses leading to modulation of gene expression, growth and cell division.

Endocrine glands involved in the synthesis and release of hormones may be influenced by environmental factors which may, through neuronal secretions, increase or decrease their activity. There is a close relationship between the neuronal and endocrine system. The complex control mechanism of the endocrine system may involve both a neural control loop and a feedback chemical control via the blood supply.

In terms of chemical structure, hormones can be divided into the following classes:-

1. Steroid Hormones e.g. oestradiol-17β
2. Amino Acid Derivatives e.g. the catecholamines
3. Peptide Hormones e.g. Insulin

Classically these hormones can be divided into two groups: (1) those which act via a receptor present on the cell surface or (2) those which combine with a receptor which is located within the cell. Recently this separation has become clouded.

It is generally agreed (Hollenberg, 1979; Catt et al., 1980) that the primary site of action of catecholamines and polypeptide hormones is the plasma membrane of target cells. The short term responses elicited by these hormones are mediated by second messengers, involving the activation of protein kinases by cyclic nucleotides. However it is possible that some of the longer term responses might involve direct action of these hormones at the nuclear level, although current evidence (Houslay and Heyworth, 1983; Kono, 1983) argues against a direct nuclear role for insulin receptor. Nevertheless the possibility cannot be totally eliminated that the mitogenic effect of insulin is mediated by direct interaction with the target cell nucleus.

Internalisation of at least a proportion of the hormone-bound plasma membrane receptors appears to be a property of most polypeptide hormone receptors (Catt _et al_., 1980). This internalisation appears to be generally associated with degradation and resynthesis or recycling of the receptors.

Classically, steroid hormones are thought to operate via a receptor molecule which is contained within the target cell i.e. steroids must enter the target cell, but recently oestradiol receptors have been reported on the surface of endometrial cells and hepatocytes (Pietras and Szego, 1977, 1979, 1984; Szego, 1984). The remainder of this thesis will be confined to the molecular mechanisms of steroid hormone action.

1.2 Steroid Hormones

1.2.1 Classes of steroid hormones.

There are six classes of steroid hormones represented by oestrogens, progestins, androgens, glucocorticoids, mineralocorticoids and the recently included vitamin D_3 (cholecalciferol) metabolites (Wecksler and Norman, 1980; Pike, 1982).

The sex steroids (Oestrogens, Progestins and Androgens) act principally on the reproductive tissues. Reproduction, however, involves a complex inter-relationship between different target organs and it seems surprising that such simple chemical compounds can produce such diverse effects on both metabolic and behavioural patterns.

1.2.2 Steroid structure.

Steroids are relatively small hydrophobic molecules derived from cholesterol. The strong hydrophobic and therefore lipophilic nature of steroids is thought to assist in the diffusion of steroids across the cell membrane.

Cholesterol biosynthesis takes place mainly in the liver and the intestine. The endocrine glands can therefore use plasma

cholesterol for synthesis of steroids, however the adrenal cortex, ovaries and other endocrine glands have the capacity to synthesise cholesterol from acetate. In the testis steroids are synthesised exclusively from acetate.

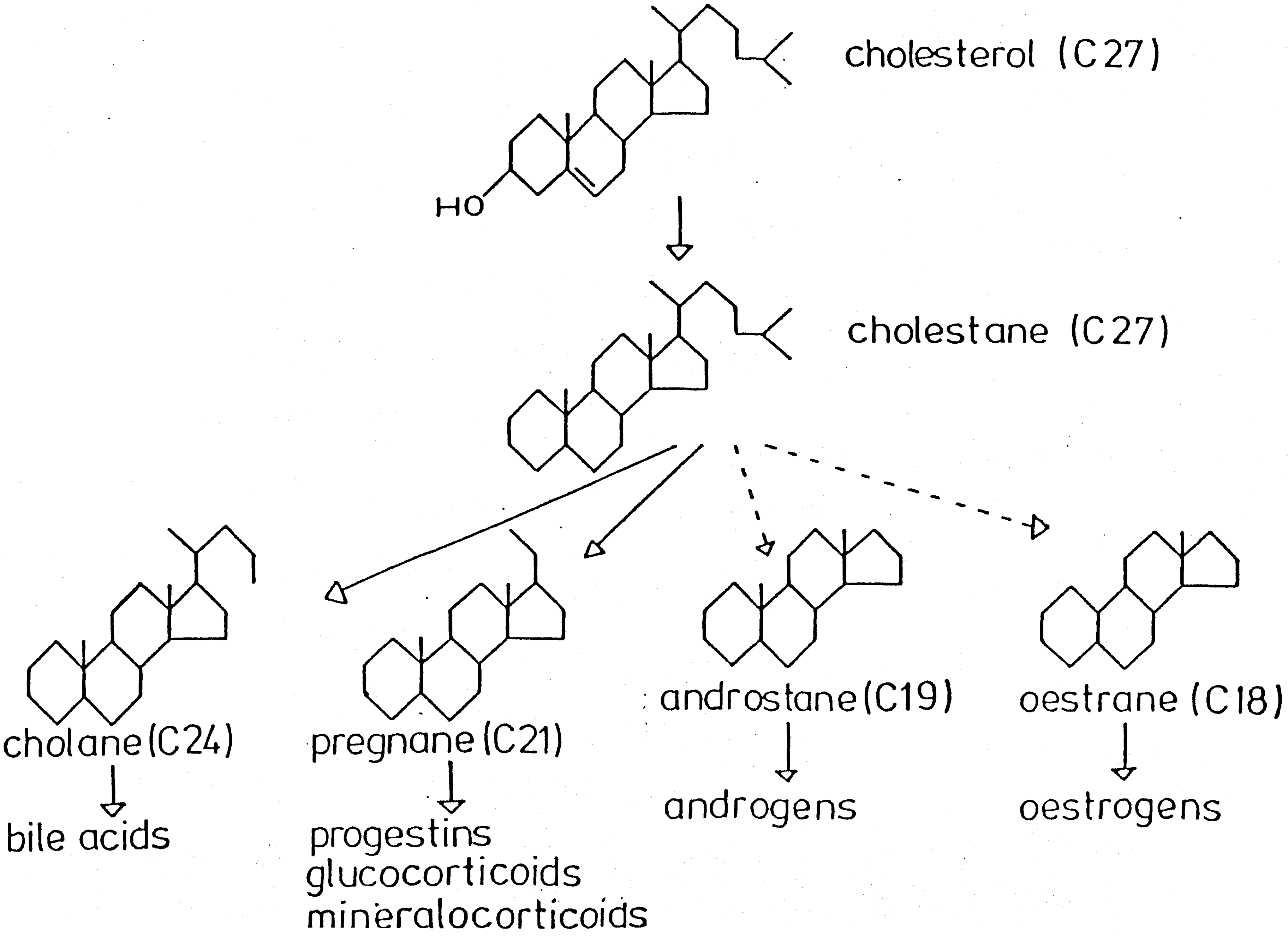
Figure 1 shows the various dehydrogenation reactions involved in the production of the final steroid structure. The steroid skeleton may be modified by various substitutions such as double bonds, hydroxyl or ketone groups, either alone or in combination. Biologically active steroids all possess an unsaturated A ring. Binding of the steroid to its specific receptor is determined by the spatial arrangement of the polar substituents. However, the nature of binding is largely non-polar due to the mainly non-polar structure of the steroid (Liao et al., 1973a). The structure and nomenclature of steroids is fully described by Gower (1979).

1.2.3 Oestrogens.

1.2.3.1 Synthesis.

Characteristically oestrogens have an A ring bearing a phenolic group in position 3 of the steroid nucleus (Fig.2). Steroidal oestrogens are 18 carbon atom compounds with substitutions at various positions in the ring structure. In premenopausal women the principal form is oestradiol-17β which is synthesised from cholesterol in the ovary by aromatisation, in the granulosa cells, of androgens produced by the theca cells as a result of stimulation by gonadotrophins from the anterior pituitary. The ovaries of post-menopausal women synthesise little oestrogen, the main source of oestrogen synthesis being the adrenal glands by the conversion of 4-androstene-3, 17- dione to oestrone (England et al., 1974; Gower and Fotherby, 1975). A similar conversion takes place in the peripheral tissues (Siiteri, 1978).

Figure 1 - Ring Structure of the Steroid Family


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

In males, oestradiol-17β is synthesised in the Leydig cells of the testes and amounts to one fifth of that in non-pregnant females (Longcope et al., 1972).

Once released into the blood stream, the activity of oestrogen has to be regulated. Consequently, oestradiol is metabolised to the less active oestrone with which it forms an equilibrium. Oestrone can be further metabolised to oestriol. Other methods of inactivation include, hydroxylation and methylation at C-2, oxidation at C-6 and hydroxylation of C-11. These conversions occur mainly in the liver, which is also responsible for the conjugation of oestrogens with glucuronic acid or sulphuric acid rendering the oestrogens more water soluble prior to excretion (see Figure 3).

1.2.3.2 Physiological action.

The principal actions of oestrogens include the following:

(1) The development of the female sex characteristics and reproductive organs at puberty such as uterus, vagina and mammary glands.

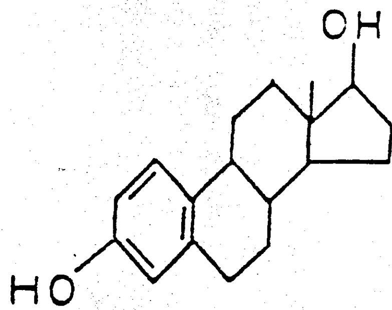
(2) During the proliferative phase of the menstrual cycle, oestrogens promote growth and development of the uterine endometrium.

(3) During pregnancy, both the glycogen and actomyosin content of the myometrium are increased by oestrogens which help in sensitising the myometrium to the stimulatory actions of oxytocin, perhaps by making available free Ca^{2+} ions (Lee and Laycock, 1978).

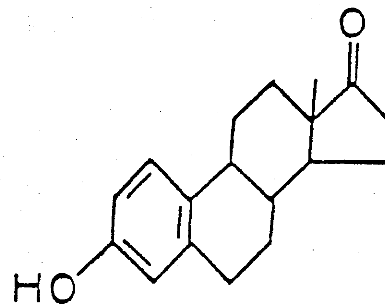
(4) The proliferation during pregnancy of the mammary duct in preparation for lactation.

(5) In combination with progesterone, oestrogens are involved in the controlled development of the foetus.

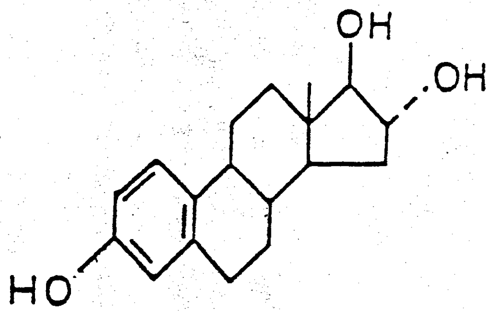
(6) Oestrogens regulate the hypothalamus and the anterior pituitary glands through positive or negative feedback loops. Oestrogens also regulate the activity of cortisol and thyroxine binding globulin by regulating their synthesis in the liver.



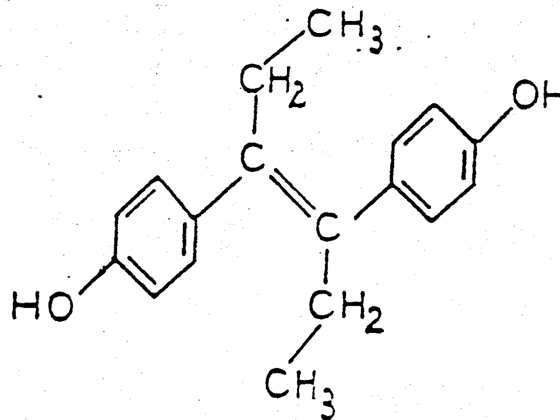
17-β oestradiol



oestrone



oestriol



diethylstilboestrol

Figure 2 - Structure of Oestrogens.

(7) The characteristic deposition of fat observed in females and calcification of epiphyseal cartilage.

(8) Some of the general metabolic effects of oestrogens include mild retention of water and sodium, lowering the plasma cholesterol concentration, and the stimulation of cervical mucosa making the mucus thinner and more alkaline for the survival and capacitation of spermatozoa.

The elucidation of the mechanism by which the above mentioned processes are brought about was greatly assisted when it became possible to designate "oestrogen target tissues" as these which contained specific oestrogen receptors. It should be noted that not all the oestrogenic responses, for example water retention, are necessarily mediated by receptor-genome interaction (Tchernitchin, 1979).

1.2.4 Anatomy of the uterus.

The uterus can be functionally divided into two tissues, the myometrium and the endometrium. The endometrium is mainly composed of epithelial and stromal cells, while the myometrium comprises mostly of smooth muscle cells.

The endometrium is firmly attached to the myometrium and undergoes cyclic changes in response to ovarian secretory activity. It is composed of two layers: the lamina basalis and the overlying lamina functionalis.

The glandular epithelium is a single layer of columnar cells, forming the lining to glandular structures. These glands grow rapidly in length during the normal oestrous cycle (see Section 1.2.5) and become distended with secretory material under the influence of progesterone (Dallenbach-Hellweg, 1981).

The epithelial cells lining the lumen of the uterus, resemble the glandular epithelium. They are very sensitive to oestrogen stimulation and during the proliferative phase of the

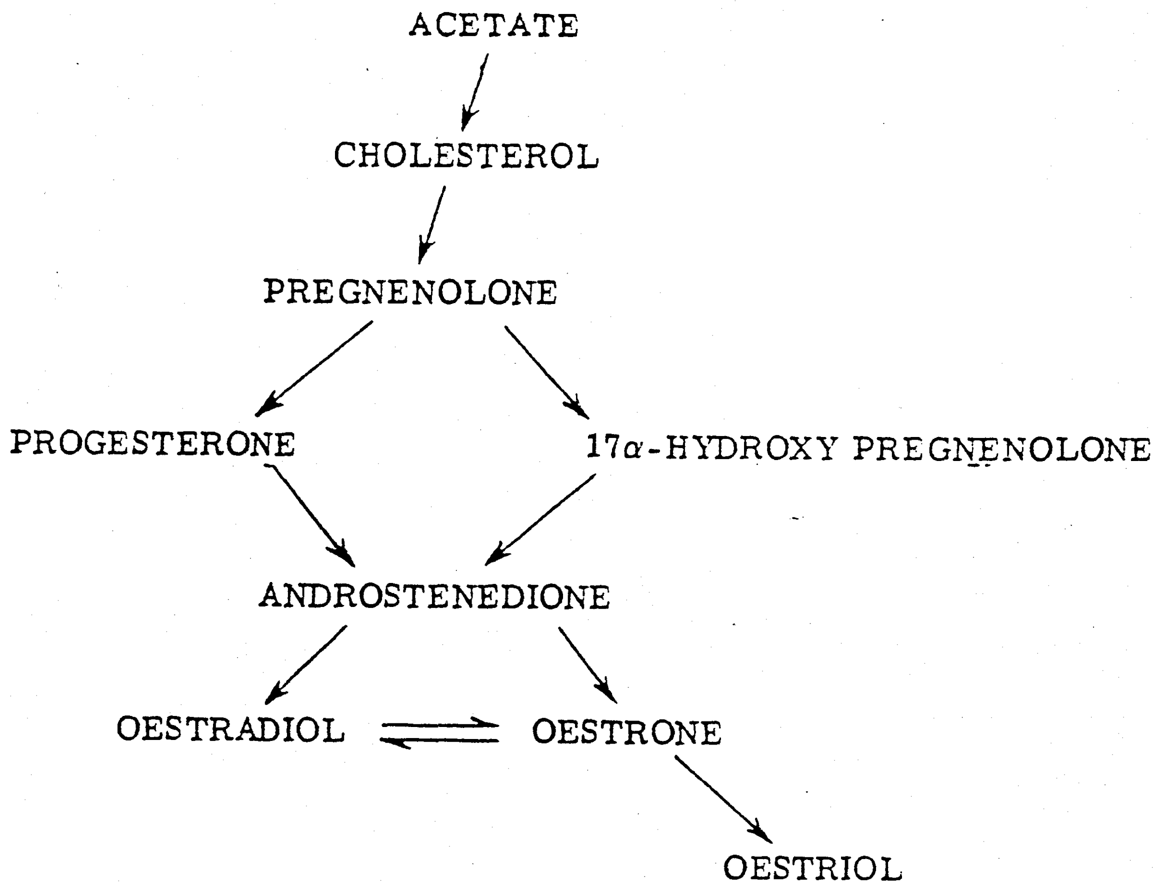


Figure 3 - Pathways Involved in Oestradiol and Oestrone Synthesis. (Adapted from Lee and Laycock, 1978).

oestrous cycle they divide in response to the increasing levels of circulating oestradiol.

The endometrial stroma consists of mesenchymal cells. In the adult, under control of progesterone, endometrial stromal cells differentiate into two forms, endometrial granulocytes and predecidual cells. These are present in roughly equal proportions (Dallenbach-Hellweg, 1981).

A variety of other minor cell types are also occasionally found in the endometrium, such as lymphocytes, mast cells, plasma cells and eosinophils (Dallenbach-Hellweg, 1981). Increased infiltration of eosinophils in response to oestrogen stimulation has some important consequences (see Section 1.3.1).

The functionalis layer contains blood vessels which differ from vessels of other organs by their unique structure and sensitivity to hormones (Dallenbach-Hellweg, 1981). Particularly obvious are spiral arterioles which branch extensively. Blood capillaries, veins and lymphatic capillaries are all to be found in the endometrium. It is thought that nerve fibres do not exist in the functionalis layer, but may occur in the lamina basalis (Dallenbach-Hellweg, 1981).

The myometrium is a massive coat of smooth muscle surrounding the endometrium and is contained within the outer sheath of the uterus (the perimetrium). The muscle fibres are separated by connective tissue. At least three layers of muscle may be distinguished, but are somewhat ill-defined owing to the presence of interconnecting bundles (Schoenberg, 1977). Within the muscle is a rich network of arteries and veins supported by dense connective tissue. During pregnancy, under the influence of oestrogen, the myometrium increases greatly in size both by cell division and by cell growth (de Brux et al., 1981). At parturition (and occasionally at other times) strong contractions of the myometrium are reinforced by the action of hormones (Sarosi et al., 1983).

1.2.5 The Oestrous Cycle.

Female sex steroids can regulate their own synthesis via the hypothalamic-pituitary axis (see Johnson and Everett, 1980). Consequently, the plasma levels of both oestrogen and progesterone change cyclically, giving rise to the oestrous or menstrual cycle In non-mated rats, the oestrous cycle is usually 4-5 days duration (Johnson and Everett, 1980), in humans the cycle lasts around 28 days. The cycle commences with a regenerative phase during which the denuded endometrium is re-epithelialised. In the following days the dominant feature is cell proliferation with development of all the individual structures, leading to an increase in thickness of the endometrium. This process is sustained under oestrogen stimulation until ovalation. The release of progesterone from the corpus luteum, which is formed after ovalation, promotes production of copious, thick, glycogen rich secretions by the 'endometrial' glands. During the first half of this phase major changes occur in the epithelium. Proliferation ceases and the cells engage in secretory activity. Spiral arteries and extracellular fibres reach their maximal development and predecidual cells and endometrial granulocytes appear. The secretory phase continues until progesterone levels decline. The decrease in progesterone levels, towards the "end" of the cycle, results in the sloughing off of the uterine endometrium, with subsequent bleeding in humans (menstruation), but not in rats, where reabsorption of the endometrium occurs.

1.2.6 Development of the Concept of Receptors and Target Tissues.

The physiological responses to oestrogens have been recognised for several years and the biochemical mechanisms by which these responses are mediated and regulated are far from understood.

A major advance was made with the assumption that receptors were involved in the action of hormones (Hechter and Halkerston, 1964).

It was assumed that the distribution of receptors determined tissue specificity and that the nature of the homrone receptor complex controlled the tissue response.

Shortly after an injection of tritium labelled oestradiol -17β, the hormone could be detected in all rat tissues (Jensen and Jacobson, 1962) but only target tissues (uterus and vagina) retain and concentrate the hormone. It was also demonstrated that the oestradiol was retained in an unmetabolised form. Thus, it was proposed that target cells contain specific receptors which combine with oestrogen to form a complex. These studies were extended by Noteboom and Gorski (1965) who reported that the oestrogen receptor is stereo-specific and probably a protein. Toft et al. (1967) identified it by sucrose density gradient analysis in wholly in vitro experiments. This also confirmed the earlier results of Talwar et al. (1964) who had shown separation of a protein bound fraction from free tritiated oestradiol using sephadex chromatography. Steroid binding molecules have been shown to be heat labile and sensitive to proteolytic enzymes confirming their proteinaceous nature (Toft and Gorski, 1966).

As detailed investigations were undertaken, it was realised that the interaction of oestrogen with uterine cells is not a simple association effect. Using autoradiographic and ultracentrifugation techniques, radioactive hormone was found to be located in two separate regions (Toft and Gorski, 1966; Toft et al., 1967). The data indicated that bound receptor was principally associated with the soluble and nuclear fractions. This led to the proposal of a two step model for the interaction of oestrogen with the uterus (Jensen et al., 1968; Gorski et al., 1968). (See Section 1.4.3.7.1).

The general model for the mechanism of action of steroid hormones considers that, once inside the cell, the steroid complexes

with a specific receptor protein. This complex then becomes activated and is able to interact with defined sites within the nucleus. Binding of receptor complexes at these defined sites leads to the specific changes in gene expression characteristic of the steroid in question.

Other target tissues identified through retention of labelled oestradiol-17β were the hypothalamus (Eisenfeld and Axelrod, 1966) and the mammary glands (Sander, 1968). Recently reports have been published which indicate that in addition to the abundance of oestrogen receptor in the target tissues, there are low concentrations of high affinity oestrogen receptor in what were previously considered non-target tissues. These include the liver (Aten et al., 1978), kidney (Li et al., 1974), adrenal glands (Muller and Wotley, 1978) and ovary (Richards et al., 1976). In the mammalian liver, for example, oestrogen enhances the production of plasma renin substrate, some blood clotting factors and some other serum proteins. In the ovary, oestrogen receptor may be involved in modulating binding of follicle stimulating hormone and, therefore, corresponding follicular development (Gorski and Gannon, 1976).

1.3 Uterotrophic Responses

A single injection of oestradiol into an immature female rat stimulates a number of biochemical and metabolic events within the uterus. These events are classified into "early" or "late" uterotrophic responses (Clark and Peck, 1979).

1.3.1 "Early" Responses.

Early uterotrophic responses include a vast array of events which occur within the first 3-4 hr after oestrogen injection and they involve the generalised initiation of metabolic and biosynthetic mechanisms of the uterus. Examples of these responses include:- hyperemia, calcium influx, histamine release, eosinophil infiltration,

increased RNA synthesis, increased uptake of RNA and protein precursors, and enhanced glucose oxidation. Some increased biosynthetic activities occur as part of the early responses to oestrogen and these are exemplified by rises in glucose-6-phosphate dehydrogenase and creatine kinase (induced protein) activities brought about by increased transcriptional activity induced by oestrogen (Kaye, 1983).

However, some of the early responses such as water inhibition, increase in vascular permeability and histamine release are not due to effects of oestrogen elicited through the oestrogen receptor system of uterine cells. These are thought to be caused by the effects of eosinophils which are attracted to the uterus by oestrogen. Eosinophils possess their own independent oestrogen receptor system (Tchernitchin, 1979; Lee, 1982).

1.3.2 "Late" Responses.

Late uterotrophic responses are associated with cellular hypertrophy and hyperplasia of the uterus. These are considered to be true growth responses. They represent the culmination of biosynthetic events, and are maximal 24-36 hrs after administration of oestrogen. At this time maximal rates of protein synthesis are observed, in addition both nucleic acid content and synthesis are elevated but the number of receptor/hormone complexes in the nucleus has declined to control levels. This suggests that the hormone has altered the uterine cells prior to 24 hrs. In fact, the receptor/hormone complex must remain in the nucleus for 6-12 hr to elicit these late responses (Clark and Peck, 1976; 1979).

1.4 Mechanism of Steroid Action

1.4.1 Plasma Transport.

Hormones are released into the blood stream by the endocrine glands. The sex steroids are then transported in the blood stream

by sex hormone binding globulin (SHBG), albumin and other plasma proteins (Clark and Peck, 1979). These transport proteins have several functions:-

(1) to protect the steroids from liver metabolism

(2) to overcome their insolubility and

(3) to provide a reservoir of available steroid.

The blood proteins bind steroid with varying affinities and the free hormone level determines the amount of steroid available to the tissue (Westphal, 1971; 1980).

1.4.2 Entry into cells.

Due to the lipophilic nature of steroid hormones it is believed that their entry into cells is by passive diffusion (Rao, 1981) which explains the entry of steroid into target and non-target tissues (Jensen and Jacobson, 1962). However the facilitated transport mechanism proposed by Milgrom et al.(1973) cannot be ruled out.

Studies of Pietras and Szego (1977) using affinity chromatography have shown the existence of oestrogen binding sites on the surface of endometrial and liver cells. Similar findings have been reported by others (O'Malley and Means, 1974; Wittliff, 1975). More recently Muller et al. (1979), Pietras and Szego (1979) and Szego (1984) have shown specific oestrogen binding sites associated with uterine plasma membranes, however the orientation of the membranes as assayed must be uncertain, and the enzyme digestion involved in the preparation of the cells could result in an abnormal distribution of receptors. The binding sites could also represent steroid metabolising enzymes.

It would appear that the major difficulty in the interpretation of results on uptake is the failure to distinguish between binding of steroids to receptor and to other proteins.

1.4.3 Steroid Receptor Molecules.

Generally speaking receptor proteins should display high affinity saturable binding for a specific hormone or biological class of hormones. This specificity enables target cells to respond to a hormonal signal without interference from other signals.

Implicit in all studies of macromolecules that bind steroids is the assumption that the binding of hormone to putative receptors must precede or accompany tissue responses, and the extent of the response should relate to some function of receptor occupancy. The demonstration of receptor dependent hormonal responses is not often met, and is the most difficult to establish. Indeed the role of hormone receptors in oestrogen activity has been questioned (Meyers, 1984).

1.4.3.1 The Oestrogen Receptor.

The oestrogen receptor was first characterised in the immature rat uterus by Toft and Gorski (1966) as a protein of molecular weight 50,000. More recently the calf uterine oestrogen receptor has been purified to homogeneity using affinity chromatography (Sica and Bresciani, 1979) and shown to have a molecular weight of 70,000. This compares with 76,200 for the rat uterine receptor (Jensen and De Sombre, 1972) and 50,000 for the receptor from human breast tumour cells (Greene et al., 1980).

Recently Sakai and Gorski (1984a) reported a molecular weight of 65,000 for the immature rat uterine receptor and also that it is possible to reversibly denature the oestrogen receptor protein using 2.3% SDS. The renatured receptor is similar to the native receptor in both affinity for oestradiol-17β and ability to bind DNA. The renaturation of cytosol and nuclear receptors to forms with properties indistinguishable from those of native receptor indicates that the

protein is not proteolytically processed to a large extent after nuclear transformation as has been suggested previously (Puca et al., 1977).

Oestrogen receptor molecules correspond to the "classical" type I binding sites reported in both the cytosol and nuclei of target tissues and have a dissocation content of 10^{-10}M for oestradiol in human tissue (McGuire and Julian, 1971). The molecules are responsible for the changes in transcriptional activity induced by oestrogen in target cells (Clark et al., 1978a). A secondary function of these molecules is to concentrate oestrogen within target cells (Clark and Peck, 1979; Leake, 1981).

Type II cytosolic binding sites have also been reported. They have a lower affinity for oestrogens (Kd 3 x 10^{-9}M in humans) and have an ill-defined cellular function. They are not involved in the changes in transcription produced by oestrogens (Clark et al., 1978a; Eriksson et al., 1978). Type II binding sites seem to be a general phenomenon and have been found in many different tissues including rat uterus (Clark et al., 1978a) chick oviduct (Smith et al., 1979) and human breast tumour (Panko et al., 1981).

Type II nuclear binding sites have also been reported (Clark and Peck, 1979). The binding is steroid and tissue specific, though the affinity for oestrogen is lower than that observed for type I nuclear binding sites. Type II nuclear binding sites are in no way related to cytosolic type II sites, and the function of these nuclear type II sites is still unclear.

Similar type II binding sites have also been reported for glucocorticoids (Barlow et al., 1979).

Recently it has become apparent that the calf uterine oestrogen receptor is a phosphoprotein. The phosphorylation state of the receptor is regulated by a kinase/phosphatase system.

Calmodulin has been shown to stimulate the phosphorylation of the protein (Auricchio et al., 1984; Migliaccio et al., 1984). It has been reported that the phosphorylation state of the receptor regulates the binding of steroid,- steroid binding is greatly enhanced when the receptor is phosphorylated (Auricchio et al., 1981; Migliaccio and Auricchio, 1981; Migliaccio et al., 1982). The phosphorylated residue on the receptor has been shown to be a tyrosine moeity (Migliaccio et al., 1984).

Protein phosphorylation/dephosphorylation is a major regulatory process of cellular activity and several peptide hormone receptors e.g. insulin (Kasuga et al., 1982) and epidermal growth factor (Cohen et al., 1980) have also been reported to be phosphoproteins. These peptide receptor molecules are also substrates for tyrosine protein kinases, a relatively unusual class of kinase.

1.4.3.2 The Progesterone Receptor.

Several lines of evidence support the notion that the native structure of the progesterone receptor of chick oviduct is a dimer of two dissimilar subunits or higher aggregates thereof (Grody et al., 1982). The 8S form of the receptor also contains proteins which do not bind progesterone (Gasc et al., 1984; Renoir et al., 1984). Transformation of this receptor by heat or salt causes a shift in sedimentation constant from 6-8S to 4S (Schrader et al., 1975; Moudgil et al., 1985). The 4S peak can then be shown using ion exchange chromatography to contain equimolar amounts of two dissimilar monomeric progesterone binding components. These proteins designated A and B possess kinetically identical hormone binding sites, but differ in their physical properties (Hansen et al., 1976). In addition the two proteins exhibit differential binding affinities for nuclear components: protein A binds with high affinity to DNA from a variety

of sources but not to chromatin, whereas B binds with high affinity to chromatin from target cells and only weakly to DNA (Vedeckis et al., 1980). Proteins A and B have been purified to apparent homogeneity and found to have molecular weights of 79,000 (Coty et al., 1979) and 108,000 (Schrader et al., 1977) respectively. The A and B subunits share a common domain of approximately 60,000 daltons (Birnbaumer et al., 1983). A similar subunit composition has been proposed for the human progesterone receptor (Lessey et al., 1983).

The presence of a dimer containing dissimilar subunits has been questioned by studies of the rabbit progesterone receptor and its biosynthesis. The protein in non-fractionated uterine cytosol was separated using electrophoresis and then transferred to nitrocellulose. The nitrocellulose was then probed using monoclonal anti-receptor antibodies. This revealed a single 110,000 dalton protein only when protease inhibitors were present during homogenisation. Smaller forms of the receptor (79,000 daltons) were present when these precautions were not observed (Loosfelt et al., 1984). The presence of a single subunit was further confirmed by translation of poly $(A)^+$ RNA from rabbit uterus in a reticulocyte lysate. Using the monoclonal anti-receptor antibody only a single protein of molecular weight 110,000 could be observed. Similar results were obtained when the rabbit progesterone receptor was purified by affinity chromatography using the anti-receptor monoclonal antibody immobilised on a column (Logeat et al., 1985).

These findings realign the progesterone receptor with the other receptors (glucocorticoid, oestrogen and androgen) for which biochemical or genetic evidence is in favour of a single functional subunit.

Both the hen oviduct progesterone receptor subunits have been reported to be phosphoproteins (Ghosh-Dastidar et al., 1984).

The subunits were phosphorylated on tyrosine residues in the presence of epidermal growth factor and the epidermal growth factor receptor. Chick oviduct progesterone receptor has also been shown to be a phosphoprotein (Dougherty et al., 1982,1984).

1.4.3.3 The Glucocorticoid Receptor.

Following electrophoresis under denaturing conditions the receptor behaves as a single polypeptide chain of molecular weight 85,000-95,000 (Rousseau, 1984). Polyclonal (Carlstedt-Duke et al., 1982) and monoclonal antibodies raised against the "non-transformed" or the "transformed" receptor have allowed one to delineate at least three distinct domains on the receptor molecule (Rousseau, 1984). These domains are:- (1) the steroid binding domain, (2) the DNA binding domain and (3) the immunoreactive domain.

The steroid binding domain is responsible for steroid binding, little else is known about this domain. Inhibition of steroid binding by pyridoxal 5'-phosphate and 12 cyclohexanedione (Di Sorbo et al., 1980) may indicate that lysine and arginine residues are involved in steroid binding.

The DNA binding domain comprises a portion of the receptor that is distinct from but is about the same size as the steroid binding domain. Its affinity for DNA is greater than that of the holo-receptor (Di Sorbo et al., 1980). The interaction of the receptor with DNA is understood in much greater detail (see Section 1.5.3).

The immunoreactive domain, which is roughly half the size of the holo-receptor, can be separated by proteolysis from the steroid and DNA binding domains. The latter domains are poorly immunogenic, probably because they are highly conserved. The immunoreactive domain is required for glucocorticoid activity and has therefore been called the specifier domain (Vedeckis, 1983).

The receptor protein can be considered as the actual mediator of glucocorticoid action, while the hormone plays the role of an allosteric ligand which promotes the "transformation" of the receptor into its DNA binding conformation. Transformation is thought to involve the dissociation of the oligomeric receptor at a rate which is temperature-dependent, into monomers that are, by definition, endowed with DNA binding activity (Vedeckis, 1983). This is in contrast to the formation of the 5S form of the oestrogen receptor which is thought to arise through dimerisation of 4S monomers (Notides et al., 1981) (see Section 1.4.3.5). The transformation event, which was first described in cell free systems, has now been shown to occur in intact cells (Munck and Foley, 1980). One consequence of receptor transformation is exposure of positive changes on the surface of the molecule which thus binds to polyanions, including DNA itself.

The glucocorticoid receptor from rat liver cytosol has also been reported to be a phosphoprotein (Houslay and Pratt, 1983; Kurl and Jacob, 1984).

It has also been reported that the glucocorticoid receptor from rat liver cytosol displays protein kinase activity but no autophosphorylation of the glucocorticoid receptor was reported from these studies (Singh and Moudgil, 1984). Dephosphorylation of the receptor may be required for complete transformation to take place (Rousseau, 1984).

1.4.3.4 The Androgen Receptor.

The size of the activated androgen receptor depends on the tissue studied. Colvard and Wilson (1981) using Dunning prostate carcinoma found that an 8S receptor was generated from a 4.5S precursor, and have since claimed that the 8S receptor is composed of an activated 4.5S receptor and a non-steroid binding protein which

renders the receptor incapable of binding nuclei (Colvard and Wilson, 1984). Using receptor from normal genital skin fibroblasts, Kovacs et al. (1983) showed that activation involved the conversion of a large complex (7S in human tissue) to a 3S molecule (in 0.3M KCl containing sucrose density gradients) which bound DNA.

Activation of the dihydrotestosterone receptor complex from rat prostate causes a decrease in the sedimentation rate from 3.8S to 3.0S (Liao, 1975). The 3S form of the receptor is believed to be the nuclear form which interacts with chromatin, inducing specific changes in gene transcription. Recently the androgen receptor has been purified from steer seminal vesicles and shown to have a molecular weight of 60,000-70,000 (Chang et al., 1982). After purification, the rat ventral prostate androgen receptor (Chang et al., 1983; Goueli et al., 1984) was shown to have a molecular weight of 85,000-87,000. The differences reported here would be due to proteolysis of the receptor during preparation or may represent a true species difference.

Target tissues for androgens are unique in that they metabolise the naturally occurring androgen, testosterone to 5α dihydrotestosterone which is the physiologically active form. The enzyme involved is 5α reductase. No such activation of other steroid ligands is known to occur.

1.4.3.5 Activation of Steroid Hormone Receptors.

At present there is disagreement over the actual site of activation. The cytosol (Jensen and De Sombre, 1972; O'Malley and Means, 1974) has been proposed which agrees with the classical two step model of steroid hormone action (see Section 1.4.3.7.1). More recently both the cytoplasm and nucleus (Linkie and Siiteri, 1978; Linkie, 1982) have been proposed, which is in agreement with the equilibrium model of Martin and Sheridan (1982) (see Section 1.4.3.7.2).

The nuclear model (see Section 1.4.3.7.3) suggests activation is a nuclear event although the recovery of oestrogen receptor in membrane fractions of cell homogenates (Szego, 1984) offers yet another possible site of activation to be considered.

All steroid receptors, including Vitamin D_3 (Brumbaugh and Hanssler, 1974) show a temperature dependent activation step which gives the receptor an increased affinity for nuclei, DNA, chromatin and other polyanions (Toft, 1972; Buller et al., 1975; Muller and Toft, 1978). Most investigators have found the change to be also dependent on changes in salt concentration or dilution. For some receptors e.g. the oestrogen receptor, activation is associated with major changes in molecular weight quaternary structure, sedimentation properties and other easily detectable physical parameters. This has become known as receptor transformation. Activation of oestrogen receptor also results in an increased affinity of interaction between the receptor and oestradiol.

Originally Williams and Gorski (1971) reported that activation was temperature sensitive. More recently Pavlick et al. (1979) and Traish et al. (1979) have reported activation at low temperature, although the rate of formation of active receptor remains temperature dependent.

The available or empty oestrogen receptor has been shown to sediment on a low ionic strength sucrose density gradient at around 8S. However the in vivo significance of this 8S receptor form has been questioned recently (King, 1984) and it may represent an in vitro artefact. The 8S form of the receptor can be dissociated into 4S monomers by centrifugation in ionic strengths greater than 0.2M KCl (Korenman and Rao, 1968).

The process of activation of the oestrogen receptor from rat uterus was shown (Notides and Nielsen, 1974, 1975) to be accompanied by an increase in sedimentation constant (in 0.4M KCl) from 4S to 5S. The 4S to 5S conversion follows second order kinetics and the 5S complex is assumed to be a dimer of a modified form of the native 4S receptor (Little et al., 1975; Notides et al., 1975, 1981). A similar change in sedimentation constant has been shown to accompany activation in other species, although, for example in human breast tumour it can only be demonstrated under very exacting conditions (Hyder and Leake, 1982).

Arginyl residues are thought to play a role in oestrogen receptor activation and transformation. Muller et al. (1983b) propose that the nuclear binding site of the oestrogen receptor contains important arginyl residues and that the integrity of a distinct set of arginyl residues in the oestrogen binding domain is required for the heat induced formation and maintenance of the receptor state with slow oestradiol dissociation. Dimerisation of the receptor subunits does not involve arginyl residues. Sulphydral groups are also thought to play an important role in the binding of oestradiol (Jensen et al., 1967; Muldoon, 1971).

In the case of the calf uterine oestrogen receptor (Muller et al., 1985) and the immature rat uterine receptor (Sakai and Gorski, 1984) it has been reported that the heat induced acquisition of the high affinity state for oestradiol does not require 4S to 5S dimerisation. These findings suggest that the 4S monomers exist in equilibrium between low affinity and high affinity conformations. Oestradiol binding to the low affinity state causes conformational changes which result in stronger interactions between the steroid and the amino acid residues of the oestrogen binding domains, thus the rate of oestradiol dissociation decreases. The formation of this

4S state with higher affinity for oestradiol is independent from receptor dimerisation. Dimerisation of the high affinity receptor may shift the equilibrium to greatly favour the high affinity state.

These data may confirm earlier observations that oestrogen receptor activation is an independent process from transformation (Bailly et al., 1980; Gschwendt and Kittstein, 1980) and that activation precedes, and is not related to transformation (Muller et al., 1983).

The use of pyridoxal phosphate in the study of the dissociation of oestradiol from activated and activated/transformed oestrogen receptor has suggested that lysine residues in the oestrogen binding domain are important in oestradiol binding. Increased oestradiol dissociation from the receptor in the presence of pyridoxal phosphate is due to alteration of these lysine residues and not disruption of dimers into monomers (Traish et al., 1980; Muldoon et al., 1980; Muller et al., 1985). The effects of pyridoxal phosphate on oestrogen receptor are reversible by the addition of agents that cause transschiffation (Muller et al., 1980).

Apart from oestrogen, activation of other steroid hormone receptor complexes does not involve an increase in sedimentation constant. Indeed, activation of the dihydrotestosterone complex of rat prostate causes a decrease in sedimentation rate from 3.8S to 3.0S (Liao, 1975). Similar decreases have been reported for the progesterone receptor complexes of hamster (Chen and Levitt, 1979), guinea pig and rabbit uterus (Saffron et al., 1976). Progesterone-receptor complex of chick oviduct shows no change in sedimentation rate after activation (Buller et al., 1975), a situation which is usually reflected in studies of activation of the other steroid-receptor complexes. It is impossible to say that dimerisation does not occur for all complexes during activation. Such dimers could

dissociate during the process of extraction and sedimentation. Equally there is much evidence that specific proteases can be closely associated with receptor, even during extraction, and that these proteases can directly reverse polymerisation of receptor (Gregory and Notides, 1982).

1.4.3.6 Inhibition of Activation.

The involvement of a low molecular weight inhibitor of activation has been reported by several groups. Sato et al., (1978a,b, 1979) have reported such an inhibitor for the oestrogen receptor in rat uterus and mouse Leydig cell tumours. Dialysis was found to increase the nuclear binding activity of oestrogen receptor. The rat liver glucocorticoid receptor complex has a similar inhibitor (Goidl et al., 1977). Low molecular weight inhibitors do seem to be a common feature for steroid receptors (Bailly et al., 1977; Shyr and Liao, 1978; Sato et al., 1979). Fishman (1981) has shown that electrolysis of rat uterus resulted in a marked increase in nuclear binding steroid receptor - a result attributed to removal of an inhibitor.

In contrast to low molecular weight inhibitors of activation, low molecular weight inhibitors of DNA or chromatin binding have also been reported (Cake et al., 1978).

Pyridoxal-5'-phosphate has been suggested as one such inhibitor (Nishigori and Toft, 1979; Muldoon and Cildowski, 1980). These factors seem to modulate the binding of activated receptor to chromatin and have been shown to be physiologically important (Disorbo et al., 1980). Recently pyridoxal 5'-phosphate has been shown to activate receptor but block subsequent DNA binding (Sekula et al., 1982). A class of macromolecular inhibitors may also regulate the binding of activated receptor to nuclei, DNA or chromatin (Milgrom and Atger, 1975; Lin and Webb, 1977; Atger and Milgrom, 1978).

Sodium molybdate has been found to inhibit the process of activation as measured by DNA binding in the case of the oestrogen

receptor (Pettersson et al., 1982; Lukola and Punnonen, 1983; Muller et al., 1983), progesterone receptor (Nishigori and Toft, 1980; Weigel et al., 1981; Chang-Ren et al., 1983; Lukola and Punnonen, 1983; Moudgil et al., 1985). Androgen receptor (Tsai and Steinberger, 1982) and glucocorticoid receptor (Norris and Kohler, 1983; Sherman et al., 1983). The ability of molybdate to inhibit DNA binding is much less for the androgen receptor than that seen for oestrogen and progesterone receptor.

The mechanism by which molybdate inhibits the DNA binding ability of the oestrogen receptor has been the subject of great debate (Grody et al., 1982; Gshwendt and Kittstein, 1983). Molybdate may stabilise the 8S form of the receptor (Grody et al., 1980; Muller et al., 1982; Muller et al., 1983a) or it may inhibit the action of a phosphatase (Barnett et al., 1980; Leach et al., 1980; Nishigori and Toft, 1980; Ruh and Ruh, 1984) or RNase and protease (Chang and Lippman 1981-82) whose activities are essential for DNA binding, or it may bind directly to the DNA binding site of the receptor (Lukola and Punnonen, 1983) or even stimulate a membrane bound guanylate cyclase (Fleming et al., 1983). Although molybdate inhibits DNA binding by receptor from rat uteri it stimulates DNA binding by rabbit oestrogen receptor. When oestrogen receptors from different breast tumours were studied both types of response were observed though the majority of samples did show inhibition of DNA binding activity (Thomas et al., 1983). The tissues which showed strong inhibition of DNA binding activity also contained a minor class of receptors which were still capable of being activated by heating in the presence of molybdate (Thomas et al., 1983).

In the case of the calf uterine oestrogen receptor two forms of the molybdate stabilised receptor can be resolved using ion-exchange chromatography (Ruh and Ruh, 1984). Evidence suggests that there is

an equilibrium between the two forms of the molybdate stabilised receptor.

These findings, especially those of Thomas _et al_. (1983) leave the precise effects of molybdate on the activation of oestrogen receptor still unresolved.

Other less commonly used inhibitors of the activation process are discussed by Grody _et al_. (1982).

1.4.3.7 Models of Steroid Hormone Action.

1.4.3.7.1 The Classical Model.

The classical "two-step" model of steroid hormone action was proposed simultaneously by Gorski and Jensen in 1968 (Gorski _et al_., 1968; Jensen _et al_., 1968). It was formed largely from the measurement of the distribution of specifically bound ^{3}H-oestradiol in the nuclear and soluble fractions of homogenised tissues. The tissues of animals having low endogeneous oestrogen levels (i.e. ovariectomised or immature animals) contained most of the available or empty receptor in the cytosol - the soluble portion of the cell homogenate remaining after high speed centrifugation. However after exposure to steroid _in vivo_, most of the hormone receptor complex could be recovered in the nuclear fraction, although 10-15% of total bound steroid always remained in the cytosol (Williams and Gorski, 1972). Very little unoccupied receptor was found in the nuclear fraction (Shyamala and Gorski, 1969).

Autoradiographic evidence was interpreted as supporting proposals that receptor with bound ligand was found in the nuclear fraction with available or empty receptor being found in the cytosol (Stumpf, 1968). Even in a cell free system, hormone receptor complexes acquire a high affinity for nuclei when incubated at 37°C but remain in the cytosol when incubated at 2°C (Jensen _et al_., 1968).

The overall ideas of this model were that:- (1) after entry into the target cell, the steroid was complexed with an "extra-nuclear" receptor, (2) this receptor was then "activated" (see Section 1.4.3.5) after which it transferred the steroid to the nuclear compartment, so called translocation, (3) subsequent nuclear binding of the receptor complex resulted in altered gene transcription.

The formal proposals of this model were widely accepted.

1.4.3.7.2 The Equilibrium Model.

Implicit in the "two-step" model is that unoccupied receptor should not be found in the nucleus - receptor should only be retained in the nucleus after it has bound steroid and then become activated. Unoccupied nuclear receptors have been reported in human mammary tumours (Zava and McGuire, 1977; Panko and McLeod, 1978), normal and malignant human endometrium (Fleming and Gurpide, 1980; Levy et al., 1980), hen liver (Mester and Banhen, 1972), rat hypothalmus (White and Lin, 1980) and the rat uterus (Thrower et al., 1981).

Sheridan et al. (1979) showed by using autoradiography, that even at $0^{o}C$, extensive nuclear localisation of specifically bound oestradiol occurred within 5 minutes. These data were in conflict with earlier work of Stumpf (1968) but were claimed to be more realistic due to the advance in methodology which had occurred in the intervening years (i.e. the thaw mount technique).

In another study (Martin and Sheridan, 1980), nuclei were prepared by an aqueous and a non-aqueous method. The aqueous method involved preparation of nuclei in normal Tris or phosphate buffers, both containing 10% glycerol and 0.01% monothioglycerol, whereas the non-aqueous method involved preparation of nuclei in 100% glycerol. When aqueous methods were used, the proportion of receptor recovered in the nuclear fraction depended on the volume of buffer used for

homogenisation. However, when non-aqueous methods were used, the majority of the unoccupied receptor was recovered in the nuclear fraction.

The study was expanded to progesterone receptor in the rat uterus (Sheridan et al., 1981) and, by using the thaw mount autoradiography technique, they showed that localisation of steroid was nuclear. In complete contrast when the tissue was processed by standard aqueous techniques the receptor was recovered mainly in the cytosolic fraction.

As a result of these studies, Martin and Sheridan (1982) have proposed that, in the intact cell, unbound steroid receptors are in equilibrium, partitioned between the nucleus and the cytoplasm according to the free water content of each compartment.

The initial idea of such an equilibrium based on the free water content of each compartment was developed by Horwitz and Moore (1974) from studies on the movement of radioactively labelled inert macromolecules in the frog oocyte which revealed that macromolecules concentrated in the nucleus because it represented a "water rich" environment when compared to the cytoplasm. DNA polymerase has been shown to leak out of the nucleus as is proposed for steroid receptors when the water content of the cellular compartments is changed during homogenisation (Forster and Gumby, 1976).

Further support for nuclear origin of receptors came from Linkie and Siiteri (1978) studying the time course of oestrogen receptor activation in the immature rat uterus. They performed a careful analysis of the nuclear forms of the receptor over a 2 hour period after the first exposure to oestrogen. As expected of a mechanism in which 4S to 5S conversion is a pre-requisite of nuclear binding,

the amount of 5S form recovered from the nuclear pellet increased from 0-40 minutes - but they could also detect 4S receptor in their nuclear pellet at all the time points. This was not casual contamination since it was maintained at about 0.18 pmol/uterus throught the first 40 minutes of the time course. At the same time the 5S:4S ratio rose from 0 to 6.41. This led the authors to suggest that the process of activation takes place in the nucleus and may require the presence of DNA as suggested by Yamamoto and Alberts (1972). Similar nuclear conversion of 4S to 5S oestrogen receptor has now been shown in other rat tissues and in other species (Linkie, 1982).

The concept that a specific high affinity cytoplasmic receptor as such, has little if any role to play in steroid hormone action, has gained popularity because of several experiments in addition to the above. Pietras and Szego (1979) showed that the use of hypotonic buffers for all cell homogenisation led to cytosol receptors but if 0.25M sucrose was included in the buffers, the majority of unoccupied receptor was recovered in the particulate fraction. This suggests that in vivo receptor may be attached loosely or otherwise to membrane components of the cell.

1.4.3.7.3 The Nuclear Model.

The specific suggestion that unoccupied steroid hormone receptors may be associated permanently with nuclear structures came from studies on the structure and function of the nuclear matrix. Specific high affinity binding sites for oestrogens and androgens were detected in the nuclear matrix of rat uterus, chicken liver and rat prostate (Barrack et al., 1977; Agutter and Birchall, 1979; Barrack and Coffey, 1980).

The nuclear matrix contains many DNA tight-binding proteins (Berezney and Coffey, 1977) and these proteins show a preference

for single stranded DNA and AT-rich DNA (Comings and Wallack, 1978), properties which have been demonstrated for steroid receptors (Hughes et al., 1981; Payvar et al., 1981; Compton et al., 1983; Sluyser, 1983). Thus the nuclear matrix would provide an ideal binding site for steroid receptors and would assist in the interaction of filled, activated receptor with specific parts of the genome.

1.4.3.7.3.1 Studies using Monoclonal Antibodies.

Although polyclonal antibodies have been raised to various oestrogen binding protein preparations, specificity for a single oestrogen binding protein has been achieved in only a few cases (e.g. Raam et al., 1982). Given the impure nature of many receptor preparations, and the general ability of steroid binding receptor subunits to react non-specifically with a wide range of macromolecules (Clark and Peck, 1979) studies of specificity must be rigorously carried out.

The development of monoclonal antibodies to steroid receptors has given another tool with which to probe the intracellular distribution of receptor. King and Greene (1984) have recently developed five monoclonal antibodies each of which recognises a sequence on one or other part of the MCF-7 cell line oestrogen receptor. These antibodies have been used to localise oestrogen receptor by an indirect immunoperoxidase technique in frozen fixed sections of human breast tumour, human and rabbit uterus and in fixed MCF-7 cell cultures. In target cells not exposed to oestrogen, oestrogen receptor is localised predominantly in the nucleus. Following short term treatment of animals or cells with physiological levels of oestradiol, little or no increase in nuclear staining occurs in either immature or ovariectomised rabbit uteri or MCF-7 cells indicating that empty oestrogen receptors are normally associated with the nucleus in the intact cell. The nuclear staining observed

in each case fulfilled all the accepted criteria for specificity (Childs, 1983; Petrusz, 1983). Specific nuclear staining was either absent or limited to a very few cells in biochemically-determined, receptor poor breast tumours, and was completely absent in non-target tissues such as colon epithelium. The rabbits used in this study were immature and 70-95% of the empty receptors were recovered in the cytosol after cell fractionation, despite their apparent nuclear localisation in the intact cell suggesting that cytosolic receptor represents an artefact of tissue disruption.

This data suggests that, in the intact cell, empty receptor is loosely attached to some component of the nucleus and that after steroid binding and activation, it merely acquires a higher affinity for the chromatin/DNA.

Data indicating a similar situation exists for the progesterone receptor have been presented by Gasc et al. (1984); Renoir and Mester (1984) and Perrot-Applomet et al. (1985).

1.4.3.7.3.2 Enucleation of cultured cells.

Using very different techniques, Welshons et al. (1984) have produced data which points to the same conclusions reached by King and Greene (1984). Using cytochalasin B induced enucleation of rat pituitary GH3 cells to obtain cytoplast and nucleoplast fractions, they have shown minimal empty receptor within the cytoplast and quantitative recovery with the nucleoplast fraction. The total recovery of protein, DNA and oestrogen receptor was always close to 100%, yet the receptor content of cytoplasts was only 5-10% of that in whole cells.

These results also strongly support the nuclear localisation of oestrogen in intact GH3 cells, but the generality of the findings from this cell line remains to be assessed in other systems using the same techniques.

These studies and the studies using monoclonal antibodies suggest that cytosolic receptor and the translocation hypothesis are probably artefacts due to cell fractionation methodology.

1.4.3.7.4 Cooperativity of Oestrogen Binding.

There is evidence to show that the "experimentally" soluble oestrogen receptor shows cooperative binding of oestrogen but only when the receptor concentration is 1nM or greater (Notides et al., 1981; Muller et al., 1984; Sakai and Gorski, 1984b). However when solubilised monomeric receptor (4S soluble in 0.4M KCl) is immobilised by binding to hydroxylapatite, subsequent oestrogen binding is not cooperative, regardless of the receptor concentration (Muller et al., 1984; Sakai and Gorski, 1984b). Like the soluble receptor, the immobilised monomeric receptor can be activated as measured by the kinetics of oestrogen dissociation. Other receptor characteristics appeared unchanged indicating retention of function criteria.

In the intact target cell, the concentration of oestrogen receptor is estimated to be 10nM (Clark and Peck, 1979), which is 10 times the concentration necessary for solubilised monomeric receptor to show cooperativity. Similar to immobilised receptor, the binding of oestrogen, as well as the response to oestrogen in target cells or tissues is non-cooperative (Williams and Gorski, 1972; Katzenellenbogen and Gorski, 1975; Kassis et al., 1984). These observations, although indirect, are consistent with the hypothesis that unoccupied receptor in addition to the activated receptor may be immobilised in vivo by binding to some nuclear or membrane component (Gorski et al., 1984). However activated receptor clearly has a much greater affinity for nuclear material as indicated by the higher salt concentrations required to extract activated receptor from target cell nuclei.

1.4.3.7.5 A New Model for Steroid Receptor Action.

The recent findings suggesting that steroid receptors may be located in the nucleus require that the accepted model of steroid receptor action must be slightly modified.

Steroid still diffuses into the cell down a concentration gradient. It may be helped into the cell and/or stored in association with the type II soluble receptor sites - assuming that they are genuine soluble proteins. Free steroid then comes into contact with empty high affinity cellular receptor which is loosely attached to the nucleus. The binding of steroid causes activation which is manifest in acquisition of a high affinity for specific nucleotide sequences, located in or close to AT-rich sequences. The kinetics of binding of the activated receptor to specific sites in the chromatin may well be aided by one or more non-histone chromosomal proteins.

1.4.3.8 Modulation of Oestrogen Receptor Levels.

The oestrogenic response within target cells is dependent on a minimum level of oestrogen receptor. Oestrogen responsive cells in a castrate or immature rat maintain sufficient levels of receptor to enable responses to be elicited. The basal level of receptor is probably controlled by genetic mechanisms that are programmed for the constitutive synthesis of "soluble" oestrogen receptor (Clark and Peck, 1979; Kassis and Gorski, 1983). However steroids can have a profound effect of oestrogen receptor levels, over and above the basal levels. It is possible that two genes coding for the oestrogen receptor exist, one of these is constitutively expressed, the other being sensitive to steroid hormones.

A general picture of the modulation of cellular oestrogen receptor levels has emerged from studies on the immature or castrate rat uterus. After injection of oestrogen there is a dose dependent depletion of "soluble" oestrogen receptors, with a concomitant

increase in nuclear receptors. This is followed by a gradual rise in unfilled "soluble" receptors (replenishment) which reach control levels by 11-16 hr, and then continue to increase or overshoot control levels (Clark and Peck, 1979). Receptor replenishment is necessary in rendering a tissue responsive to subsequent oestrogen administration and is therefore an important element in target organ function (Kassis and Gorski, 1983).

Replenishment after a single injection of oestradiol-17β is due to both recycling and resynthesis of receptor (Kassis and Gorski, 1983). However, replenishment of "soluble" unoccupied receptor lags behind nuclear receptor loss, and thus total receptor content is decreased 2-6 hr after oestradiol injection (Mester and Baulieu, 1975; Zava et al., 1976; Kassis and Gorski, 1981). This loss of receptor has been termed "processing" and, has been reported to occur both in vitro and in vivo. Processing refers to the loss of detectable binding of steroid and therefore may not be due to loss of receptor protein, but rather a change in binding properties of the receptor. Oestrogen receptor processing has been extensively studied in the MCF-7 breast cancer cell line in which processing appears to both correlate with, and be essential for induction of the progesterone receptor (Edwards et al., 1979; Kassis and Gorski, 1983).

In the rat uterus, oestrogen receptor processing also occurs, but many studies using short acting oestrogens such as oestradiol-16α have concluded that processing is not a prerequisite for some oestrogenic responses. Short acting oestrogens stimulate early oestrogenic responses (e.g. water inhibition, which is not a receptor mediated event and induced protein synthesis, which is a receptor mediated event) but not long term responses (DNA synthesis) (Clark et al., 1977). These responses depend on recycling though it is

possible that "replenishment" of available receptor involves the production of functional receptor from a pool of inactive receptor precursors. These observations have led some authors to conclude that receptor processing in uterine cells need not be directly involved in the oestrogen response pathway but may have some alternative function (Kassis and Gorski, 1983).

Inhibitor studies suggest that progesterone may induce the synthesis of an oestrogen receptor regulatory factor which causes a rapid loss of occupied nuclear receptor (Evans and Leavitt, 1980; Evans et al., 1980). It has been suggested that this rapid loss of receptor may occur through a dephosphorylation-inactivation mechanism. Auricchio et al. (1981) have also suggested that the receptor is processed or inactivated by dephosphorylation in the nucleus, then reactivated by phosphorylation. Whether these in vitro activities are equivalent to processing and reversal of processing in the cell remains to be proven. These ideas suggest that receptor processing is due to inactivation rather than degradation. However, it has been reported Cidlowski and Muldoon (1976) that cycloheximide can block receptor replenishment, suggesting that protein synthesis may be required. It is possible however that it is the synthesis of a factor required to "activate" inactive receptor which is blocked. Data produced from such studies are difficult to interpret because of the highly toxic nature of the metabolic inhibitors used.

Anti-oestrogens cause prolonged retention of nuclear receptor and delay replenishment. It has been proposed that anti-oestrogens delay replenishment by interfering with recycling of the oestrogen receptor and Migliaccio and Auricchio (1981) have shown in their in vitro system that the anti-oestrogen/oestrogen receptor complexes are not inactivated.

These observations have led Kassis and Gorski (1983) to propose a model for receptor replenishment, which is based on the assumption that three forms of the oestrogen receptor exist. These are:-

(1) A form with high affinity for steroid, which is functionally activated by binding steroid.

(2) A form with low affinity for steroid which is functionally inactive.

(3) An "activated" form of the receptor which has a high affinity for steroid and an increased affinity for DNA, chromatin proteins, nuclei etc.

1.4.3.9 Hormonal Control of Oestrogen and Progesterone Receptor Levels.

Treatment of rat uterus with oestradiol results in the induction of oestrogen receptors. This is considered to be a marker of oestrogen action (Clark and Peck, 1979). Oestrogen also induces increased levels of "soluble" progesterone receptor in uterine cells (Clark and Peck, 1979). Increased cellular progesterone receptor levels confer increased sensitivity of target cells to progesterone (Clark and Peck, 1979). Furthermore, an established effect of progesterone is to decrease cellular levels of both oestrogen and progesterone receptors (Clark and Peck, 1979). Thus oestradiol and progesterone have antagonistic effects on the cellular levels of both their receptors. The modulation of progesterone receptor levels during the oestrous cycle is a major factor in the functional state of differentiation of uterine cells (Clark and Peck, 1979). Induction of progesterone receptor represents one of the few specific markers of oestrogen action, which can be used to distinguish oestrogen stimulation from the effects of other hormones.

1.4.3.10 Anti-Oestrogens.

Anti-oestrogens are typically non-steroidal compounds which prevent oestrogens from expressing their full effects on oestrogen target tissues. As such they antagonise a variety of oestrogen dependent processes, including uterine growth and growth of oestrogen dependent mammary tumours (Katzenellenbogen et al., 1979). Examples of these compounds are Nafoxidine and Tamoxifen. In vivo tamoxifen is metabolised to the more active derivative 4-hydroxytamoxifen, which is thought to be the physiologically active form though several other metabolites are under investigation.

The properties of anti-oestrogens show a wide species variation. In the rat uterus these compounds are partial oestrogen agonists - partial antagonists. The uterus is a complex organ containing several groups of functionally different cells, and these different cell populations show different responses to anti-oestrogens (Clark et al., 1978b; Jordan and Dix, 1979; Dix and Jordan, 1980; Martin, 1981). Most studies in the rat indicate that whilst anti-oestrogens can increase the overall DNA content of the uterus, the luminal epithelial cells undergo prolonged hypertrophy but not hyperplasia. The situation in the oestrogen withdrawn chick oviduct appears to be less complex with anti-oestrogens demonstrating only oestrogen antagonism (Sutherland, 1981; Mester et al., 1981). In the mouse, tamoxifen is fully oestrogenic (Lee, 1974).

1.4.3.10.1 Mechanism of Action of Anti-Oestrogens.

Conceivably the antagonistic action of an anti-oestrogen could take place at any of the stages of oestrogen interaction with the receptor mechanisms of target cells or at hypothetical control points post receptor action.

Much of the evidence available suggests that anti-oestrogens act at three main points:- (1) they compete for available receptor; (2) they appear to alter the association of the receptor ligand complex and the nuclear binding sites; and (3) they interfere with the regeneration of "soluble" receptor.

Generally speaking anti-oestrogens have a lower affinity for the oestrogen receptor than oestradiol but, the active metabolite- 4-hydroxytamoxifen has an affinity for the receptor which is comparable to that of oestradiol (Borgna and Rochefort, 1981; Fabian et al., 1981).

Studies on the binding of anti-oestrogens/oestrogens to the oestrogen receptor have shown that anti-oestrogens bind directly to the "soluble" receptor with the binding of oestrogen and anti-oestrogen being mutally competitive, indicating that the agonist and antagonist bind to the same site or closely interacting sites. The rates of association of agonists and antagonists are similar while the rates of dissociation may be slower e.g. 4-hydroxytamoxifen, or faster e.g. tamoxifen than oestradiol, leading to dissociation constants for the oestrogen receptor which are greater or less than that for oestradiol (Capony and Rochefort, 1978; Katzenellenbogen et al., 1978; Borgna and Rochefort, 1980; Mester et al., 1981). It appears likely that there are few differences between oestrogens and anti-oestrogens in their kinetic interaction with soluble oestrogen receptor. However differences in the physicochemical characteristics of the molybdate stabilised oestrogen receptor when bound by oestrogen and anti-oestrogen have been reported (Ruh et al., 1983; Keene et al., 1984).
Previously two forms of the molybdate stabilised calf uterine oestrogen receptor had been reported which could be separated by DEAE-sephadex chromatography. When the molybdate stabilised oestrogen receptor

was labelled with the high affinity anti-oestrogen H1285, only one of these forms was present. This suggests the initial interaction of anti-oestrogen with the molybdate stabilised receptor may be different from that of oestradiol.

Several recent studies indicate tht the high affinity anti-oestrogenic ligands 4-hydroxytamoxifen and CI 628M can activate the oestrogen receptor as assessed by standard criteria (Katzenellenbogen et al., 1981; Mester et al., 1981; Rochefort and Borgna, 1981). However there is lack of agreement on the affect of "heat activation" on the dissociation kinetics of anti-oestrogen/oestrogen receptor complexes. Rochefort and Borgna (1981) found that activation by heating decreased the dissociation rate for oestradiol but not that of 4-hydroxytamoxifen, whilst Katzenellenbogen et al. (1981) found that similar activation produced a decreased rate of dissociation for both oestradiol and CI 628M under similar conditions. This could of course be a difference in the behaviour of the two anti-oestrogens.

After injection of anti-oestrogen into immature rat, the anti-oestrogen/oestrogen receptor complex is retained in the nucleus for a much longer period of time than oestradiol/oestrogen receptor complex. This prolonged retention results in reduced replenishment of "soluble" receptors hence the "soluble" receptor levels are depleted for a longer period of time (Rochefort and Capony, 1972; Clark et al., 1973; Katzenellenbogen et al., 1979). During this period of depleted "soluble" receptor, the uterus is incapable of responding to oestradiol, as monitored by synthesis of the oestrogen induced protein or by uterine weight gain (Katzenellenbogen and Ferguson, 1975). Such data led Clark et al. (1974) to postulate that oestrogen antagonism was primarily due to inhibition of "soluble" receptor replenishment. However depletion of "soluble" receptor levels and

apparent lack of replenishment are not confined to antagonists, but can be induced by high doses of synthetic oestrogens that give full uterotrophic responses (Katzenellenbogen et al., 1977; Jordan et al., 1978). This and subsequent work by Clark et al. (1978b)make it seem likely that receptor replenishment is impaired in anti-oestrogen treated rats and chicks, probably as a result of the inability of the nuclear anti-oestrogen receptor complex to fully stimulate synthesis of new oestrogen receptor.

Differences in the binding of anti-oestrogen and oestrogen receptor complexes to chromatin have been reported. Baudendistal and Ruh (1976) showed that anti-oestrogen receptor complexes were completely extractable with 0.3M KCl or 50mM spermine, whereas oestradiol receptor complexes demonstrate a salt resistant form. Differential extraction of oestradiol and anti-oestrogen receptor complexes was also reported using actinomycin D or ethidium bromide. The salt resistant receptor complex was assumed to be bound at a limited number of "acceptor" sites, binding at which was a requisite for true uterine growth (Clark and Peck, 1976). Anti-oestrogens were considered not to induce this type of binding, this did not give true uterine growth. However, the cis isomer of clomiphene which is capable of eliciting a full uterotrophic response (Jordan et al., 1981) is unable to form salt resistant nuclear receptor sites (Ruh and Baudendistal,1977) shedding doubt on the significance of salt resistant sites in mediating oestrogen action.

The binding of ^{3}H-oestradiol and ^{3}H-H1285 (a high affinity anti-oestrogen) receptor complexes to rabbit uterine chromatin has been studied by Singh et al. (1984). They report enhanced binding of "oestrogen" receptor complexes to chromatin binding sites exposed by various guanidine hydrochloride concentrations. The binding of

"anti-oestrogen" receptor complexes to these sites was markedly decreased as was the affinity of the interaction. Thus the differences in the physiological and physiochemical properties of oestrogens and anti-oestrogens may be related to their differential interaction with uterine chromatin subfractions.

In more sophisticated studies on the nature of nuclear receptor chromatin interactions in hen oviduct nuclei, Massol et al. (1978) were able to identify a specific 13-14S peak of oestradiol binding following moderate digestion of nuclei with micrococcal nuclease. This specific binding peak was absent in oestrogen withdrawn immature chick oviducts and could not be induced with tamoxifen, either alone or in combination with oestradiol (Lebeau et al., 1981), despite the fact that tamoxifen and oestradiol when administered at appropriate doses can induce similar levels of nuclear oestrogen receptor in the chick oviduct (Sutherland et al., 1977). These data have been confirmed using 4-hydroxytamoxifen and they provide strong evidence that in the chick oviduct, where these compounds are pure antagonists, the oestrogen receptor complex and the anti-oestrogen receptor complex do not bind to the same nuclear acceptor sites. At concentrations above 1μM, tamoxifen has been shown to be cytotoxic for both oestrogen receptor positive and negative mammary carcinoma cell lines, i.e. at these high concentrations tamoxifen's cytotoxic effects occur via receptor and non-receptor mediated events (Taylor et al., 1984).

Specific anti-oestrogen binding sites have been reported in all tissues of the rat (Sudo et al., 1983). These sites are not competable by oestrogens and the binding of anti-oestrogens is of high affinity (dissociation constant 1-3nM). Oestrogenic stimulation of the specific anti-oestrogen binding sites in rat uterus and liver has been reported by Winneker and Clark (1983). Specific anti-oestrogen

binding sites have also been reported in the human breast cancer MCF-7 cell line cytosol (Faye et al., 1983). In the case of the specific anti-oestrogen binding sites present in all rat tissues, Sudo et al. (1983) postulated that because of their wide distribution they may represent binding sites for other natural ligands which bear a structural resemblance to anti-oestrogenic molecules. Subsequently Brandes et al. (1985) have shown that the anti-oestrogen binding sites present on rat liver microsomes may be a histamine like receptor which mediates cell growth. Histamine formation has been implicated in certain types of rapid tissue growth (Szego, 1965).

1.5 Receptor Chromatin Interaction

1.5.1 Nuclear Binding.

Many oestrogen receptor mediated responses are known to involve changes in the expression of specific genes (Gorski and Gannon, 1976).

Generally speaking nuclear binding can be divided into two types. Extraction of nuclei with 0.3M-0.4M KCl does not remove all of the nuclear bound oestrogen (Puca and Bresciani, 1969; Mester and Baulieu, 1975; Clark and Peck, 1979). Love et al. (1983) have shown that in human endometrium, only 40% of the nuclear receptor can be released in this way, thus nuclear binding can be divided into a form which is salt extractable and a form which is resistant to extraction with salt. This would suggest that some oestrogen receptor complexes are bound more tightly than others. Clark and Peck (1976) have also shown that only a limited number of nuclear binding sites (1000-3000 per cell) are involved in the production of maximal uterine growth. These sites are retained for longer than 4-6 hr in the nucleus and are equal in number to the sites resistant to salt extraction. They propose that these are the true acceptor sites for oestrogen receptor complex.

Characteristics expected of an acceptor site are:- (1) high affinity binding; (2) a limited number of binding sites must exist; (3) steroid specificity.

The major stumbling block to universal acceptance of the acceptor site model is the disagreement between authors regarding the binding of receptor complexes to purified nuclei or chromatin. Many authors have shown that nuclear binding of steroid receptor complexes is a saturable phenomenon (Allerga et al., 1971; Fang and Liao, 1971; Mainwaring and Peterken, 1971; Higgens et al., 1973; Buller et al., 1975) whereas others claim that limited numbers of specific nuclear sites do not exist (Chamness et al., 1973, 1976; Andre and Rochefort, 1975), although Yamamoto and Alberts (1974, 1975) have suggested that specific binding sites could be masked by the very large amount of low affinity binding which occurs to DNA. However despite the problem with in vitro binding systems, it is possible to show that a limited number of nuclear binding sites must be involved in the events leading to maximal uterine growth in the rat, and to suggest that these nuclear sites may represent nuclear acceptor sites (Clark et al., 1978c).

Recently good evidence has been presented to show that, under defined conditions, saturable binding of steroid receptor complex to target cell nuclei can be demonstrated and that unsaturable binding is mainly a result of the use of poor methodology in the binding assay (Spelsberg et al., 1976a; Kon and Spelsberg, 1982). The inclusion of a salt concentration which is almost physiological in these binding assays is necessary to facilitate the study of tissue specific saturable binding.

Such saturation analyses can be used to calculate the total number of specific binding sites per nucleus that are available to steroid receptor complex. The number of binding sites present are much higher than is consistent with the activation of a small number of genes

(Clark and Peck, 1979; Leake, 1981). Kon et al. (1980) estimated 2,500 sites/cell for the hen oviduct. Mulvihill and Palmiter (1977) estimated 10,000 sites/cell for the chick oviduct and Anderson et al. (1975) 6,000 sites/cell for the rat uterus.

Analysis of the saturable binding of progesterone receptor complexes to oviduct nuclei led Spelsberg (1976) to conclude that there were, in fact, several classes of nuclear "acceptor" sites with differing affinities for the hormone receptor complex. The highest affinity class (approx. 100 sites/cell) were fully saturated before any physiological changes were observed. The Kd for these was 10^{-12}M and they would be fully saturated at physiological plasma steroid concentrations. The next two classes, in terms of binding affinities, constitute 900 and 4,000 sites/cell for oestrogen receptor and 1,000 and 10,000 sites/cell for progesterone receptor (Thrall et al., 1978). When these two classes of "acceptor" are filled maximum physiological responses ensue. These calculations involve the critical assumption that only one hormone receptor complex is bound to one "acceptor" site at any one instant. In fact the total number of acceptor sites which must be filled in order to get full physiological response to oestrogen-receptor complex has been calculated as only 1,000-2,000 sites/cell (Clark and Peck, 1979).

In the light of the finding of these multiple classes of nuclear binding sites it is not surprising that "acceptor" activity has been reported in more than one chromatin protein fraction (Spelsberg, 1982; Spelsberg et al., 1983).

1.5.1.1 The role of Nuclear Components in Nuclear Binding.

Over the years each component of the nucleus has been proposed as the specific nuclear acceptor site, principally because it has been isolated in association with steroid receptor complex.

The nuclear components proposed have included:- ribonucleoproteins (Liao et al., 1973b; Liang and Liao, 1974), the

nuclear envelope (Jackson and Chalkley, 1974; Smith and Van Holt, 1981), the nuclear matrix (Barrack and Coffey, 1980, 1982; Barrack, 1983; Colvard and Wilson, 1984), histone proteins (King and Gordon, 1967), non-histone chromatin proteins (King and Gordon, 1972; Puca et al., 1974, 1975; Mainwaring et al., 1976; Thrall et al., 1978; Ruh et al., 1981; Ruh and Spelsberg, 1983; Spelsberg et al., 1984) and DNA (Higgins et al., 1973; Yamamoto and Alberts, 1974, 1975; Payvar et al., 1981, 1983).

It is, however, the DNA of the activated genes and the possible role of chromatin proteins that has attracted most attention and the evidence for their involvement will be considered in turn.

1.5.2 General Nuclear Control.

A large proportion of the DNA of an organism is beyond doubt potentially transcribable, yet it has been recognised for many years that all cells and organisms exercise strict control over which genes are expressed and at which time. The phenomenon of cell differentiation results in a specialised cell expressing only a very small subset of the tens of thousands of genes at its disposal. The mechanisms which allow the cell to select which genes to express, and when, are of fundamental importance in molecular biology.

1.5.2.1 Role of Nucleotide Sequences.

In prokaryotes, transcription is controlled by regions of DNA on the upstream, or 5' side of structural genes. These regulatory or promoter regions are composed of a set of DNA sequence elements whose function, in most cases, has been clearly demonstrated. As a logical extension of this knowledge, the attempt to identify transcriptional control elements in eukaryotes has also been focussed with considerable success on the DNA sequences upstream of structural genes. The upstream control elements of eukaryotic genes are classified into three types

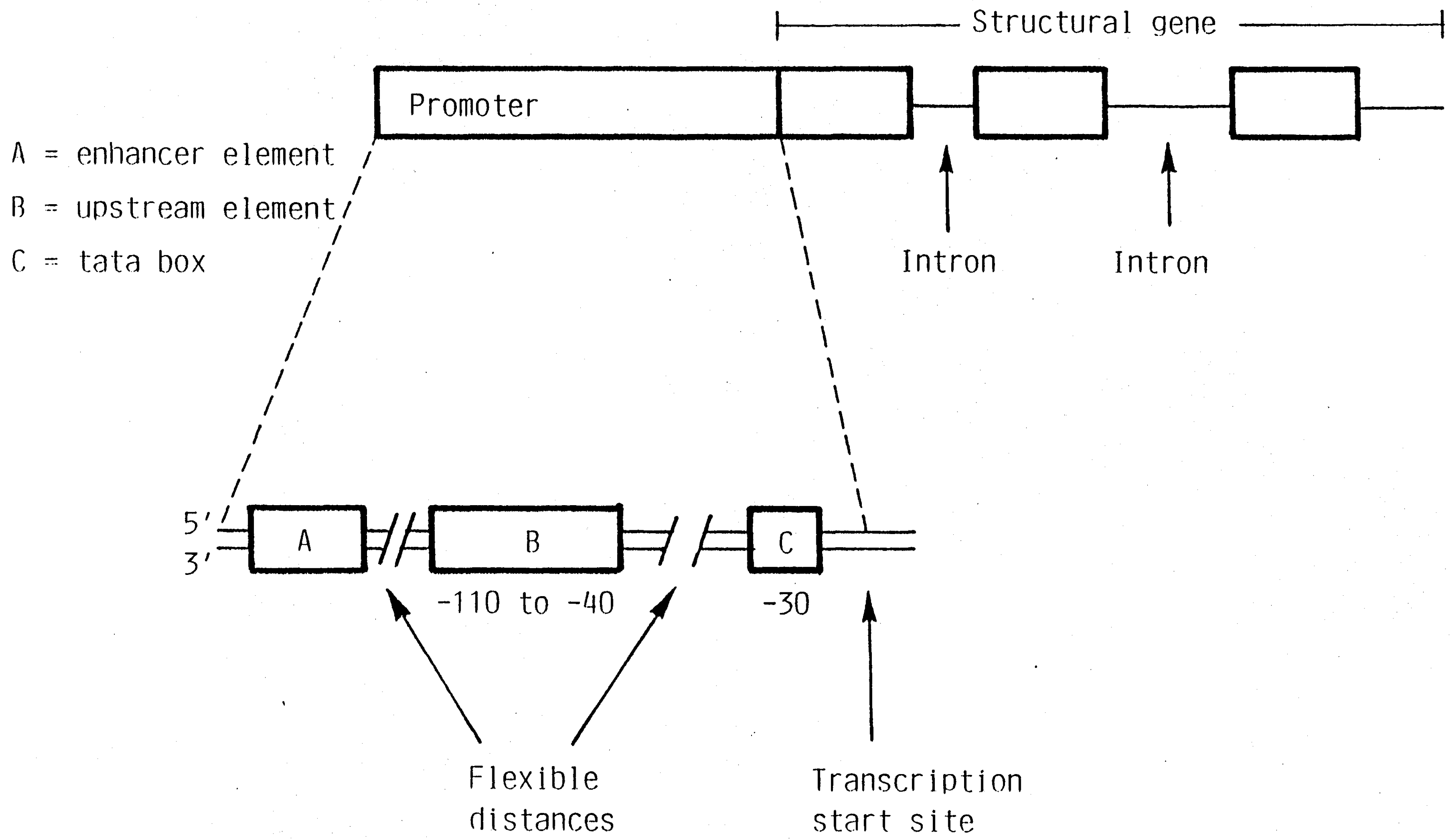
based on their function, their sequence characteristics and their position relative to the start of transcription (Chambon *et al*., 1984). (See Figure 4).

One type termed a TATA box element, is involved in fixing the start site of transcription to a point 30bp downstream from its own position. A second type, the upstream element, is a broad class of sequences at a variable distance from the transcription start site that seem to be important in determining the level of transcription. These elements have previously been referred to as G-C rich elements. Finally enhancer elements which were first reported in mammalian viruses, seem to be able to stimulate gene transcription from considerable distances and have been postulated to be tissue specific modulators of transcription for some mammalian genes (Picard and Schaffner, 1984; Walker *et al*., 1984).

The role of the TATA box is reasonably clear but the occurrence of the other two types of sequences is of great interest.

Examples of such sequences are short stretches of DNA amidst the 5' hypersensitive sites of the genes for the heat shock proteins of molecular weight 83,000 and 70,000 in *Drosophila*, which are found to be resistant to exonuclease digestion. The resistant regions cover both the TATA box sequence and an upstream control element. These DNA sequences are considered to represent binding sites for regulatory proteins involved in the activation of the adjacent gene (Pelham, 1982; Parker and Topol, 1984; Wu, 1984; Bienz, 1985). The pattern of resistance to nuclease digestion before and after induction of gene expression suggests that the heat shock genes are activated by the sequential binding of at least two non-histone proteins. Binding of one in the TATA box region may establish the transcriptional potential of heat shock genes, with the potential being realised during heat

Figure 4 - Features of Eukaryotic Protein Coding Genes. (Adapted from Reudelhuber, 1984)



Structural gene
Promoter
A = enhancer element
B = upstream element
C = tata box
Intron
Intron
5'
3'
A
B
C
-110 to -40
-30
Flexible distances
Transcription start site

shock activation by the additional binding of a second protein. The fact that a second binding site is only found in active genes, argues that the second protein is a positive activator of transcription. Purification of this second protein is currently being attempted (Wu, 1985). Binding of these protein factors is envisaged to create deoxyribonuclease I (DNase I) hypersensitive sites which are thought to be characteristic of genes available for transcription. Binding of steroid receptors to DNA sequences is discussed more fully in Section 1.5.3.

Further examples of such upstream sequences are those present in the 5' flanking sequence of a human gene for metallothionein (Karin et al., 1984). Deletion experiments have defined two stretches of DNA lying close to the promoter of the gene, which separately mediate the induction of the gene by heavy metals, particularly cadmium and by glucocorticoids. The element responsible for induction by cadmium is duplicated, yet a single copy is fully functional. The element responsible for induction by glucocorticoids is coincident with the DNA-binding site for the glucocorticoid hormone receptor. Both of these elements can activate heterologous promoters located at least 600bp away suggesting they operate as "enhancer like" elements. Further evidence that upstream binding sites for steroid hormone receptors activating enhancer sequences is presented in Section 1.5.3.

There is now growing evidence that control elements that are downstream i.e. within the coding region, of a structural gene are also important in regulating the expression of some genes. Such an element has been reported in the adult β-globin gene (Charney et al., 1984; Wright et al., 1984), this gene also has a full complement of functionally important upstream control elements. Further examples include the Xenopus 5S ribosomal RNA gene (Schlissel and Brown, 1984) and some mouse immunoglobulin genes (Picard and Schaffner, 1984).

Especially relevant to steroid hormone action is the report by Moore et al. (1985), that the first intron of the human growth hormone gene contains a binding site for the glucocorticoid receptor. This binding was selectively inhibited by methylation of two short, symmetrically arranged clusters of guanine residues within the binding site.

1.5.2.2 DNA Base Methylation.

A feature of eukaryotic DNA which has long held attractions as a possible signal for the control of gene expression is the variable extent to which its bases, in particular cytosine, are methylated. Two to seven percent of cytosines in mammalian DNA occur as 5-methylcytosine, and 90% of these occur as the dinucleotide CpG. The extent to which a gene is methylated can be partially determined by digestion with paired isoschizomeric restriction endonucleases which recognise the same nucleotide sequence but are differently affected by its methylation.

Using this approach, it has been shown that globin genes are undermethylated in tissues expressing them (Waalwijk and Flavell, 1978; McGhee and Ginder, 1979; Shen and Mariatis, 1980; Van der Ploeg and Flavell, 1980). This negative correlation between gene expression and under methylation is also seen in the case of the chicken egg white protein genes (Mandel and Chambon, 1979) the mouse immunoglobulin genes (Rogers and Wall, 1981) the mouse α-fetoprotein genes (Andrews et al., 1982) and the human growth hormone gene (Hjelle et al., 1982). Stumpf et al. (1983) have shown that the 3' end of ovalbumin gene has a region greatly enriched in methylation and that this region is specifically undermethylated in the oviduct where the gene is expressed. Thus it appears that site specific undermethylation is associated with gene expression, but this is not the only factor involved as a gene can be undermethylated and not expressed, as is the case with the Morris Hepatoma α2u-globin gene (Nakhasi et al., 1982). This is confirmed

by Wilks et al. (1982) who have shown that oestrogen causes the specific undermethylation of a sequence near the 5' end of the chicken vitellogenin gene. However it does this in two target tissues of the hormone, the liver where the gene is expressed and in the oviduct where it is not. It also appears that the demethylation event occurs subsequent to the onset of transcription, so it seems that it is a consequence rather than a cause of transcriptional activity. In contrast this demethylation event does not occur in the oestrogen inducible vitellogenin genes in Xenopus, which are expressed when fully methylated (Gerber-Huber et al., 1983). As a result the role of methylation in oestrogen induced, and indeed gene expression in general is still unresolved. One possible answer is that not all the methyl groups are involved in gene expression. Perhaps the loss of a single methyl group is sufficient to "switch on" a gene. Such a loss would be difficult to detect.

1.5.2.3 Chromatin Conformation and the Availability of Genes for Transcription.

The best evidence that the conformation, packaging or composition of chromatin exerts an influence on gene transcription comes from the studies using DNase I and micrococcal endonuclease. Genes available for transcription are preferentially digested by these enzymes and by implication are part of a chromatin arrangement more available to enzyme interaction. Thus an increase in the DNase I sensitivity of the four Xenopus vitellogenin genes can be demonstrated in oestrogen treated liver cells. No such increase occurs in non-target tissues (Gerber-Huber et al., 1981). Similar changes have been reported for the chicken vitellogenin genes (Dimitriadis and Tata, 1982; Williams and Tata, 1983). The egg white protein genes of the chick oviduct after exposure to oestrogen show a similar increase (Garel and Axel, 1977; Lawson et al., 1980; Fritton et al., 1983). These changes do not occur in non-target

tissues. The ovalbumin gene is coordinately expressed with two pseudogenes X and Y and the three are linked in a 100Kb domain of DNA. The whole of this domain is preferentially sensitive to DNase I (Lawson et al., 1982), with a sharp change in sensitivity at either end. Repetitive DNA elements of the CR1 family are located in opposite orientations at or very near the transition points and Stumpf et al. (1983), have suggested that repetitive regions may play a role in defining the ends of DNase I sensitive regions.

As with methylation, the increase in the DNase I sensitivity of active genes is related to their availability for transcription and does not necessarily imply that they are being transcribed.

In addition to the general DNase I sensitivity of transcribable genes, there are often narrow regions of 50-400bp which are especially sensitive to digestion. These so called hypersensitive sites are often located at the 5' end of genes and their occurrence may be related to the level of gene transcription (Fritton et al., 1983).

The features of the chromatin of active genes which make them more DNase sensitive are not known in detail but are dependent on the specific occurrence in their constituent nucleosomes of two of the high mobility group (HMG) non-histone proteins, HMG14 and HMG17 (Weisbrod and Weintraub, 1981). Differential DNase sensitivity is lost when non-histone proteins are removed but is restored by the addition of HMG14 and HMG17 (Weisbrod and Weintraub, 1979). Other proteins which have been associated with active chromatin include ubiquitin-conjugated histone H2A (Levinger and Varshavsky, 1982) and acetylated histones (Nelson et al., 1980). Nucleosomes which are deficient in histones H2A and H2B have been shown to be heavily enriched in active genes and also able to selectively bind RNA polymerase II (Baer and Rhodes, 1983). Numerous reports have shown that oestrogen action is accompanied by qualitative

and quantitative changes in chromatin proteins (Teng and Hamilton, 1969; Barker, 1971; Cohen and Hamilton, 1975), including HMG protein accumulation (Teng and Teng, 1981) and very rapid hormone induced changes in histone acetylation (Pasqualini et al., 1983). The role that these and other chromatin components might play in oestrogen control of gene expression is unclear.

1.5.3 Binding to DNA.

As a result of early investigations, it has been reported that a variety of steroid receptor complexes bind to DNA (Baxter et al., 1972; King and Gordon, 1972; Musliner and Chader, 1972; Spelsberg et al., 1971, 1976; Toft, 1972; Yamamoto and Alberts, 1974, 1975; Rousseau et al., 1975; Simons et al., 1976; Bugany and Beato, 1977; Thanki et al., 1978; Kallos and Hollander, 1978). There have been reports of specific interaction in terms of preference of steroid receptors for substituted over unsubstituted DNA (Andre et al., 1976; Kallos and Hollander, 1978), for eukaryote over prokaryote DNA (Clemens and Kleinsmith, 1972) and finally for native DNA over RNA (Toft, 1972, Yamamoto and Alberts, 1974). However this binding is of relatively low affinity (Buller and O'Malley, 1976) and shows no species specificity although there is preferential binding to AT-rich DNA (Kallos and Hollander, 1978; Kallos et al., 1978). Although these studies may explain in part the large number of apparent nuclear binding sites, they do nothing to reveal how, in some target tissues, the hormone-receptor complex is capable of selectively activating a small number of specific genes. Nevertheless, the interaction with DNA would appear to be important. Thus in the human breast cancer cell line MFC-7, in which oestrogens induce the synthesis of a glycoprotein, the incorporation of bromodeoxyuridine into DNA, inhibits the induction. Since the synthesis of other proteins is unaffected, it seems likely that the inhibition is due to interference with chromatin recognition sites, rather than with

RNA transcription (Garcia et al., 1981).

The first good evidence that steroid receptor complexes might specifically associate with the genes that they activate comes from studies on the insect hormone ecdysterone. Both Gronemeyer and Pongs (1980) and Dworniczak (1983) used photoactivation to cross-link the hormone to the giant polytene chromosomes of Drosophila salivary glands. This allowed the bound complexes to withstand the preparation of chromosome squashes. They then used immunofluorescent labelling to show that the hormone was specifically localised at chromosome puffs which were known to correspond to ecdysterone induced genes. Further work by Schallman and Pongs (1982) showed that by crosslinking covalently bound ecdysterone to its receptor protein that both the hormone and receptor protein were bound to the activated genes.

These studies with ecdysterone have not identified whether the hormone receptor binding site is on the DNA or some other component of the polytene chromosome. They do indicate however, that at least in this system, there are highly specific binding sites. It is tempting to speculate that, in addition to a general relatively low affinity for all DNA, steroid hormone receptor complexes might possess a high affinity for specific nucleotide sequences associated with the genes they activate. In recent years the isolation of hormone responsive genes, together with the increased availability of purified receptor proteins and monoclonal antibodies to receptor protein, has allowed a search for such binding sites. The following approaches have been used:-

1. DNA Binding Studies - The affinity of a DNA fragment for the hormone-receptor complex has been monitored by its ability to displace the complex from DNA-cellulose (Mullvihill et al., 1982), by the ability of a receptor to retain a DNA fragment on a nitrocellulose filter (Hughes et al., 1981) and by the ability of the receptor complex to protect a region of the gene from nuclease digestion (Scheidereit

et al., 1983).

2. Gene Transfer Experiments - The relevance of specific nucleotide sequences to hormonal control of gene expression has been tested by introducing the gene into cells which contain functional receptor for the hormone concerned but do not normally express the gene (Buetti, 1981; Kurtz, 1981). In this way it has been shown that sequences 5' to a number of genes could be associated with the control of their expression. Furthermore, these sequences have subsequently been fused to genes not normally influenced by hormones, with the result that the gene becomes responsive (Chandler et al., 1983; Hynes et al., 1983). Fragmentation and selective mutation of the active sequences can be employed to define more precisely the nucleotides involved.

3. Search for consensus sequences - Sequences derived by the approaches in (1) and (2) can be searched for in similar locations in other genes activated by the same hormone. This may lead to the identification of a consensus sequences for the binding of that hormone-receptor complex (Mulvihill et al., 1982; Scheidereit et al., 1983).

Unfortunately the use of the above systems to analyse the binding of oestrogen-receptor complexes to the genes they activate has lagged behind that of other hormones. The greatest progress has been made with genes which are responsive to glucocorticoids and/or progesterone.

Some of the most striking advances have been made with the mouse mammary tumour virus (MMTV), a retrovirus which causes mammary carcinoma in mice and which will also replicate in rodent cells in tissue culture. Glucocorticoids dramatically enhance the rate of virus replication, and the tissue culture system serves as an excellent model

for the study of steroid hormone action.

Several groups have demonstrated that rat liver glucocorticoid receptor binds specifically to at least five widely separated regions on pure pro-viral DNA (Payvar et al., 1982, 1983; Gustafsson et al., 1984). One of these specific binding domains, which itself contains at least two distinct receptor binding sites, resides within a fragment of viral DNA that maps -110 to -449bp upstream of the initiation site for MMTV RNA trasncription. Four other binding domains lie downstream of the promoter and within the MMTV primary transcription unit. Chimaeric genes containing restriction fragments from these binding domains fused to various marker genes have been used to identify regions associated with the response to the hormone. Recently Hynes et al. (1983) have shown that the enhanced expression by glucocorticoids was associated with that portion lying 0 to -202bp upstream of the transcription initiation site. Furthermore Scheidereit et al. (1983) have shown using monoclonal antibodies, that partially purified receptor selectively protects two areas within the sequence from digestion by deoxyribonuclease I. These areas are -72 to -124 and -163 to -192bp upstream of the initiation site. They share some sequence homology, both with each other, and with the 5' flanking sequences of other glucocorticoid-regulated genes, but a clear consensus sequence does not emerge from this study. Recently Cato et al. (1984) have shown that a region 2.6Kb upstream of the rabbit uteroglobin gene recognises the glucocorticoid receptor. The region contains three binding sites, and all three show homology with the glucocorticoid receptor binding sites reported for other genes. These elements may be responsible for the glucocorticoid regulation of uteroglobin gene expression by acting over a relatively long stretch of nucleotide sequence.

The binding of progesterone receptor complexes to the fowl egg white protein genes has also been extensively studied. As with the binding of glucocorticoids to MMTV DNA, there appear to be multiple progesterone binding sites (Mulvihill et al., 1982; Compton et al., 1983, 1984). Mulvihill et al. (1982) identified a binding site located 250-300bp upstream of the initiation site of the ovalbumin gene. This region showed considerable sequence homology with the 5' flanking regions of genes for the other egg white proteins conalbumin and ovomucoid as well as with two ovalbumin pseudogenes. By comparing these homologies they proposed a 19bp progesterone binding consensus sequence. Compton et al. (1984), have demonstrated preferential binding of the progesterone receptor complex to a region 150 to 190bp upstream of the ovalbumin gene. Dean et al. (1983) have also shown that a segment of DNA containing this sequence (95 to 222bp upstream of the ovalbumin gene) when fused to a marker gene and used in gene transfer experiments, caused the marker gene to become progesterone responsive.

Renkowitz et al. (1984) have shown that deletion of sequences from the chicken lysozyme promoter renders the gene insensitive to both glucocorticoids and progesterone. They were able to detect a strong glucocorticoid receptor complex binding site between -74 and -39bp upstream of the promoter and a weaker binding site between -208 and -161bp upstream of the gene. Subsequent work by von der Ahe et al. (1985) has shown that deletion of 44bp from the chicken lysozyme promoter (-208 to -164 upstream of the initiation site) results in co-ordinate loss of both glucocorticoid and progesterone dependent gene expression. Finally, by studying the promoter regions of both the chicken lysozyme and MMTV genes, they showed that purified glucocorticoid receptor from rat liver and progesterone receptor from rabbit uterus yield similar or overlapping exonuclease III foot-prints, implying that

at least some of the binding sites for these two steroid receptor complexes may be the same.

Similar studies involving oestrogen receptor complexes have recently shown that both a DNA sequence upstream of the chicken vitellogenin II gene (Jost et al., 1984) and a region at least 1Kb upstream from the rat prolactin gene (Maurer, 1985) selectively bind oestradiol receptor complex.

In summary, from these studies it seems that the signal for the binding of a hormone receptor complex to the gene it activates may be encoded in the DNA, however the sequence homology so far detected between different binding sites is not good and more may be involved than the base sequence. In the well characterised systems studied to date, there appears to be multiple binding sites which can be either upstream of, and within the gene. There is some evidence that the binding sites may function in a manner analogous to viral enhancers of transcription. Enhancers can act over several kilobases regardless of their orientation. Hynes et al. (1983) have shown that the MMTV glucocorticoid responsive region can function 500bp from the viral promoter and Chandler et al. (1983) have shown that it will work in both orientations. Further support for this idea comes from Wilks et al. (1982), who show that oestrogen receptor complexes bind to a region of the chicken vitellogenin II gene which contains the oestrogen-dependent methylation site and contains a binding site that shows considerable homology with an enhancer of the virus SV-40 (Jost et al., 1984).

However the affinity of the hormone receptor complexes for the specific DNA sequences isolated so far is only 10-40 times greater than that for a heterologous DNA such as total calf thymus DNA. This seems inadequate to provide a mechanism for the specific activation of

a small number of genes and is certainly too little to allow rapid attraction of incoming steroid receptor complexes. This implies that some other recognition signal must be involved. It is very likely that DNA is an essential component of a three dimensional acceptor site but it does not offer the exclusive determinant of steroid receptor complex binding. Previous work has shown that treatment of nuclei with DNase causes release of bound receptor (King and Gordon, 1972) implying that DNA is involved in the binding. However it has also been shown that proteases can destroy binding activity (Puca et al., 1974), which implies that nuclear proteins also have a role to play in the binding of steroid receptor complexes.

1.5.4 The Role of Chromatin Proteins.

Chromosomal proteins are likely to be involved in the availability of a gene to hormone activation. In any differentiated tissue there are genes that are permanently "switched off", genes that are always available for transcription and genes that can be activated. The chromosomal proteins are assumed to be strongly involved in this process and in the case of hormone activated genes, may well be associated with the differential effects of the hormone on different target tissues. It also seems likely that chromosomal proteins may be involved directly in the nuclear binding process.

1.5.4.1 Non Histone Chromatin Proteins.

By convention non-histone chromatin proteins are considered to be acidic in nature, but work has been published which reports binding activity to be present in both basic non-histone chromatin proteins (Puca et al., 1974, 1975; Mainwaring et al., 1976) and acidic non-histone chromatin proteins (e.g. Spelsberg, 1982; Spelsberg et al., 1983).

1.5.4.1.1 Acidic Proteins.

To date the best characterised system is the interaction of progesterone receptor complexes with the avian oviduct (Spelsberg, 1982; Spelsberg et al., 1983). The "acceptor" function is tightly bound to DNA and can be destroyed by proteases (Spelsberg et al., 1984). The protein fraction is confined to chromatin and is more active in target tissues than non-target tissues (O'Malley et al., 1972; Chytil, 1975; Pikler et al., 1976; Spelsberg and Toft, 1976). Deproteinisation of chromatin to remove histones and some non-histone proteins reveals that the "acceptor" activity is present in all hen tissues but is masked in non-target tissues, primarily by non-histone proteins. Indeed, even in target tissue chromatin approximately 70% of the high affinity "acceptor" sites are also masked (Spelsberg, 1982; Spelsberg et al., 1983). Using chromatin dissociation and reconstitution techniques it has been possible to transfer "acceptor" activity from target to non-target tissues (Spelsberg et al., 1971, 1972). Recently Spelsberg et al. (1984) have performed reconstitution studies and presented data to support the involvement of specific chromatin proteins in "acceptor" site function. Furthermore they show that the "acceptor" protein fraction will only interact with a limited number of specific DNA sequences in the avian genome. This is supported by the observation that it is not possible to reconstitute native like acceptor activity if the oviduct "acceptor" protein fraction is re-annealed to bacterial DNA. The transfer of "acceptor" activity from target to non-target tissue has also been reported by Klyzsejko-Stefanowicz et al. (1976) when studying the interaction of androgen receptor with various male rat tissues including prostate. More recently Ross and Ruh (1984) report the reconstitution of native like acceptor activity when studying the interaction of oestradiol-receptor complexes with calf uterine chromatin.

However the techniques of chromatin fractionation and reconstitution have been heavily criticised by a number of groups (Zasloff and Felsenfeld, 1976; Fuhner and Fasman, 1979; Stein, 1979).

Characterisation of the protein fraction responsible for "acceptor" activity in the avian oviduct has revealed that the bulk of "acceptor" activity falls into a molecular weight range of 14,000-18,000. Using isoelectric focussing two primary peaks of activity could be resolved, one which focussed at a pH range of 5.0-5.5 and the other over a broader pH range of 6.0-7.0 (Spelsberg, 1982; Spelsberg et al., 1983).

Ruh et al. (1981) have shown that oestradiol-receptor complexes binding to calf uterine chromatin can be resolved into three components by selective deproteinisation of the chromatin using guanidine hydrochloride. Using different techniques, similar results have been reported for progesterone receptor complexes binding to chick oviduct chromatin (Thrall et al., 1978). The majority of the receptor complex binding in both cases was resistant to 0.4M KCl extraction and this is in agreement with a similar report by Perry and Lopez (1978) regarding the binding of oestrogen-receptor complex to sheep hypothalamic chromatin. These findings that acidic proteins seem to constitute a necessary component of "acceptor" activity is consistent with the findings of Spelsberg et al. (1975, 1976b, 1979) in the chick oviduct system, Klyzsejko-Stefanowicz et al. (1976) in the rat prostate and Tsai et al. (1980) in rat sertoli cells.

Using similar extraction procedures to Ruh et al. (1981), Ruh and Spelsberg (1983) studied the binding of oestradiol-receptor complexes to hen oviduct chromatin. The peak of acceptor activity was uncovered by 5M guanidine hydrochloride. This was similar to the concentration required to uncover "acceptor" activity for progesterone receptor complex binding and this led to the conclusion that the "acceptor"

protein fractions for each receptor complex may have similar properties. Earlier work (Kon and Spelsberg, 1982) had shown that oestrogen-receptor complexes and progesterone-receptor complexes did not compete for the same nuclear binding sites. In this study oestrogen-receptor complex binding to purified hen oviduct nuclei was assessed. A single class of binding site was detected (Kd = 1.8×10^{-10}M, with 3000-5000 binding sites/nucleus). This Kd value compares well with the value of 1.5×10^{-10}M, which was the value measured by Ruh and Spelsberg(1983) when studying the binding of oestrogen-receptor complexes to guanidine hydrochloride extracted hen oviduct chromatin. This study also indicated that a large percentage of the "acceptor" sites for oestrogen-receptor complexes were masked in the nuclei of the avian oviduct as had been shown previously for the progesterone receptor (Spelsberg, 1982; Spelsberg et al., 1983).

1.5.4.1.2 Basic Proteins.

Reports in the literature have been far less extensive concerning the role of basic non-histone chromatin proteins in acceptor activity. Puca et al. (1974, 1975) have reported a tightly bound chromatin protein fraction which is extractable in 2M NaCl from calf uterine nuclei which exhibits "acceptor" activity for the oestrogen receptor complex. The binding activity is destroyed by proteases confirming its protein nature. The basic nature of the protein was established using hydroxylapatite chromatography. The assay of binding activity was carried out by immobilising the various fractions obtained after exposing the calf uterine nuclei to 2M NaCl on sepharose 4B and eluting $^{3}H\text{-}E_2$ labelled oestrogen receptor complex through the nuclear extract sepharose resins. Receptor complex binding was saturable and displayed a Kd of 3×10^{-9}M. Formation of receptor complex/acceptor protein complexes in solution, followed by sephadex G-100 chromatoraphy allowed a molecular weight of

85,000 to be estimated for the acceptor protein at neutral pH and almost physiological ionic strength. DNA immobilised on polymers could efficiently bind the acceptor proteins, which in turn could then specifically bind the receptor complex. This is not unexpected as the proteins were presumably bound to DNA in the nucleus before disruption of the chromatin.

The advantages of forming an insoluble matrix suitable for affinity chromatography as has been done by Puca et al.(1974) are threefold:- (1) Bulk preparation of stable acceptor matrix, (2) Extreme analytical precision, (3) Experimental flexibility.

Mainwaring et al.(1976) have reported two distinct sets of binding sites for androgen receptor complexes present in the 2M NaCl extract of rat prostate nuclei. Prior to analysis, the nuclear extracts were immobilised on sepharose 2B essentially as outlined by Puca et al. (1974). These binding sites comprised:- (1) a small number of specific high affinity sites, (2) a large number of low affinity non-specific sites. The high affinity sites were shown to be a fraction of the basic but non-histone proteins using both Bio-Rex-70 ion exchange chromatography and isoelectric focussing. The acceptor protein was estimated to have a molecular weight of 70,000 as shown by gel exclusion chromatography. The apparent Kd for the interaction of the high affinity sites with androgen receptor complex was 2.5×10^{-10}M. The low affinity, non-specific sites were recovered with the acidic non-histone proteins.

As in the case of the work of Puca et al. (1974, 1975) the interaction between receptor complex and acceptor site were studied under conditions far removed from their authentic environment in the intact cell, especially in respect of the complete absence of DNA.

Care must be exercised in the classification of these acceptor proteins. The classification appears to be clearly that they are either

basic or acidic in charge. This nomenclature refers to the proteins after they have been removed from the chromatin, and thus may not be a realistic picture of their overall charge when they are associated with chromatin. Thus proteins which carry an overall acidic or basic charge after extraction may not carry this charge in vivo. How much the charge on the protein changes after extraction will be governed by the nature of its attachment to chromatin in vivo i.e. ionic or hydrophobic, although the former is the most likely. Changes in the overall charge of a protein may also be introduced by any conformational changes which occur during its extraction.

1.5.4.1.3 Histone Proteins.

Several reports have appeared in the literature which suggest the interaction between steroid hormone receptor complexes and histones is completely non-specific (King et al., 1969; Steggles and King, 1970; Puca et al., 1974, 1975).

In contrast, Kallos et al. (1981) report that oestrogen receptor complexes from rabbit uterus can interact selectively with purified histones with the strongest binding being to histones H2A and H2B with histone H1 only displaying weak binding activity.

It is difficult to rule out a modulating role for histones in regulating steroid hormone mediated gene expression in view of the data of Kallos et al.(1981) and that of Ruh et al . (1981) who showed that a major fraction of acceptor activity was found in only partially dehistonised calf uterine chromatin but it is probable that histones are involved in general control mechanisms, with more selective and specific controls involving non-histone proteins.

1.5.4.2 The Nuclear Matrix.

The nuclear matrix is the structure which remains after nuclei have been sequentially extracted with high salt buffers and detergent and then digested with DNase and RNase. It represents 2% of the total nuclear

phospholipid and RNA, 0.1% of the total nuclear DNA and 10% of the total nuclear protein (Barrack and Coffey, 1982). It consists of a net-work of thin proteinaceous fibres together with structural elements of the pore complexes, lamina, the internal net-work and nucleolus. The matrix has been shown to have a key role in DNA replication (Bucholtz, 1981; Hunt and Vogelstein, 1981) and in hnRNA production and processing (Jackson et al., 1981). DNA is arranged in supercoiled loops anchored to the matrix, and studies have shown that genes are randomly arranged within these loops. Indeed it has been shown that transcriptionally active genes are closely associated with the matrix. Thus, Robinson et al. (1982, 1983) have shown that ovalbumin and conalbumin genes are preferentially associated with the nuclear matrix in oviduct cells. Furthermore ovalbumin genes selectively dissociate from the oviduct nuclear matrix during oestrogen withdrawal and reassociate with it after re-stimulation (Robinson et al., 1983).

The concept that steroid hormone activated genes are attached to the nuclear matrix forces a re-assessment of the reports that steroid-receptor complexes bind to the matrix. Many groups have reported that a substantial component of nuclear hormone-receptor complexes cannot be extracted with 0.3 - 0.6M KCl, and some have presented data that these residual complexes are bound to the nuclear matrix (Barrack et al., 1977; Barrack and Coffey, 1980; Barrack and Coffey, 1982; Barrack, 1983). Androgen receptor has also been reported associated with the nuclear matrix of normal and hyperplastic human prostate (Donnelly et al., 1984). It would seem a possibility worthy of further investigation that the steroid, its receptor protein, the gene to be activated and the nuclear matrix are all part of a complex in which the gene takes up a new conformation with increased accessibility for transcription.

1.6 The Distribution of Receptor Acceptor Complexes within Chromatin.

At present little is known about the intranuclear interactions in which steroid receptors are involved and even less is known about the intranuclear location of these interactions. Attempts to answer these questions have utilised chromatin fractionation techniques which separate transcriptionally active and inactive regions using either mechanical (Levy and Baxter, 1976; de Boer et al., 1978; Franeschi and Kim, 1979) or enzymatic (Hemminiki, 1977; Senior and Frankel, 1978; Rennie, 1979; Scott and Frankel, 1980; Thomas and Bell, 1983; Pratt et al., 1984) disruption of chromatin. However the results have not been unambiguous. Oestrogen receptors from chick-oviduct (Franeschi and Kim, 1979) and rat uterus (de Boer et al., 1978) have been reported to associated predominantly with fast sedimenting chromatin which is considered to be inactive. Similar observations have been reported for androgen receptors in prostate chromatin (Rennie, 1979) and glucocorticoid receptors in putuitary chromatin (Levy and Baxter, 1976). Conversely other investigators have reported that transcriptionally active regions of chromatin are enriched in oestrogen receptors (Hemminiki, 1977; Hemminiki and Vaukhonen, 1977; Scott and Frankel, 1980). Pratt et al. (1984) have also shown that oestrogen receptor complexes bind to calf uterine chromatin at pre-existing nuclease hypersensitive sites which are known to occur in regulatory regions (often at the 5' end of actively transcribed genes). Bruchovsky et al. (1981) have reported that 95% of nuclear androgen receptors are associated with linker DNA.

In the case of rat prostate chromatin, Davies and Thomas (1984) show that, after controlled digestion of prostate nuclei by micrococcal nuclease, the majority of androgen receptor complexes could be recovered in an oligonucleosome fraction which was transcriptionally active and only represented 10% of the genome. A similar report that

glucocorticoid receptors are preferentially associated with a fraction of rat thymocyte chromatin which is transcriptionaly active, has been made by Thomas and Bell (1983).

Having studied the intra-nuclear distribution of rat uterine oestrogen-receptor complexes Pavlick and Katzenellenbogen (1982) propose a model where the majority of receptors are located in a fraction of chromatin which is transcriptionally inactive,with only a small minority of receptors being associated with transcriptionally active chromatin. Data presented by de Boer et al. (1984) studying oestrogen receptor binding to chicken target cell nuclei support the above model.

Since a close correlation exists between the concentration of nuclear bound oestrogen receptor complex and synthesis of oestrogen induced protein in the rat uterus (Katzenellenbogen and Gorski, 1971, 1975) and the number of RNA initiation sites in the chick oviduct (Kalimi et al., 1976), it would seen reasonable that the bulk of oestrogen-receptor complexes in the nucleus have a productive role in the mechanism of steroid action. Conversely it has been suggested that only a small number of oestrogen-receptor complexes productively bind to high affinity sites on the genome, while the remainder bind to lower affinity non-productive sites (Yamamoto and Alberts, 1975). Those binding to productive sites could possibly be those associated with active chromatin or the 10-20% which are released from chromatin more easily than DNA is liberated. Schoenberg and Clark (1981) report such a fraction of receptor complexes, which are released in this way and presumed to be associated with active chromatin. The remainder of receptor complexes are associated with sites in the nucleus which are not nuclease sensitive.

1.7 Relevant Techniques

1.7.1 Affinity Chromatography.

Classical procedures of protein separation and purification are generally based on the relatively small differences in the physico-

chemical properties of proteins in the mixture. They are hence unselective, tedious and of poor resolution.

Affinity chromatography occupies a unique place in separation technology since it is the only technique which enables purification of almost any biomolecule on the basis of its biological function or individual chemical structure.

Affinity chromatography is a type of adsorption chromatography in which the molecule to be purified is specifically and reversibly adsorbed by a complementary binding substance (ligand) immobilised on an insoluble support (matrix). For example, specific adsorbants can be used to purify enzymes, antibodies, nucleic acids and cofactors, vitamins, repressors, transport, drug or hormone binding receptor proteins. Purification is often of the order of several thousand-fold and recoveries of the active material are generally very high. Many spectacular separations have been achieved in a single step allowing immense time-saving over less selective multi-stage procedures. Affinity chromatography has a concentrating effect which enables large volumes to be conveniently processed. The high selectivities of the separations derive from the natural specificities of the interacting molecules. For this reason affinity chromatography can be used for:-

(1) purifying substances from complex biological mixtures,

(2) separating native from denatured forms of the same substance,

(3) removing small amounts of biological material from large amounts of contaminating substances.

The first application of affinity chromatography was the selective adsorption of amylase onto insoluble starch in 1910. The complex organic chemistry required to synthesise a reliable matrix and to attach ligands covalently, prevented the technique from becoming generally established in biological laboratories. However Axen et al. (1967) reported that molecules containing primary amino groups could be coupled

to polysaccharide matrices activated by cyanogen bromide. This represented the beginning of affinity chromatography as a routine separation technique.

Examples of purifications achieved using affinity chromatography have included, avidin from crude egg white (Cuatrecassas and Wilchek, 1968), neuramidase from Vibrio cholerae (Cuatrecassas and Illiano, 1971), Staphylococcal nuclease (Cuatrecassas et al., 1968), vertebrate collagenases (Bauer et al., 1971). Especially relevant to the actions of oestrogens has been the purification of the receptor for oestradiol-17β from calf uterus by Sica and Bresciani (1979).

A slightly different use of affinity chromatography has been made by Puca et al. (1974, 1975) and Mainwaring et al. (1976). The object of these studies was not to purify a specific molecule but to demonstrate the existence of specific nuclear binding sites for both oestrogen and androgen receptor complexes. By coupling crude extracts of target cell nuclei to sepharose, they were able to probe these affinity resins with labelled steroid hormone receptor complex and establish the existence of specific binding sites (see Section 1.5.4.1.2).

1.7.2 Protein Blotting.

Over the years sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has become one of the most extensively used methods in the many facets of molecular biology. The visual evaluation of the composition of a protein sample is quite often the final step, the ultimate assay in a particular experiment. Protein blotting is an extension to SDS-PAGE and is rapidly becoming widely accepted as the optimal means of identifying and characterising proteins in complex mixtures.

In principle, a protein mixture is separated into its constituents using a gel system of choice, most commonly SDS-PAGE (Laemmli, 1970); however, non-denaturing gels, urea containing gels, acrylamide or agarose gels as well as isoelectric focussing and two-dimensional gels

have all been used successfully (Gershoni and Palade, 1983), After electrophoresis, the separated proteins are eluted from the gel and adsorbed onto an immobilising matrix, under conditions that maintain the electrophoretic pattern. Elution can be accomplished electrophoretically or by diffusion or convection. Elution is most efficiently performed electrophoretically. The main problem encountered during this phase of blotting is that high molecular weight proteins migrate out of the gel more slow. than do low molecular weight proteins, thus creating a biased plot. This problem can be overcome in several ways including altering the buffer composition (Gershoni and Palade, 1982; Nielsen et al., 1982), using reversibly crosslinked gels (Renart et al., 1979) or composite agarose/ acrylamide gels (Elkon et al., 1984) or using an apparatus that generates a gradient in the electric field (Gershoni et al., in press).

A variety of immobilising matrices exist, such as nitrocellulose membrane filters, nylon based membranes and diazotised papers; all have advantages and disadvantages, the ultimate blotting matrix has yet to be developed. The protein should be adsorbed to the immobilising matrix strongly enough to allow the blot to be thoroughly washed so that any non-specifically bound probe can be removed. The problem of non-specific background is common to all the procedures published so far. The background can be minimised by quenching i.e. blocking all the unoccupied potential binding sites of the blot. This is usually achieved by preincubating the blot in a solution containing an "inert" protein such as bovine serum albumin (BSA) (Towbin et al., 1979). Another approach has been to decrease the stickiness of the probe by including non-ionic detergents in the various solutions, Tween 20 is particularly good for this (Batteiger et al., 1982).

The blot i.e. immobilising matrix containing the transferred proteins, is then reacted with an appropriate probe or probes usually with continual rocking or shaking. Non-specifically bound probe is then

removed by exhaustive washing of the blot. The most popular probes used are either radioactively labelled, or conjugated to enzyme systems to enable their detection.

The most common application of protein blotting, "immuno-overlays" has been developed for the detection of an antigen with a specific antibody. This process is straightforward and well established. However, blots can be used in the analysis of glycoproteins and various receptors, as well as studies of protein-ligand association.

1.8 Aims

1. To obtain a reasonably pure preparation of myometrial nuclei.
2. Fractionate the nuclei, immobilise the fractions on sepharose 4B and probe the various nuclear fractions for the ability to specifically interact with activated, labelled oestrogen receptor from various sources.
3. Characterise the binding activity of the appropriate nuclear fractions with respect to affinity, saturability and specificity of binding by the steroid receptor complex.
   The chemical nature of any high affinity binding fractions was also to be investigated.

2.1 Materials

2.1.1 Fine Chemicals.

Fine chemicals were obtained as follows:-

Chemical	Supplier
Bovine Serum Albumin (BSA) - (Fraction V)	Sigma, London
Charcoal (Norit A - activated) - (untreated powder)	Sigma, London
Coomassie Brilliant Blue R-250 -	Sigma, London
Deoxyribonucleic Acid (DNA) - (Calf Thymus type V - sodium salt, highly polymerised)	Sigma, London
Dextran T-70 -	Pharmacia, Sweden
Di-isopropyl Fluorophosphate (DFP)	Sigma, London
Dithiothreitol (DTT) -	Koch Light Laboratories, Colnbrook, England
Methanol, ethanol, acetone, isopropanol -	Fisons, England
β -mercaptoethanol -	Reidel - De Haen AG, Seelze - Hannover
Potassium chloride -	Koch Light Laboratories, Colnbrook, England and BDH AnalaR Grade
Ribonucleic acid (RNA) - (Yeast)	Sigma, London
Sepharose 4B (CN-Br activated) -	Sigma, London
Sucrose -	Koch Light Laboratories, Colnbrook, England
Triton X-100 (purified) -	Koch Light Laboratories, Colnbrook, England

Tween-20 - Aldrich Chemical Co.,
Gillingham, Dorset

All other chemicals used were, whenever possible, AnalaR grade reagents.

2.1.2 Buffers.

N-2-hydroxypiperazine-N'-2-ethane sulphonic acid (HEPES) was obtained from BDH Chemicals Ltd., Poole, Dorset.

TRIS (hydroxymethyl) aminoethane was obtained from Sigma, London and the Boehringer Corporation (London) Ltd..

2.1.3 Radiochemicals.

The following radiolabelled compounds were obtained from Amersham International, Amersham, Bucks.

[1,2,6,7-^{3}H]corticosterone (80-105Ci/mmol)

[1,2,4,5,7-^{3}H] dexamethasone (78Ci/mmol)

5α-dihydro [1,2,4,5,6,7-^{3}H] testosterone (5α-DHT) (104Ci/mmol)

[17α-methyl-^{3}H] mibolerone (7,17α-dimethyl-19-nor-testosterone)(76Ci/mmol)

[2,4,6,7-^{3}H] oestradiol (E_2)(101-104Ci/mol)

ORG-2058 (16α-ethyl-21-hydroxy-19-nor [6,7-^{3}H] Pregn-4-ene-3,20-Dione)(45Ci/mmol)

[N-methyl-^{3}H] tamoxifen (Trans 1-(p-β dimethyl-aminoethyloxyphenyl)-1,2-diphenyl (but-1-ene)(89Ci/mmol)

[1,2,6,7-^{3}H] testosterone(80-105Ci/mmol)

16α-^{125}I iodooestradiol (approx. 2000Ci/mmol)and ^{125}I(Carrier free

Unlabelled dexamethasone, 5α-DHT, and E_2 and were obtained from Sigma, London.

Unlabelled ORG 2058 and mibolerone were obtained from Amersham International, Amersham, Bucks.

Unlabelled tamoxifen was obtained from I.C.I. Pharmaceuticals

Macclesfield, Cheshire.

2.1.4 Scintillation Materials.

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

Toluene (AnalaR Grade)

2,5, diphenoxazole (PPO)

1,4-di(2-(5,phenyloxazolyl)) benzene (POPOP)

Triton X-100 (Scintillation grade) was obtained from Rohm and Haas, Croydon, England.

2.1.5 Autoradiography Materials.

The following materials were obtained from Kodak.

Kodak LX-24 X-ray developer

Kodak FX-40 X-ray liquid fixer

Kodak X-OMAT AR film

2.1.6 Enzymes.

Deoxyribonuclease I from Bovine Pancreas (1400-1800 units/mg)

Ribonuclease type A from Bovine Pancreas (3000-4000 units/mg)

Trypsin - TPCK from Bovine Pancreas (180-220 units/mg) were all obtained from Worthington, U.S.A.

Papain type III from Papaya Latex (16-40 units/mg) was obtained from Sigma, London.

Pronase (Streptomyces griseus) (approx. 6 units/mg) was obtained from Boehringer Mannheim, West Germany.

Protease S.aureus (Staphyloccus aureus V8)(570 units/mg) was obtained from Miles Scientific, U.S.A.

2.1.7 Livestock.

Rats were all Albino Wistar (Glasgow University Colony), Immature females were (16-23 days old) and mature were

more than 60 days old. Prostatic tissue was obtained from adult males weighing approximately 250g.

Liver tissue for the glucocorticoid work was obtained from females weighing approximately 150g.

2.1.8 Human Tissue.

Human breast tissue was kindly supplied by surgical staff at various hospitals in the West of Scotland.

Samples of human myometrium and endometrium were obtained from several gynaecologists working in the:-

Victoria Infirmary, Glasgow

Western Infirmary, Glasgow

Royal Infirmary, Glasgow

Southern General Hospital, Glasgow

David Elder Infirmary, Glasgow.

2.1.9 Miscellaneous.

Plastic disposable mini columns were obtained from the Amicon Corporation, Massachusetts, U.S.A.

Glass/glass tissue grinders were obtained from Kontes, U.S.A. or Cowie Scientific, Middlesborough, England.

Teflon/Glass homogenisers were obtained from Jencons (Scientific) Ltd., Leighton Buzzard, Beds.

Nitrocellulose was obtained from Schleicher and Schull, West Germany.

Protein A from *Staphyloccus aureus* was obtained from Sigma, London.

Before use, all glassware was washed and rinsed in glass distilled water.

2.2 Methods

2.2.1 Human Tissue Handling.

2.2.1.1 Tissue Collection.

Tissue was collected fresh from the operating theatre. In the case of breast tissue, collection was into an empty sterile container which was then transported to the laboratory on ice. In the case of myometrium, the tissue was obtained at hysterectomy. The uterus was collected from the operating theatre and transported to the hospital Pathology Department on ice. Sections of myometrium were then cut by a pathologist. These sections were then transported to the laboratory on ice for analysis. Endometrial tissue was obtained at dilation and curretage, transferred to an empty sterile container, then transported to the laboratory on ice for analysis.

2.2.1.2 Tissue Storage.

Whenever possible, tissue was used fresh, but when this was not possible the tissue was stored at -20°C in a medium of 50% (v:v) glycerol, 0.25M sucrose, 1.5mM $MgCl_2$, 10mM HEPES (N-2-hydroxyl-piperazine-N'-2-ethane sulphonic acid) pH7.4. Before use, the tissue was rehydrated in 0.25M sucrose, 1.5mM $MgCl_2$, 10mM HEPES pH7.4 for 20-30 minutes at 0-4°C. The properties of this storage medium have been described elsewhere (Crawford et al., 1984).

2.2.2 Purification of Human Myometrial Nuclei.

Myometrial tissue was dissected free of connective and other non muscle tissue before nuclear purification was commenced. The basic procedure was a modified version of that described by Widnell and Tata (1964).

Myometrial tissue was minced into very small pieces using both scissors and scalpel. These small pieces were then homogenised in 3-4 volumes of 0.32M sucrose/1.5mM $MgCl_2$/0.2%

Triton X-100 using 3 x 20 second bursts of an Ultra turrax homogeniser (Model TP 18/2). The homogenate was cooled on ice between each burst. The homogenate was then passed through 4 layers of cheese-cloth, and the clear filtrate rehomogenised using a teflon/glass homogeniser (2-3 strokes). The volume of this clear homogenate was then doubled using 0.32M sucrose/1.5mM $MgCl_2$ and the sucrose concentration reduced to 0.25M using distilled water. The homogenate was then layered over 10ml of 0.32M sucrose/1.5mM $MgCl_2$ in 4-6 batches and centrifuged at 700g for 10 minutes using a JS 13 rotor in a Beckman J2-21 centrifuge at 4^oC. The crude nuclear pellet obtained was resuspended in 25ml of 2.4M sucrose/1.5mM $MgCl_2$ using a teflon/glass homogeniser (1-2 strokes) and then centrifuged at 50,000g for 60 minutes using a JA 20.1 rotor in a Beckman J2-21 centrifuge at 4^oC. The final nuclear pellet was taken up in 1.0ml of 0.32M sucrose/1.5mM $MgCl_2$ and washed once using this medium before final resuspension in 1.0ml of the same medium.

2.2.2.1 Assessment of the Purity of the Nuclear Preparation.

The purity of the nuclear preparation was assessed in two ways. The first involved the use of phase contrast microscopy and the second by calculating the DNA: Protein ratio of the final nuclear pellet (see Sections 2.9 and 2.10).

2.2.2.2 Fractionation of Human Myometrial Nuclei using 2M NaCl.

After purification the nuclei were "pelleted" by centrifugation at 2000g and resuspended in 10-15 volumes of 50mM sodium phosphate buffer pH7.1/2M NaCl/10mM di-isopropyl fluorophosphate (DFP). DFP was included to prevent degradation of protein by serine proteases. The nuclei were incubated for 2 hours at 4^oC under these conditions, with occasional mixing. The extract was then centrifuged at 48,000g for 30 minutes at 4^oC. The precipitate (Fraction A) was "resuspended" in 25mM sodium phosphate buffer pH7.1.

The supernatant was dialysed overnight against 5-10 volumes of 25mM sodium phosphate buffer pH7.1 at $4^{o}C$ with several changes of buffer. The contents of the dialysis bag were then centrifuged at 46,000g for 30 minutes at $4^{o}C$. The pellet (Fraction C) was taken up in 25mM sodium phosphate buffer pH7.1/2M NaCl. Centrifugation was carried out using a Beckman J2-21 centrifuge.

The above procedure separates nuclear material on the basis of solubility in, or resistance to 2M NaCl. Fraction A represents the material insoluble in 2M NaCl. The supernatant from this step represents the nuclear material solubilised by 2M NaCl. Dialysis separates this material into Fraction B, the material which remains in solution at low ionic strength and Fraction C, the material soluble in 2M NaCl.

The three nuclear fractions were then assayed for protein DNA and RNA content (see Sections 2.9, 2.10 and 2.11).

2.2.2.3 Extraction of Myometrial Nuclei with 0.1M H_2SO_4.

The purified nuclei were "pelleted" as described in Section 2.2.2.2. They were then resuspended in 10-15 volumes of 0.1M H_2SO_4/ 5mM dithiothreitol (DTT)/10mM DFP at $4^{o}C$ for 20 minutes. The extract was then centrifuged at 40,000g for 30 minutes at $4^{o}C$. The supernatant was removed at the extracted proteins precipitated at $-20^{o}C$ with 4 volumes of ethanol for 50 minutes. The precipitated protein was collected by centrifugation at 40,000g for 30 minutes at $4^{o}C$. The pellet of precipitated protein was taken up in 25mM sodium phosphate buffer pH7.1/1.0M KCl.

The protein, DNA and RNA content of the final pellet was then assayed (see Sections 2.9, 2.10 and 2.11).

Centrifugation was carried out using a Beckman J2-21 centrifuge.

2.2.2.4 Electrophoretic Analysis of the Nuclear Fractions.

2.2.2.4.1 Nuclear Fractions B and C.

These nuclear fractions were subjected to sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (P.A.G.E.) as described by Laemmli (1970). Analysis was carried out using slab polyacrylamide gels of 12.5% and 15%. In all cases a stacking gel of 5.3% was used. Slab gels were run at 50-60mA in an apparatus which incorporated a cooling system.

Gels were stained with Coomassie Blue (0.04% Brilliant Blue R 250/25% isopropanol/10% acetic acid) overnight and destained with 10% acetic acid/10% methanol in distilled water.

The protein present in nuclear Fraction B was precipitated by 4 volumes of acetone at -20°C for 1 hour. The protein was then collected by centrifugation and taken up in sample buffer.

Nuclear Fraction C was taken up in sample buffer. A small volume was then acetone precipitated to enable a protein determination to be carried out.

2.2.2.4.2 0.1M H_2SO_4 Nuclear Extract.

This was carried out essentially as described by Panyim and Chalkley (1969).

Electrophoresis was carried out on 15% polyacrylamide gels in the presence of 6M urea. Pre-electrophoresis was carried out for approximately 11 hours. The nuclear extract was taken up in 0.9M acetic acid/15% sucrose. The tank buffer used was 0.9M acetic acid. Electrophoresis was carried out at 1.5mA/gel for approximately 2½ hours.

Staining and destaining was carried out essentially as described in Section 2.2.2.4.1 with destaining being achieved using 10% acetic acid.

2.2.2.5 Immobilisation of Nuclear Fractions on CN-Br activated Sepharose 4B.

Nuclear fractions were prepared as described previously (see Sections 2.2.2.2 and 2.2.2.3). Prior to coupling, CN-Br activated Sepharose 4B was allowed to swell in 1mM HCl for 20-30 minutes. Typically 500mg of Sepharose was used for each column. After swelling the Sepharose was collected on a sintered glass filter and washed with a further 40ml of 1mM HCl. This was followed by washing with 5-10ml of 25mM sodium phosphate buffer pH7.1/0.1M KCl. The Sepharose was then immediately transferred to the appropriate nuclear fraction. The nuclear extract was then incubated with the Sepharose overnight at 4°C with continuous end-over-end mixing. The remaining unreacted groups on the Sepharose were then blocked by incubating the Sepharose resin with 0.2M glycine pH9.4 at room temperature for 2 hours. Again end-over-end mixing was employed throughout.

The Sepharose resin was then washed to remove any proteins which were not covalently bound. This was achieved using high and low pH washes in the presence of 0.5M NaCl. Initially the resin was washed with 20ml of 25mM sodium phosphate buffer pH7.1/0.5M NaCl, this was followed by 20ml of 0.1M sodium acetate buffer pH4.0/0.5M NaCl, then finally by 20ml of 25mM sodium phosphate buffer pH7.1/ 0.5M NaCl. To remove the NaCl the Sepharose resin was washed with 100ml of 20mM HEPES pH7.4/1.5mM EDTA (HE buffer). The Sepharose was then stored in 20mM HEPES pH7.4/1.5mM EDTA/0.12M KCl (HE/0.12M KCl). Control columns were prepared by coupling glycine to the Sepharose alone.

To check on the efficiency of protein coupling to the CN-Br activated Sepharose 4B, the supernatant from the initial coupling incubation and subsequent washes were assayed for protein

content (see Section 2.9). Usually 70-80% of protein present in solution initially was coupled to the Sepharose.

2.2.2.6 Pouring of Sepharose Resins in to Columns.

Plastic disposable mini-columns were used throughout. The columns were poured under gravity at $4^{o}C$ using HE/0.12M KCl as buffer. Before use, each column was washed exhaustively with at least 100mls of HE/0.12M KCl.

2.2.2.7 Buffers used in Binding Assays.

The running buffer used in all cases was HE/0.12M KCl. To elute labelled receptor complex bound to the columns the buffer was changed to HE/2M KCl. The columns were then thoroughly washed with HE/0.12M KCl.

2.2.3 Labelling of Various Tissue Cytosols with ^{3}H-oestradiol-17β.

2.2.3.1 Preparation of ^{3}H-oestradiol and other Radioactive Steroid Solutions.

Stock ^{3}H-labelled oestradiol-17β was prepared at a concentration of $5 \times 10^{-7}M$ in absolute alcohol and stored at $-20^{o}C$. The other ^{3}H-labelled steroids were prepared at the same concentration in absolute alcohol, and stored at $-20^{o}C$.

Solutions of non-radioactive steroids were also prepared in absolute alcohol usually at a concentration of $5 \times 10^{-4}M$ and stored at $-20^{o}C$.

2.2.3.2 Immature Rat Uterine Cytosol.

Immature female rats (16-23 days) were anaesthetised using chloroform and killed by cervical dislocation. The uteri were removed, dissected free from any adhering fat and mesentery and placed in 20mM HEPES pH7.4/1.5M EDTA/0.25mM DTT (HED buffer) at $4^{o}C$. The uteri were then homogenised (1.5 uteri / ml) in HED buffer using a glass/glass tissue grinder. The homogenate was then made 10mM with respect to di-isopropyl fluorophosphate (DEP) (using a stock solution of 1M in isopropyl alcohol) and centrifuged at 2000g in a Beckman J2-21 centrifuge at $4^{o}C$. The

resultant supernatant ("cytosol") was retained in this crude form for the labelling of receptor. The cytosol was then labelled overnight at $4^{o}C$ with $5 \times 10^{-9}M$ ^{3}H-oestradiol 17-β (H). A 200 fold excess of diethylstilboestrol (DES) was included in a separate incubation to determine non-specific binding - (H+C). The final alcohol concentration in the H was 0.01% and in the H+C 0.02%.

Following this overnight incubation the cytosol was incubated at $37^{o}C$ for 20 minutes to ensure activation of the receptor had taken place (Notides et al., 1981).

2.2.3.3 Removal of Unbound Steroid from the Cytosol.

This was achieved using 0.25% Dextran Coated Charcoal solution (0.25% w/v Norit A Charcoal, 0.0025% w/v dextran T70 in 0.25M sucrose 1.5mM EDTA, 10mM HEPES pH7.4).

The same volume of DCC solution as cytosol to be "stripped" was centrifuged at 1000g for 5 minutes to give a charcoal pellet. The supernatant was poured off and the cytosol added to the charcoal pellet. After resuspension, stripping was continued for 15 minutes at $0^{o}C$ with periodic mixing of the tubes. At the end of this time, the charcoal was pelleted by centrifugation at 1000g for 5 minutes. The "stripped" cytosol was then transferred to a clean tube.

10µl aliquots of the H and H+C were then placed in plastic scintillation vials, 10ml of Triton toluene scintillant (1400ml toluene/PPO (2,5-diphenyloxazole), (5g/1)POPOP (1,4-di-(2-(5-phenyloxazole))-benzene), (0.24g/1): 600ml Triton X-100: 200ml absolute alcohol) was added to each vial and the vials counted in a Searle MKII Liquid Scintillation Analyser. The counting efficiency was calculated to be approximately 30%. The concentration of receptor in the cytosol could thus be calculated, and the appropriate volume (calculated as number of specific cpm) to be applied to the Sepharose column could be determined.

2.2.3.4 Human Myometrial Cytosol.

Myometrial tissue was minced very finely using both scissors and scalpels. The tissue was then homogenised in HED/0.12M KCl at a concentration of 100mg/ml. Homogenisation was carried out using an Ultra-Turrax homogeniser (model TP 18/2). The homogenate was then passed through 4 layers of cheese-cloth, and the "filtrate" further homogenised using a glass/glass tissue grinder. This final homogenate was then made 10mM with respect to DFP. The remaining procedures were as described in Sections 2.2.3.2 and 2.2.3.3.

2.2.3.5 Human Breast Tumour Cytosol.

Tissue from tumours, previously shown to be oestrogen receptor positive, was homogenised in HED/0.12M KCl at a concentration of 50mg/ml. Homogenisation was carried out using an Ultra-Turrax homogeniser (model TP 18/2), followed by the use of a glass/glass tissue grinder. The homogenate was then made 10mM with respect to DFP. The remaining procedures were as described in Sections 2.2.3.2 and 2.2.3.3.

2.2.3.6 Preparation of ^{3}H-Oestradiol 17-β labelled Oestrogen Receptor in an Unactivated Form.

Immature rat uterine cytosol was prepared as described in Section 2.2.3.2 except for the inclusion of 20mM sodium molybdate which has been shown to inhibit the process of activation (Muller et al., 1983a).

2.2.4 Assay of the Various Nuclear Extract/Sepharose Columns for the Ability to Bind Activated ^{3}H-Oestradiol 17-β labelled Oestrogen Receptor.

Binding assays were carried out at 4°C using the buffers described in Section 2.2.2.7.

The volume of cytosol to be applied to the column (whether H or H+C) was calculated as described in Section 2.2.3.3. About

10,000 cpm were normally applied to each column. Before applying the sample, the flow rate of the column was recorded. After the sample had been applied, 10 fractions each approximately 1ml in volume were collected in plastic scintillation vials. The eluting buffer was then changed from HE/0.12M KCl to HE/2M KCl and 6 fractions, each approximately 1ml in volume, were collected in plastic scintillation vials. The columns were then washed with approximately 10ml of HE/0.12M KCl before further use. 10ml of Triton toluene scintillant was added to each vial and the vials counted in a Searle MKII Liquid Scintillation Analyser at about 30% efficiency.

2.2.4.1 Calculation of Results.

Specifically bound cpm were calculated as the difference between the cpm eluted with HE/2M KCl after cytosol labelled in the presence of $^3H\ E_2$ alone (H) was eluted through the columns and the cpm eluted with HE/2M KCl after cytosol labelled with $^3H\ E_2$ plus a 200 fold excess of DES (H+C) had been eluted through the column. Binding was expressed as f mol bound/mg of protein immobilised on the column.

2.3 The Effect of Enzymic Digestion of Binding Activity

2.3.1 Preparation of Nuclear Extract/Sepharose Resins.

Both the 2M NaCl soluble nuclear material (Fraction C)/ Sepharose resin and the 0.1M H_2SO_4 nuclear extract/Sepharose resin were prepared as described in Sections 2.2.2.2 and 2.2.2.3 respectively.

2.3.2 Preparation of Enzyme Solutions.

Solutions of enzyme (1mg/ml) were made fresh before use, Papain was supplied already in solution. DNase and RNase were dissolved in 20mM HEPES pH7.4 supplemented with 1mM $MgCl_2$.

Trypsin was dissolved in HE buffer and in 20mM HEPES pH8.0.

Protease S.aureus was dissolved in 50mM Tris/HCl pH7.8.

Papain was activated at room temperature for 30 minutes in 20mM HEPES pH6.4/55mM cysteine-HCl/11mM EDTA/66μM mercaptoethanol before use.

Pronase was dissolved in HE buffer.

2.3.3 Digestion of the nuclear extract/Sepharose resins with various enzymes.

100mg of the appropriate nuclear extract/Sepharose resin was placed in a total volume of 1.0ml of each of the above enzyme solutions (1mg enzyme/ml), in a siliconised Eppendorf tube. The digestion reaction was carried out at 37°C for 2 hours with continuous end-over-end mixing. Following this incubation serine protease were inhibited by adding di-isopropyl fluorophosphate to a final concentration of 10mM.

2.3.3.1 Washing of the nuclear extract/Sepharose resins after digestion.

After digestion, the nuclear extract/Sepharose resins were collected on a sintered glass filter and washed with at least 100ml of HE buffer to remove the digestive enzymes.

2.3.3.2 Storage of the digested nuclear extract/Sepharose resins.

After washing, 10mg amounts of the digested nuclear extract/ Sepharose resins were placed in siliconised Eppendorf rubes and 0.2ml of HE/0.12M KCl added. The Eppendorf tubes were then stored at 4°C until binding assays were carried out.

2.3.4 Assay of the ability of digested nuclear extract/Sepharose resin to bind labelled activated oestrogen receptor.

Immature rat uterine cytosol was labelled and "stripped" as described in Sections 2.2.3.2 and 2.2.3.3.

A volume of cytosol corresponding to 10,000 cpm was placed in each Eppendorf tube and the total volume made up to 1.0ml using HE/0.12M KCl. The tubes were then incubated at 4°C for 60 minutes with continuous end-over-end mixing. At the end of this period the

nuclear extract/Sepharose resin was pelleted by centrifugation. 0.8ml of the supernatant was then placed in a plastic scintillation vial, 10ml of Triton toluene scintillant added and the vials counted in a Searle MKII Liquid Scintillation Analyser at about 30% efficiency. Each binding assay was carried out in triplicate.

2.3.4.1 Expression of results.

Controls were performed by using nuclear extract/Sepharose resin which had not been digested in any way. Binding activities measured after digestion of the nuclear extract Sepharose resin are expressed as a percentage of this control.

2.4 Saturation analysis of the binding activity

2.4.1 Preparation of nuclear extract/Sepharose resins.

Both the 2M NaCl soluble nuclear material (Fraction C)/ Sepharose resin and the 0.1M H_2SO_4 nuclear extract/Sepharose resin were prepared as described in Sections 2.2.2.2 and 2.2.2.3 respectively.

2.4.2 Methodology of the saturation binding assay.

After preparation the nuclear extract/Sepharose resin, 20mg amounts were weighed into siliconised Eppendorf tubes and 0.2ml of HE/0.12M KCl added. The tubes were then stored at $4^{o}C$ until a binding assay was performed.

Immature rat uterine cytosol was "labelled" and "stripped" as described in Sections 2.2.3.2 and 2.2.3.3.

An increasing range of cpm (corresponding to an increasing amount of labelled activated oestrogen receptor) was added to each set of assay tubes. The range of cpm added was usually 1,000 cpm-30,000 cpm. This gives a range of 1.5×10^{-11}M to 4.5×10^{-10}M $^{3}H\ E_2$ (receptor bound). The total volume in each assay tube was then made up to 1.0ml using HE/0.12M KCl. The tubes were then incubated at $4^{o}C$ for 60 minutes with continuous end-over-end mixing. After this incubation the nuclear extract/Sepharose resin was

"pelleted" by centrifugation. 0.8ml of the supernatant was placed in a plastic scintillation vial, 10ml of Triton toluene scintillant added, and the vials counted in a Searle MKII Liquid Scintillation Analyser at about 30% efficiency.

2.4.3 Expression of results.

The results were analysed using the method of Scatchard (1949).

2.5 Labelling of immature rat uterine cytosol with various ^{3}H-compounds at a concentration of $5 \times 10^{-9}M$

2.5.1 ORG 2058.

Labelling was carried out essentially as described in Section 2.2.3.2 except for the inclusion of 10% glycerol in the homogenisation buffer. This has been shown to enhance the stability of the progesterone receptor. ORG 2058 was used as the ligand to probe for progesterone receptor as it does not cross react with the glucocorticoid receptor. Stripping of the cytosol was carried out as described in Section 2.2.3.3. A 200 fold excess of non radioactive ORG 2058 was used instead of DES. Stock solutions of the ligands were prepared as described for oestradiol-17β in Section 2.2.3.1.

2.5.2 Tamoxifen.

Cytosol was "labelled" and "stripped" with ^{3}H-tamoxifen ($\pm$ a 200 fold excess of unlabelled tamoxifen) as described in Sections 2.2.3.2 and 2.2.3.3 using "hot" and "cold" tamoxifen solutions prepared exactly as described for oestradiol-17β in Section 2.2.3.1.

2.5.3 5α-dihydrotestosterone, mibolerone.

"Labelling" and "stripping" were carried out essentially as described in Sections 2.2.3.2 and 2.2.3.3 with a 200 fold excess

of the appropriate non-radioactive ligand being used instead of DES. Stock solutions were prepared as described for oestradiol-17β in Section 2.2.3.1. Where appropriate, see text, glycerol (10%) and molybdate (20mM) were included in the homogenisation buffer.

2.5.4 Dexamethasone.

"Labelling" and "stripping" was carried out essentialy as described in Sections 2.2.3.2 and 2.2.3.3 with a 200 fold excess of the appropriate ligand being used instead of DES. Stock solutions were prepared as described for oestradiol-17β in Section 2.2.3.1.

2.5.5 With various ligands in the presence of DES.

The cytosol was labelled with the following ligands both alone and in the presence of a 200 fold excess of cold DES overnight at 4°C:-

Corticosterone, mibolerone, testosterone and
5α DHT.

Stripping was carried out as described in Section 2.2.3.3.

2.5.6. Application of the cytosols prepared in Sections 2.5.1-2.5.5 to the nuclear extract/Sepharose resin columns.

Application was essentially as described in Section 2.2.4 but the specific activity of each radioactive ligand was taken into account when the volume of cytosol to be applied was being calculated. This was to ensure that the amount of protein-bound ligand applied was comparable to that applied when ^{3}H E_2 labelled oestrogen receptor was applied to the column.

Binding to the nuclear extract/Sepharose resins was expressed as f mol bound/mg of protein bound to the column.

2.5.7 Labelling of female rat liver cytosol with ^{3}H-Dexamethasone.

A female rat (approximately 150g) was anaesthetised using chloroform and killed by cervical dislocation. The liver was removed and washed on ice with HE/0.12M KCl, then minced using scissors.

Homogenisation was then carried out in approximately 10ml of HED/0.12M KCl using a teflon/glass homogeniser. The homogenate was then made 10mM with respect to DFP. The homogenate was then centrifuged at 4°C at 50,000g for 15 minutes. The fat layer was then carefully removed and the resultant cytosol was then labelled overnight at 4°C with either 5×10^{-9}M ^{3}H-dexamethasone alone (H) or 5×10^{-9}M ^{3}H-dexamethasone plus a 200 fold excess of non-radioactive dexamethasone (H+C).

After incubation, stripping of the cytosol was carried out as described in Section 2.2.3.3. The stripped cytosol was then applied to the nuclear extract/Sepharose column as described in Section 2.5.5.

2.5.8 Labelling of rat prostatic cytosol with ^{3}H mibolerone or 5α dihydrotestosterone.

Ventral prostates were removed from male rats (approximately 250g) which had been anaesthetised using chloroform and killed by cervical dislocation. After washing on ice using HE/0.12M KCl, homogenisation was carried out in HED/0.12M KCl/10% (v/v) glycerol using a glass/glass tissue grinder at 75mg prostate/ml. The homogenate was then made 10mM with respect to DFP. The homogenate was then spun at 46,000g at 4°C for 15 minutes.

The fat layer was then carefully removed and the resultant cytosol was then labelled overnight at 4°C with either (5×10^{-9}M) ^{3}H-5αdihydro-testosterone or ^{3}H-mibolerone. In each case a 200 fold excess of the appropriate non-radioactive ligand was included in the H+C incubation.

"Stripping" of the cytosol was carried out as described in Section 2.2.3.3. The stripped cytosol was then applied to the nuclear extract/Sepharose resin as described in Section 2.5.5. In some cases 20mM molybdate was included in the homogenising buffer.

2.6 Measurement of possible competition between progesterone receptors and oestrogen receptors for nuclear binding sites.

Parallel columns of nuclear extract/Sepharose resin were set up. Binding of activated ^{3}H oestradiol-17β labelled oestrogen receptor was then measured either alone or in the presence of cold ORG 2058 labelled progesterone receptor. The difference in bound label was assumed to represent the level of competition.

The reciprocal experiment was performed by measuring the binding of ^{3}H-ORG 2058 labelled progesterone receptor either alone or in the presence of excess cold DES labelled oestrogen receptor. Again the difference in bound label was assumed to represent the level of competition.

Immature rat uterus was used as a source of receptor.

Binding to the nuclear extract/Sepharose columns in all cases was expressed as f mol bound/mg of protein immobilised on the column.

2.7 Protein Blotting Experiments

2.7.1 Fractionation of Human Myometrial Nuclei using 2M NaCl.

This was carried out essentially as described in Section 2.2.2.2. After nuclear Fractions B and C had been obtained they were treated as follows:-

An aliquot of nuclear Fraction B was retained for protein assay and the remainder was precipitated using 4 volumes of acetone at -20°C for approximately 1 hour. The precipitate was collected by centrifugation and boiled for 5 minutes in Laemmli sample buffer (62.5mM Tris HCl pH6.8/2% SDS/5% 2-mercaptoethanol/10% sucrose).

Nuclear Fraction C was taken up in Laemmli sample buffer by boiling for 5 minutes. A small aliquot was removed and the protein precipitated using 4 volumes of acetone at -20°C for approximately 1 hour. The precipitate was collected by centrifugation

and taken up in 25mM sodium phosphate buffer pH7.1/2M NaCl. A protein assay was then carried out.

2.7.2 SDS polyacrylamide gel electrophoresis of Nuclear Fractions B and C.

This was carried out essentially as described by Laemmli (1970). The main separating gel contained 12.5% acrylamide and the stacking gel contained 5.3% acrylamide. The gels run were of the slab type. Electrophoresis was carried out at a constant current of 50-60mA using electrophoresis apparatus which incorporated a cooling system.

Gels were stained overnight in 0.04% Coomassie Blue (0.04% Brilliant Blue R-250/25% isopropanol/10% acetic acid) and destained using 10% acetic acid/15% isopropanol/75% distilled water.

2.7.3 Transfer of Electrophoretically separated Proteins onto Nitrocellulose.

The transfer of proteins to nitrocellulose was achieved electrophoretically at a constant current of 40mA overnight at room temperature using a Bio Rad Laboratories Trans blot TM cell and using 25mM Tris pH8.3/0.192M glycine/20% (v/v) methanol/2% SDS as electrode buffer.

After transfer the remaining unreacted groups on the nitrocellulose were blocked by incubating the nitrocellulose for 2 hours at room temperature in 20mM HEPES pH7.4/0.12M KCl/0.5% Tween-20 with constant shaking. Finally the nitrocellulose was washed for approximately 10 minutes in HE/0.12M KCl.

2.7.4 Labelling of Immature Rat Uterine Cytosol with ^{125}I-iodo oestradiol.

This was carried out as described in Section 2.2.3.2 with 5×10^{-9}M ^{3}H-oestradiol being replaced with 5×10^{-9}M ^{125}I-oestradiol-17β. The H+C incubation was omitted.

Stripping was carried out as described in Section 2.2.3.3.

To determine how many cpm were being applied to the nitrocellulose, 10µl of the cytosol were counted using an LKB WALLAC 1275 min-gamma counter.

2.7.5 Incubation of Cytosol with the Nitrocellulose.

The nitrocellulose was placed in a tailor-made polythene bag, double sealed on 3 sides. The $^{125}I\text{-}E_2$ labelled cytosol was then added to the bag and the fourth side of the bag sealed. The cytosol was then smoothed over the whole of the nitrocellulose before incubation at 37°C for 40 minutes with constant gentle shaking.

At the end of this incubation, the nitrocellulose was removed from the bag and washed at room temperature with 50ml of 20mM HEPES pH7.4/0.12M KCl/0.5% Tween-20 for 20 minutes with continuous shaking. This buffer was replaced with 30ml of HE/0.12M KCl and the above washing repeated. This buffer was subsequently changed a further 3 times.

The nitrocellulose was then air dried and subjected to autoradiography at -70°C against an intensifying screen to enhance the sensitivity of the procedure.

2.7.6 Protein Blot using Rabbit Antiserum raised against Oestrogen Receptor.

2.7.6.1 Nuclear Fractions B and C.

Fractionation of human myometrial nuclei with 2M NaCl was carried out as described in Section 2.2.2.2.

Electrophoresis and transfer of the protein onto nitrocellulose were carried out as described in Sections 2.7.2 and 2.7.3 respectively. After transfer the remaining active groups were blocked using 20mM HEPES pH7.4/0.12M KCl/3% BSA/0.1% azide (blotting buffer) for 2 hours at room temperature, with continuous shaking. The nitrocellulose was then washed with HE/0.12M KCl at

room temperature for 10 minutes.

Immature rat uterine cytosol was "labelled" with 5 x 10^{-9}M cold E_2 essentially as described in Section 2.2.3.2 and "stripped" before use as described in Section 2.2.3.3.

The cytosol was then incubated with the nitrocellulose essentially as described in Section 2.7.5 with the incubation being at 37°C for 60 minutes.

At the end of this incubation the nitrocellulose was washed with 6 changes of 20mM HEPES pH7.4/0.12M KCl (washing buffer) over a period of 60 minutes at room temperature.

After this washing, blotting buffer containing rabbit antiserum raised against oestrogen receptor was added to the nitrocellulose, and the incubation continued at room temperature for 60 minutes with continuous shaking. The antiserum was preblocked in blotting buffer overnight at 4°C before use, as it had been shown previously to cross-react with serum albumin.

The nitrocellulose was then washed with 6 changes of washing buffer over a 60 minute period at room temperature.

^{125}I-labelled protein A (approximately 80,000 cpm) was then added in blotting buffer to the nitrocellulose and the incubation continued for 60 minutes at room temperature.

^{125}I-labelled protein A was prepared by dissolving 1mg in 1ml of 20mM Tris/HCl buffer pH7.2 containing 150mM NaCl and labelling continued for 15 minutes at room temperature with Iodogen (1mg) and 300-500μCi of Na^{125}I (Salacinski et al., 1981). ^{125}I-Iodide was removed by gel filtration on Sephadex G-25. Fractions containing the labelled protein A were pooled, divided into small samples, and kept at -20°C until use.

Finally the nitrocellulose was washed with 6 changes of washing buffer over a 60 minute period at room temperature.

The nitrocellulose was then air dried and subjected to autoradiography at $-70^{o}C$ against an intensifying screen to enhance the sensitivity of the procedure.

Two types of control experiment were performed. The first omitted the addition of immature rat uterine cytosol i.e. activated occupied oestrogen receptor, the rest of the procedures were as above. In the second control experiment antiserum was replaced by normal rabbit serum, again the rest of the procedures were as above.

2.7.6.2 0.1M H_2SO_4 Nuclear Extract.

Fractionation of human myometrial nuclei was carried out using 0.1M H_2SO_4 as described in Section 2.2.2.3. The precipitated protein was then taken up in Laemmli sample buffer.

Electrophoresis and transfer of the proteins onto nitrocellulose were carried out as described in Sections 2.7.2 and 2.7.3.

The remaining procedures were carried out as described in Section 2.7.6.1.

2.8 Assay of Binding activity present in various Immature Female Rat Tissues

Nuclei were purified from kidney, liver, spleen and uterus essentially as described in Section 2.2.2. The fractions of nuclear proteins were then prepared on the basis of initial solubility in or resistance to 2M NaCl as described in Section 2.2.2.2. In each case the appropriate Fraction C was then coupled to CN-Br activated Sepharose 4B as described in Section 2.2.2.5. The nuclear extract/ Sepharose resin complexes were then poured into columns as described in Section 2.2.2.6.

2.8.1 Assay of binding Activity.

Immature rat uterus was used as the source of oestrogen receptor as described in Section 2.2.3.2 and Section 2.2.3.3.

Binding was expressed as f mol bound/mg of protein immobilised on the column.

2.9 Protein Determination

This was carried out using both the micro and standard Bradford assays (Bio Rad Laboratories Ltd., West Germany).

2.10 DNA Determination

DNA was determined by the modification by Katzenellenbogen and Leake (1974) of the method of Burton (1956).

2.11 RNA Determination

RNA was determined by the Orcinol reaction as described by Plummer (1978).

3. Results

3.1 Assessment of Nuclear Purity and Composition of Nuclear Fractions

3.1.1 Phase Contrast Microscopy.

An example of a final nuclear pellet under the phase contrast microscope is shown in Figure 5 . Many of the nuclei are long and thin, reflecting the fact they have come from the long thin muscle cells of the myometrium. The nuclei are in the main intact but look slightly damaged. The techniques required to disrupt muscle tissue are fairly harsh. Nuclear preprations which contained mainly ruptured nuclei i.e. masses of chromatin could be seen under the microscope, were not used for subsequent protein extraction. The yield of pure nuclei varied from preparation to preparation but was usually between 30 and 35% as assessed by recovery of DNA from the initial homogenate. This relatively low and variable recovery was probably due to the fibrous nature of myometrial tissue which also varied from sample to sample.

3.1.2 DNA:Protein Ratio.

The protein and DNA content of the final nuclear pellet was determined as described in Sections 2.9 and 2.10 respectively. The values were then expressed as a ratio. The ratio of DNA:Protein was found to be in the range of 0.5-0.6.

The non-histone protein content of metabolically active nuclei is much higher than the non-histone protein content of less active nuclei (Allfrey, 1971). Myometrial cells are fairly inactive, except during pregnancy and as such will have a lower non-histone protein content. The DNA:Protein ratio of the purified nuclei reflects the lower level of non-histone protein found in these nuclei.

A DNA:Protein ratio within this range and the appearance of the nuclei under the phase contrast microscope were considered adequate for the nuclei to be used for subsequent protein extraction. The integrity

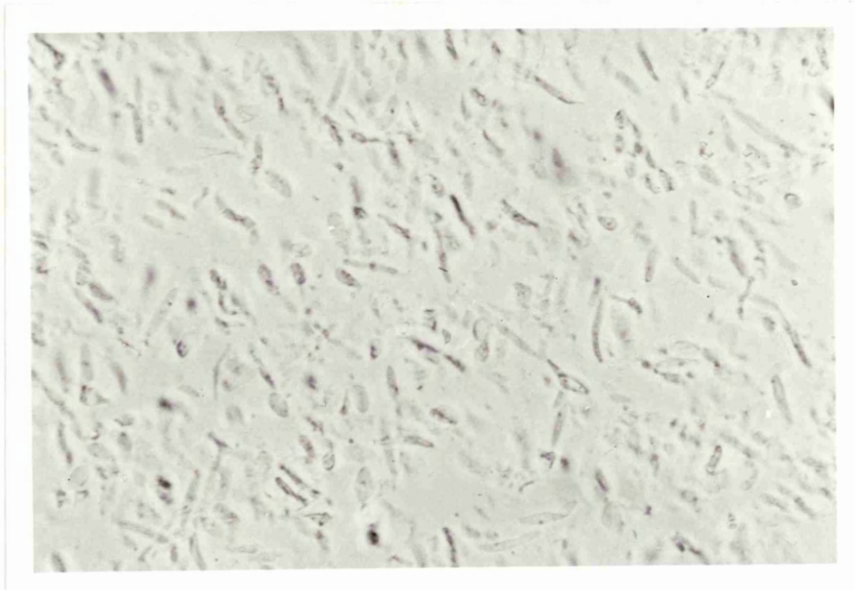


Figure 5 - Phase contrast photograph of human myometrial nuclei purified as described in Section 2.2.2. (Magnification x 400).

of each nuclear preparation to be used for protein extraction was assessed using the phase contrast microscope. Nuclear preparations containing large numbers of ruptured nuclei were discarded.

3.1.3 Composition of the Nuclear Fractions A, B and C.

The typical composition of the nuclear fractions after the human myometrial nuclei had been exposed to 2M NaCl as described in Section 2.2.2.2 is shown in Table 1. Protein, DNA and RNA determinations were carried out as described in Sections 2.9, 2.10 and 2.11 respectively.

NaCl Extract

The majority (range 0.6mg-1.0mg) of protein was recovered in nuclear Fraction C (2M NaCl soluble material), with approximately equal amounts of protein being recovered in the other two fractions. However, the insoluble material remaining after the nuclei had been exposed to 2M NaCl (Fraction A), was very sticky and impossible to redissolve. Subsequent analysis by the Lowry method (Lowry et al., 1951) of this "particulate" fraction revealed significant amounts of protein remaining in this fraction (up to 400μg). The Bradford protein assay proved unreliable for particulate proteins and the conditions required to solubilise these proteins e.g. alkali or detergents subsequently interfered with the assay.

From the initial experiments (using the Bradford assay the recovery of protein seemed on the low side when compared to the recovery of DNA (see Table 1). On the basis of the protein and DNA content of pure nuclei, it would be expected that the amount of protein recovered should be greater than the amount of DNA recovered. The same situation was still observed following assay of Fraction A by the Lowry method, suggesting that further protein may still be trapped and unassayable in Fraction A.

The bulk of DNA was recovered in nuclear Fraction A, with lesser amounts in nuclear Fractions C and B respectively.

TABLE 1

Nuclear Fraction/ Constituent	0.1M H_2SO_4 Nuclear Extract	A	B	C
Protein (mg)	1.3 ± 0.5	0.26 ± 0.14	0.30 ± 0.13	0.8 ± 0.2
DNA (mg)	0	1.5 ± 0.3	0.05 ± 0.01	0.4 ± 0.05
RNA (mg)	0.085 ± 0.02	2.0 ± 0.2	1.5 ± 0.2	0.23 ± 0.03

Means of 4 Exps. ± SD

Table 1 - The constituents of the various human myometrial nuclear fractions. Protein,DNA and RNA were determined as described in Sections 2.9, 2.10 and 2.11 respectively. In each case the starting material was 12.5g of human myometrium.

The bulk of RNA was recovered between nuclear Fractions A and B, with a much lesser amount being recovered in Fraction C.

0.1M H_2SO_4 Nuclear Extract

The typical composition of the extract after human myometrial nuclei had been exposed to 0.1M H_2SO_4 as described in Section 2.2.2.3 is shown in Table 1 . The extract contains no DNA and only low levels of RNA.

3.1.4 Electrophoretic Analysis.

Fractions A, B and C.

Due to the "sticky" nature of Fraction A, this nuclear fraction was not subjected to electrophoretic analysis. Further, the high DNA content of this fraction would have interfered with the proper running of an electrophoretic gel.

Figure 6 shows nuclear Fractions B and C subjected to electrophoresis as described by Laemmli (1970) on a 12.5% acrylamide gel.

The predominant features of Fraction C are the bands corresponding to the core histone proteins. These bands are absent in Fraction B. Fraction C also contains numerous other higher molecular weight bands.

Fraction B contains many bands, the majority of which correspond to molecular weights greater than those of the core histone proteins.

Figure 7 shows the 0.1M H_2SO_4 nuclear extract electrophoresed as described by Panyim and Chalkley (1969). As with Fraction C, this nuclear fraction contains the core histone proteins and numerous other higher molecular weight proteins.

Figure 8 shows the 0.1M H_2SO_4 nuclear extract electrophoresed as described by Laemmli (1970) on a 12.5% acrylamide gel. The major bands correspond to the core histone proteins, with a cluster of bands present at a molecular weight of approximately 28,000 - 32,000.

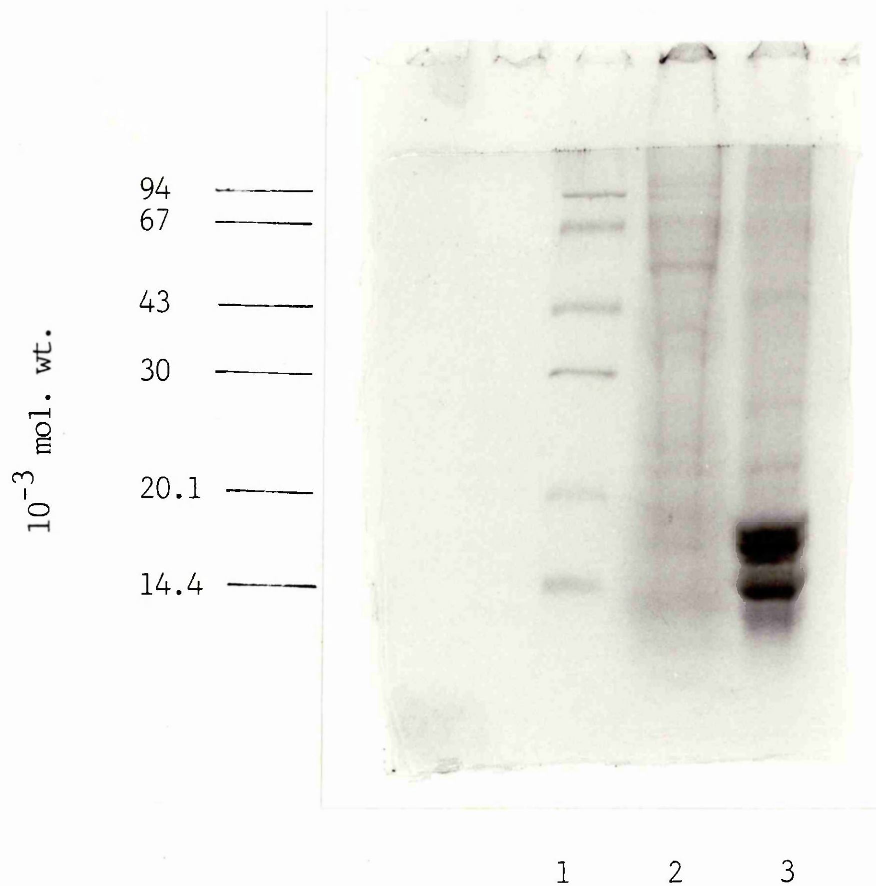


Figure 6 - SDS polyacrylamide gel electrophoresis of nuclear Fraction B (20µg, track 2) and nuclear Fraction C (20µg, track 3) on a 12.5% acrylamide gel as described by Laemmli (1970). After electrophoresis the gel was processed as described in Section 2.2.2.4.1. Track 1 standard molecular weight proteins. Phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soyabean trypsin inhibitor (20,100) and α-lactalbumin (14,400).

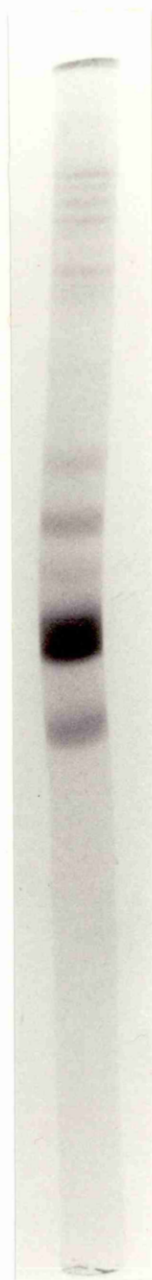


Figure 7 - 0.1M H_2SO_4 nuclear extract (15μg) electrophoresed as described by Panyim and Chalkley (1969) (See Section 2.2.2.4.2). After electrophoresis the gel was processed as described in Section 2.2.2.4.2.

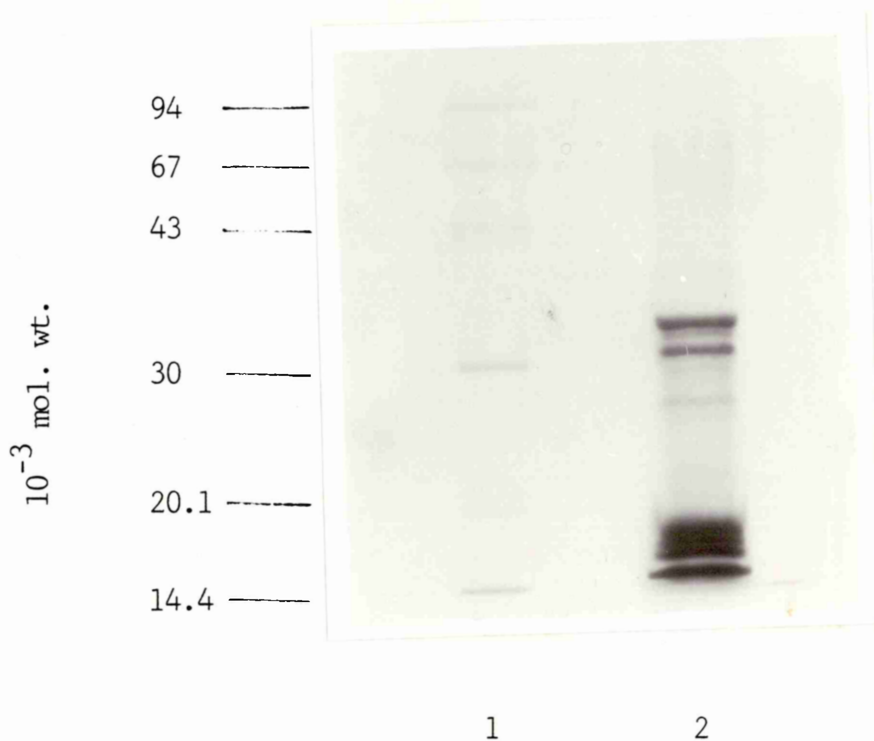


Figure 8 - SDS polyacrylamide gel electrophoresis of the 0.1M H_2SO_4 nuclear extract (20μg) (track 2) on a 12.5% acrylamide gel as described by Laemmli (1970) After electrophoresis the gel was processed as described in Section 2.2.2.4.1.

Track 1 standard molecular weight proteins as described in Figure 6.

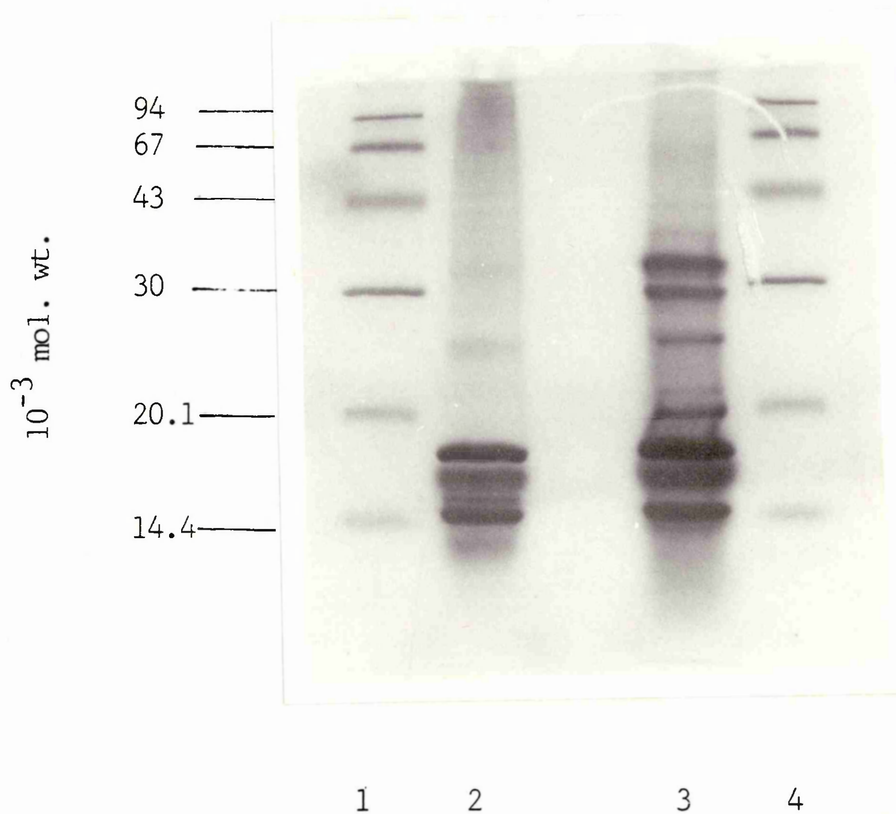


Figure 9 - SDS polyacrylamide gel electrophoresis of nuclear Fraction C (20μg)(track 2) and the 0.1M H_2SO_4 nuclear extract (20μg) (track 3) on a 15% acrylamide gel as described by Laemmli (1970). After electrophoresis the gel was processed as described in Section 2.2.2.4.1.

Tracks 1 and 4 standard molecular weight proteins as described in Figure 6.

Figure 9 shows the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C electrophoresed as described by Laemmli (1970) on a 15% acrylamide gel. As can be seen, both fractions contain the same pattern of core histone bands. Both fractions also contain a common band at a molecular weight of approximately 28,000 with numerous other high molecular weight bands being present.

3.1.5 Discussion.

Many published methods for the purification of mammalian nuclei work well with certain types of tissue but not with others. The chief difficulty in purifying nuclei lies in the initial disruption of the tissue, and the conditions under which it is carried out. A balance must be achieved between disrupting sufficient tissue to release the maximum number of nuclei possible without further damaging the already released nuclei any more than is necessary. In this study initial homogenisation was achieved using a fairly rough technique (Ultra Turrax homogeniser). The remaining fibrous debris was then separated from the "homogenate" using cheesecloth and the resultant solution rehomogenised using a teflon/glass homogeniser to disrupt any remaining intact cells. The inclusion of 0.2% triton X-100 in the homogenisation medium proved successful in eliminating problems caused by erythrocyte contamination. The use of these two methods of homogenisation minimised the damage caused to the released nuclei. However, as already mentioned in Section 3.1.1, the yield of nuclei was never very high. One problem often observed in the purification of nuclei is that of clumping, but the inclusion of 1.5mM $MgCl_2$ in the homogenising medium seems to minimise this problem as well as helping to preserve the integrity of the nuclei.

The inclusion of triton X-100 in the homogenisation medium will destroy cell membranes and also strip off the outer nuclear membranes. The use of such detergents will also disrupt intracellular membranes releasing

the enzyme components of the lysosomes, hence some damage to nuclear components must be anticipated under these circumstances (Hyodo and Ono, 1970).

No perfect method is available for the isolation of nuclei and, as such, each method available must be considered something of a compromise. The suitability of some methods to certain tissues must also be borne in mind as must the initial reason for isolating the nuclei. If metabolic studies are to be performed, the nuclei must be isolated in such a way that metabolic activity is preserved i.e. use of isotonic, sucrose containing media. However, if metabolic activity is not under investigation but purity and ease of preparation are important, procedures such as that of Higashi *et al*. (1966) which involve the use of citric acid may still be relevant. Dense sucrose methods have been used to isolate nuclei for metabolic studies, but they are perhaps best suited to the study of nucleic acids or other chemical constituents of the nucleus.

Assessing the purity of a nuclear purification is fraught with difficulty. Several tests of composition, and determination of enzymic activity, have been used as means of assessing the purity of nuclear components. All systems have built within them the hazards inherent in cell fractionation and differential centrifugation. Enzyme relocation, activation or destruction may modify the results obtained. Ascertaining yields on the basis of nuclear DNA has the problem of heterogeneity of the cell population in the original material as well as potential losses of DNA through lysis or experimental manipulation. Use of enzymic assays particularly those designated to detect components of the endoplasmic reticulum must take into consideration the outer nuclear envelope as a component of the endoplasmic reticulum. Probably the most successful means of assessing the relative purity of a nuclear preparation with respect to cytoplasmic constituent contaminants, is electron

microscopy. However using this technique alone is not enough, and the best idea of purity and integrity is achieved through the use of microscopic techniques in conjunction with some biochemical parameter.

The composition of the various nuclear fractions after the nuclei had been exposed to 2M NaCl indicates that under the conditions of extraction not all of the protein is dissociated from chromatin (see Table 1). A substantial amount remains associated with the material which is not solubilised under these conditions. This is not surprising as more severe extraction conditions have been reported to be required for complete dissociation of proteins from chromatin (e.g. Levy et al., 1972; Van den Broek, 1973). Under these conditions 90-95% of the proteins present in chromatin was released. It has also been reported that 2M NaCl in conjunction with 5M urea fully dissociates histone protein but only 15% of the non-histone protein from rat liver chromatin (Wilhelm et al., 1972). Similar treatment of chromatin from rat livers bearing chemically induced tumours with 2.5M NaCl in conjunction with 5M urea releases only 20% of the non-histone proteins as well as the histone protein fraction (Chiu et al., 1975). The use of 0.1M H_2SO_4 was more efficient in releasing proteins from chromatin, as suggested by the consistently higher protein content of the 0.1M H_2SO_4 nuclear extract when compared to nuclear Fractions B and C.

Electrophoretic analysis of both nuclear Fraction C and the 0.1M H_2SO_4 nuclear extract (see Figure 9) shows that their major constituents are the histone proteins,with numerous minor bands corresponding to non-histone proteins. This is what would have been expected from previous studies(e.g. Levy et al., 1972). The loading of samples onto gels presented problems since the nuclear histone proteins represent nearly 60% of total chromosomal proteins (Peterson and McConkey, 1976). Hence the majority of any sample loaded for electrophoresis

corresponds to histone protein, with the remainder of the sample corresponding to many non-histone proteins which are only present in small amounts. The use of 2-dimensional gel electrophoresis as described by O'Farrell (1977) would have given better resolution of the components of these nuclear extracts but for the purposes of the present work analysis using SDS PAGE was considered sufficient.

3.2 Assay of the Various Nuclear Fractions Abilities to Bind Activated $^{3}H\text{-}E_2$ labelled Oestrogen Receptor.

3.2.1 Labelling of various cytosols.

Various tissues were used as a source of oestrogen receptor for these studies.

Human Breast Tumour (oestrogen receptor positive), Human myometrial and immature rat uterine cytosols were prepared as described in Sections 2.2.3.5, 2.2.3.4 and 2.2.3.2 respectively. Labelling and DCC "stripping" were carried out as described in Sections 2.2.3.2 and 2.2.3.3 respectively.

The labelling pattern obtained with these different cytosols is presented in Table 2.

In these labelling experiments, the cytosol which shows the greatest difference in CPM between the "H" and "H+C" incubations i.e. competition, was considered to contain the largest number of specific binding sites i.e. oestrogen receptor molecules. All cytosols were of similar protein content. As can be seen in Table 2 the best source of oestrogen receptor is immature rat uterus.

This may reflect the fact that this tissue is probably the easiest to prepare a "cytosol" from, the other two being rather fibrous. Consequently the techniques required to disrupt them are more severe and probably result in damage to the native receptor.

3.2.2 The elution of $^{3}H\text{-}E_2$ labelled immature rat uterine cytosol through the various nuclear extract sepharose derivatives.

Immature rat uterine cytosol (1-2mg protein/ml) was prepared, labelled and DCC stripped as described in Sections 2.2.3.2 and 2.2.3.3 respectively. It was then eluted through the various nuclear extract/ sepharose columns as described in Section 2.2.4. The composition of each affinity resin is indicated in Table 3 .

TABLE 2

Source of Cytosol	10µl "H"	10µl "H+C"
Immature Rat Uterus 1-2mg/ml	1600 ± 200	150 ± 50
Human Breast Tumour 1-3mg/ml	550 ± 50	100 ± 30
Human Myometrium 1.8 - 2.4mg/ml	300 ± 50	120 ± 40

Means of 3 Exps. ± SD

Table 2 - The labelling pattern obtained when different tissue cytosols were labelled with $5x10^{-9}M$ $^{3}H-E_2$ both alone and in the presence of a 200 fold excess of cold DES. Labelling and stripping were carried out as described in Section 2.2.3.2 and 2.2.3.3.

The cytosols were prepared as described in Sections 2.2.3.2, 2.2.3.4 and 2.2.3.5.

Values quoted are cpm. Breast tumour samples were pre-selected as those being oestrogen receptor positive. Aliquots of cytosol were counted as described in Section 2.2.3.3.

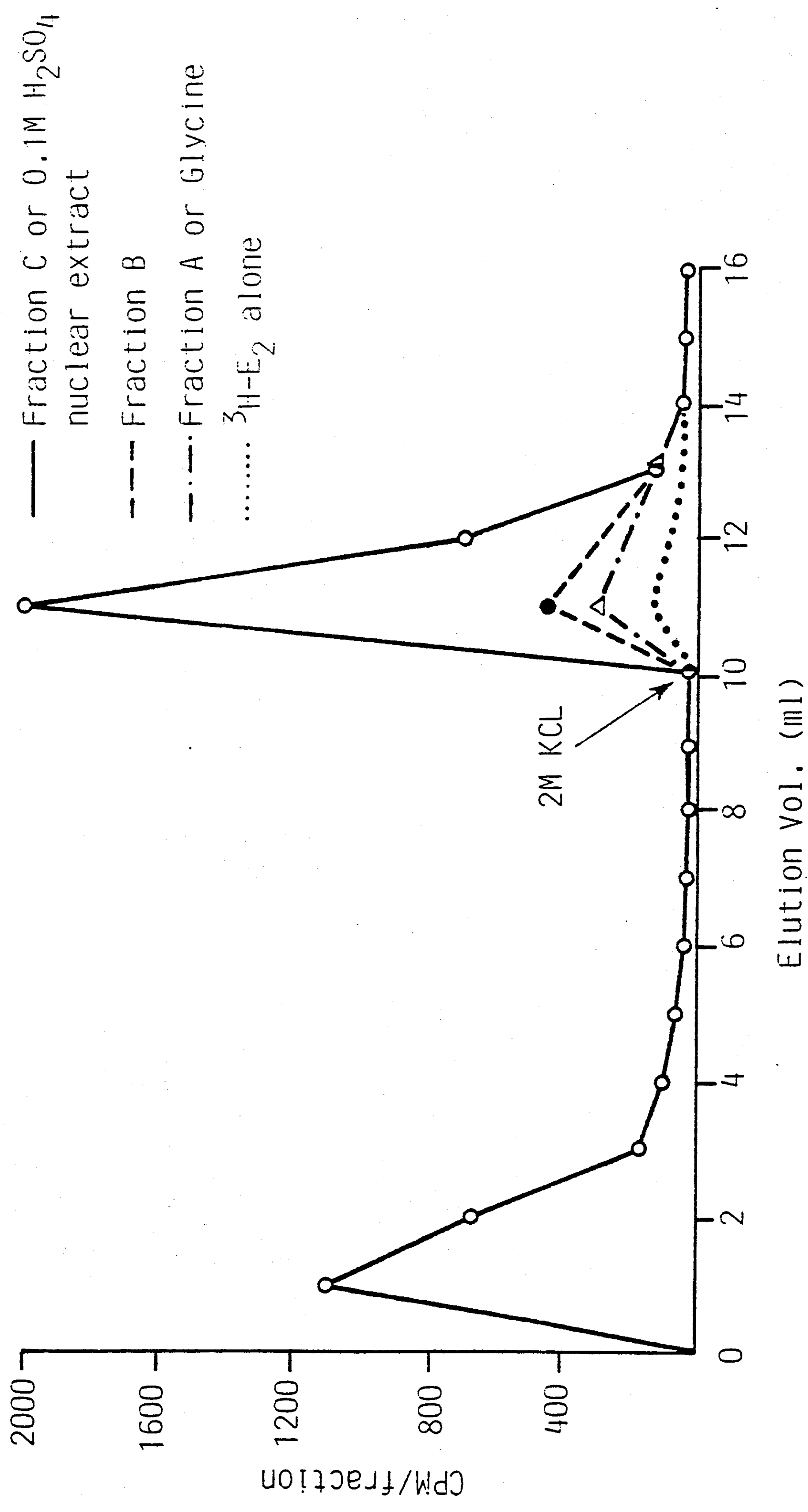
The elution profiles obtained from this experiment are presented in Figure 10.

As can be seen from Figure 10, in all cases a peak of radioactivity passes through the column and is not retained. However when HE/2M KCl is applied to the columns differences emerge. The largest peak of radioactivity (labelled oestrogen receptor) is released from either the 2M NaCl soluble nuclear material (nuclear Fraction C) or the 0.1M H_2SO_4 nuclear extract columns. Lesser amounts of radioactivity (labelled oestrogen receptor) are released from the other nuclear fraction columns (Fraction A and B). A smaller peak of radioactivity is released from the control column which only has the α-amino acid glycine coupled to the sepharose.

These results suggest that nuclear Fraction C and the 0.1M H_2SO_4 nuclear extracts possess an increased ability to bind activated ^3H-E_2 labelled oestrogen receptor.

A more important observation however, is that when an equivalent amount of free ^3H-E_2 in HED buffer is eluted through the various nuclear extract/sepharose columns, no large peak of radioactivity was released on the application of 2M KCl. This observation answers several questions. The first of these is that in order to observe binding of ^3H-E_2 to the nuclear extract sepharose columns, the ligand must be bound by the oestrogen receptor molecule. This was further confirmed by labelling oestrogen receptor negative human breast tumour cytosols with 5 x10^{-9} ^3H-E_2 and eluting them through the various nuclear extracts. When 2M KCl was applied, no large peak of radioactivity was released. This suggests the presence of the oestrogen receptor is necessary to observe high levels of binding and also indicates that the binding cannot be induced by other cellular proteins which bind ^3H-E_2. The second question this observation answers is that the binding is not due to either the

Figure 10 - The Elution Profiles obtained when ^{3}H-E_2 labelled Immature Rat Uterine Cytosol (1-2mg protein/ml) was run on the various nuclear extract/sepharose columns as described in Section 2.2.4. 7,000 cpm were applied to each column. The composition of each column is given in Table 3. 7,000 cpm of ^{3}H-oestradiol in HED buffer were applied to all columns in a separate experiment.



Fraction C or 0.1M H_2SO_4
nuclear extract
Fraction B
Fraction A or Glycine
$^3H-E_2$ alone
2M KCL
CPM/fraction
2000
1600
1200
800
400
0
2
4
6
8
10
12
14
16
Elution Vol. (ml)

binding of free ligand in the cytosol applied to the column or binding of ligand which has become dissociated from receptor during equilibration of cytosol with the column. Further, free ligand is not being retained through binding to oestrogen receptor spuriously incorporated into the protein initially immobilised onto the sepharose 4B resin. An observation which also supports these ideas is that eluting cold DES in HED buffer through the columns prior to the application of labelled cytosol did not reduce the observed binding of $^{3}H-E_2$ labelled activated oestrogen receptor to any extent.

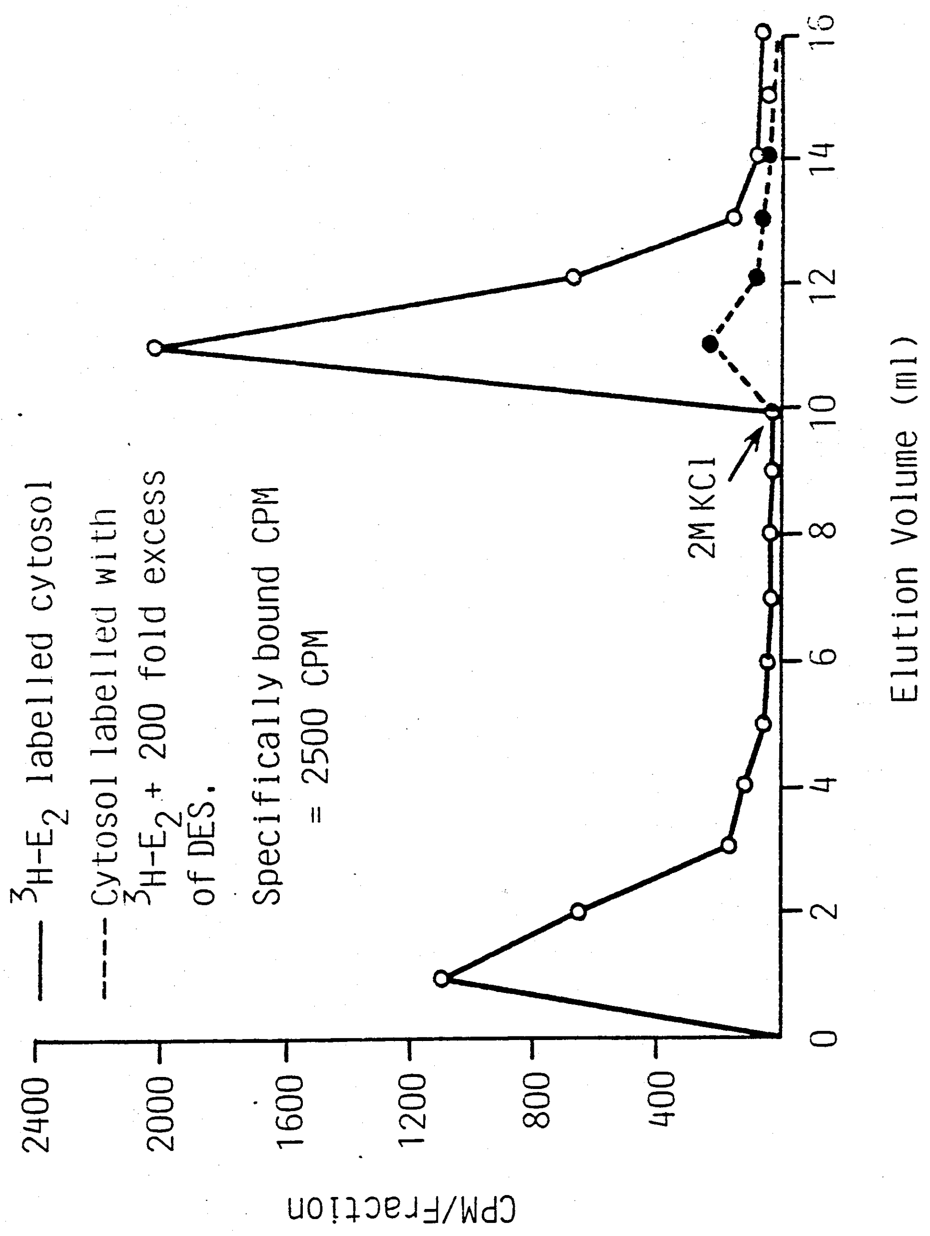
From this experiment is appears that the bulk of oestrogen receptor binding activity is extracted into both the 2M NaCl soluble nuclear material (Fraction C) and the 0.1M H_2SO_4 nuclear extract. Consequently the majority of experiments concentrated on these two nuclear fractions.

The next question to be answered concerns the specificity of the binding activity which is being observed, i.e. how much of the binding activity is due to oestrogen binding proteins which are, nevertheless, different from normal receptor - defined in terms of affinity and binding capacity. To answer this question immature rat uterine cytosol labelled with $^{3}H-E_2$ either alone or in the presence of a 200 fold excess of cold DES was applied to the nuclear extract/sepharose columns.

The elution profile obtained when the above cytosols were applied to a nuclear Fraction C/sepharose column is presented in Figure 11.

As can be seen the majority of binding observed in the presence of $^{3}H-E_2$ alone is due to the binding of activated $^{3}H-E_2$ labelled oestrogen receptor. In the presence of excess cold DES occupied oestrogen receptor, the levels of $^{3}H-E_2$ binding to the column are much lower. This level of competition strongly suggests that the binding activity observed is specific and represents the binding of activated $^{3}H-E_2$ labelled oestrogen receptor to defined sites present on the nuclear Fraction C/sepharose column.

Figure 11 - The Elution Profile obtained when ^{3}H-E_2 labelled Immature Rat Uterine Cytosol (1-2mg protein/ml) was applied to a Nuclear Fraction C/sepharose column as described in Section 2.2.4. 7,000 cpm of both the H and H+C cytosols were applied. The difference in observed binding between the two cytosols represents the specific binding of labelled, activated oestrogen receptor. The column contained 1,000μg of immobilised protein.



^{3}H-E_2 labelled cytosol
Cytosol labelled with ^{3}H-E_2 + 200 fold excess of DES.
Specifically bound CPM = 2500 CPM
2M KCl
CPM/Fraction
2400
2000
1600
1200
800
400
0
2
4
6
8
10
12
14
16
Elution Volume (ml)

TABLE 3

Nuclear Fraction Coupled to Sepharose 4B	f mol (^3H-E_2 labelled receptor) specifically bound/mg of immobilised protein	
Fraction A (2M NaCl insoluble material) (500μg protein coupled)	15 $\pm$ 1.5	(3)
Fraction B (material soluble at low Ionic strength) (600μg protein coupled)	24 $\pm$ 4.0	(3)
Fraction C (2M NaCl soluble material) (1000μg protein coupled)	40 $\pm$ 2.5	(3)
0.1M H_2SO_4 extract (900μg protein coupled)	43 $\pm$ 1.5	(3)
Control Column (Glycine coupled)	4 $\pm$ 1	(3)
	Mean $\pm$ SD	No. Expts.

Table 3 - The abilities of the various nuclear fraction/sepharose resins to specifically bind activated ^3H-E_2 labelled receptor complexes. Specific binding was assessed as described in Section 2.2.4.1. 7,000 cpm of both the H and H+C cytosols were applied in each case.

Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. Columns were run as described in Section 2.2.4.

The same experiment was repeated with the other nuclear extract/sepharose resins and the results are presented in Table 3.

The results in Table 3 confirm earlier observations that nuclear Fraction C and the 0.1M H_2SO_4 extract possess the greatest ability to specifically bind activated $^3H\text{-}E_2$ labelled oestrogen receptor.

3.2.2.1 Elution of various oestrogen receptor containing tissue cytosols through a nuclear Fraction C sepharose column.

Cytosols were prepared as described in Section 3.2.1. These were eluted through a nuclear extract Fraction C/sepharose column as described in Section 2.2.4. The results from these experiments are presented in Table 4.

As can be seen there is variation in the amount (units) of activated $^3H\text{-}E_2$ labelled oestrogen receptor from these different sources bound to the nuclear Fraction C/sepharose resin. The concentration of specific receptor applied to the column from each source does not reflect the amount retained by the column. The binding induced by activated $^3H\text{-}E_2$ labelled human myometrial oestrogen receptor is much lower than the receptor from human breast tumour cytosol and immature rat uterine cytosol, which can induce similar levels of specific binding to the nuclear Fraction C/sepharose resin. Again, this may be a reflection on the homogenisation techniques which are required to disrupt the myometrial tissue, possibly causing some damage to the acceptor binding domain of the receptor.

The levels of binding which are induced by oestrogen receptor from immature rat uterus and oestrogen receptor positive human breast tumour are comparable. However since not all breast tumour cytosols were capable of inducing specific binding, and immature rat uterus represents a more "convenient" source of oestrogen receptor it was used for all subsequent studies.

TABLE 4

Source of Oestrogen Receptor	f mol Specifically bound ($^3H-E_2$ labelled oestrogen receptor)/mg immobilised protein
Human breast tumour cytosol (1-3mg/ml)	36 $\pm$ 3.0
Human myometrial cytosol (1.8-2.4mg/ml)	15 $\pm$ 1.5
Immature rat uterine cytosol (1-2mg/ml)	40 $\pm$ 2.5

Means of 3 exps. $\pm$ SD

Table 4 - The ability of activated oestrogen receptor from various sources to induce binding to the nuclear Fraction C/sepharose resin. The cytosols were prepared as described in Sections 2.2.3.2, 2.2.3.4, 2.2.3.5 and eluted through the column as described in Section 2.2.4. Specific binding of labelled oestrogen receptor complex was calculated as described in Section 2.2.4.1.The nuclear extract sepharose column contained 1000μg of immobilised protein.

7,000 cpm of both the H and H+C cytosols were applied in each case.

3.2.2.2 Effect of assay conditions on binding activity.

The results from these studies are presented in Tables 5 and 6.

From these tables the binding activity appears to be reasonably stable at a salt concentration between 0.12M and 0.15M KCl but increasing the KCl concentration to 0.2M results in a sharp drop of 50-60% in the observed binding activity. This drop is slightly more pronounced with the 0.1M H_2SO_4 nuclear extract/sepharose resin. Increasing the KCl concentration further to 0.5M, results in a loss of approximately 90% of observed binding activity with both the nuclear Fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins. Consequently all future binding assays were carried out in the KCl range of 0.12M - 0.15M, usually at a KCl concentration of 0.12M, which is approaching physiological ionic strength _in vivo_.

Figure 12 shows the effect of the KCl concentration on the bound/free (B/F) values for the observed interaction of $^3H-E_2$ labelled activated oestrogen receptor with the nuclear extract sepharose resins. As can be seen, the B/F values are very sensitive to the KCl concentration in the range of 0.12M - 0.2M, a finding suggested by the data in Tables 5 and 6. These data suggest that the interaction of the oestrogen receptor complex with the nuclear extract/sepharose resin is very sensitive to the KCl concentration within this range.

As shown in Tables 5 and 6, the presence or absence of either EDTA or DTT in the binding assay buffer had no effect on the observed binding activity hence the buffer of choice for future binding assays was HE/0.12M KCl.

3.2.2.3 The effect of sodium molybdate on the observed binding activity.

Immature rat uterine cytosol was prepared and labelled $\pm$ 20mM sodium molybdate as described in Sections 2.2.3.2 and 2.2.3.6. "Stripping" was carried out as described in Section 2.2.3.3. The cytosols were then

TABLE 5

Eluting Buffers Used to run Column	f mol Specifically bound (^{3}H-E$_2$ labelled oestrogen receptor)/ mg immobilised protein
HE/0.12M KCl	210 ± 10
HED/0.12M KCl	200 ± 10
HE/0.15M KCl	200 ± 7
HE/0.2M KCl	107 ± 6
HE/0.5M KCl	12 ± 4
20mM HEPES pH7.4/0.12M KCl	195 ± 6
HE/0.12M KCl	210 ± 10

Means of 3 Exps. ± SD

Table 5 - The influence of assay conditions on the levels of specific binding of activated oestrogen receptor to nuclear Fraction C/sepharose resin. Experiments were carried out using columns containing 500mg of sepharose resin with 500µg of immobilised protein. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 10,000 cpm of both the H and H+C cytosols were applied in each case. Specific binding was calculated as described in Section 2.2.4.1.

TABLE 6

Eluting Buffer used to run Column	$\frac{\text{f mol Specifically bound } (^3H\text{-}E_2 \text{ labelled oestrogen receptor})}{\text{mg immobilised protein}}$
HE/0.12M KCl	65 ± 6
HED/0.12M KCl	64 ± 5
HE/0.15M KCl	70 ± 5
HE/0.2M KCl	25 ± 1.5
HE/0.5M KCl	5 ± 1.5
20mM HEPES pH7.4 0.12M KCl	61 ± 3
HE/0.12M KCl	64 ± 1.5

Means of 3 Exps. ± SD

Table 6 - The influence of assay conditions on the levels of specific binding of activated oestrogen receptor to the 0.1M H_2SO_4 nuclear extract/sepharose resin. Experiments were carried out using columns containing 500mg of sepharose resin with 900μg of immobilised protein. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 10,000 cpm of both the H and H+C cytosols were applied in each case specific binding was calculated as described in Section 2.2.4.1.

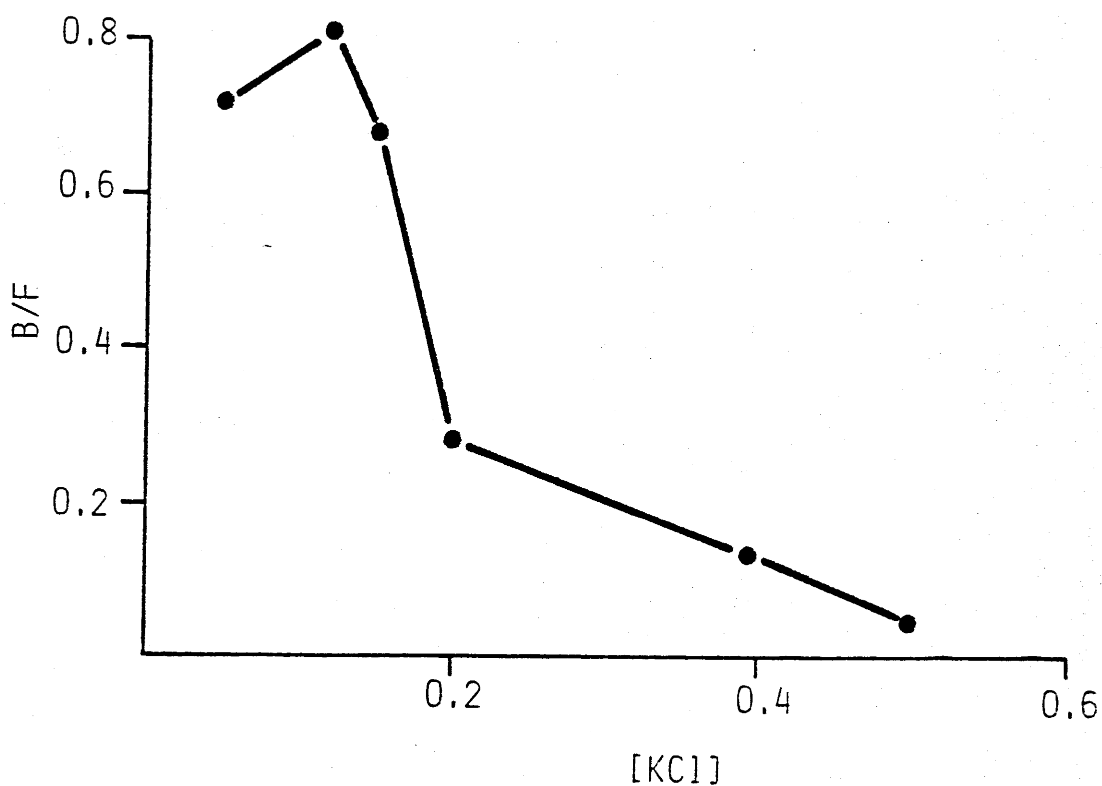


Figure 12 - The effect of KCl concentration on the B/F ratio measured from the specific binding of $^{3}H-E_2$ labelled activated, oestrogen receptor to the nuclear Fraction C/sepharose resin. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 10,000 cpm of cytosol (H and H+C) were used for each point. Columns of nuclear Fraction C/sepharose resin (300ug immobilised protein/500mg resin) were used.

eluted through parallel nuclear extract/sepharose resin columns as described in Section 2.2.4.

The results from these studies are presented in Table 7.

As can be seen, the inclusion of 20mM sodium molybdate in the homogenisation buffer has no effect on the subsequent binding of $^3H-E_2$ labelled oestrogen receptor to both nuclear Fraction C and the 0.1M H_2SO_4 nuclear extract sepharose resins made from human myometrium.

Previous reports have shown that molybdate inhibits the activation of oestrogen receptor (e.g. Muller et al., 1983a),as assessed by its ability to bind to DNA or nuclei. As such the data in Table 7 is somewhat difficult to reconcile.

However, earlier experiments studying the binding of immature rat uterine oestrogen receptor, prepared in the presence of 20mM sodium molybdate, to the 0.1M H_2SO_4 nuclear extract/sepharose resin prepared from immature rat uterine nuclei showed that, in this case, the presence of 20mM sodium molybdate in the homogenisation buffer resulted in a decrease of approximately 50% in the observed binding activity. However, possible species differences after exposure of oestrogen receptor to molybdate seem unlikely.

3.2.2.4 Further studies of the binding of receptor to nuclear Fractions B and C.

The initial studies on the binding of activated $^3H-E_2$ labelled oestrogen receptor were carried out by coupling the entire nuclear fraction to sepharose, with the result that the various nuclear extract resins did not have equal amounts of protein immobilised on them.

For these studies, the separation of nuclear Fractions B and C was carried out as carefully as possible during the preparation. Finally equal amounts of both fractions (0.3mg) were coupled to sepharose 4B and multiple parallel affinity columns were set up and run as described in Section 2.2.4.

TABLE 7

Buffer used for Receptor Preparation	f mol Specifically bound ($^3H-E_2$ labelled oestrogen receptor)/mg immobilised protein
Nuclear Fraction C	
HED/0.12M KCl	212 ± 9
HED/0.12M KCl/20mM Sodium molybdate	232 ± 8
0.1M H_2SO_4 Nuclear Extract	
HED/0.12M KCl	63 ± 6
HED/0.12M KCl/20mM sodium molybdate	66 ± 4

Means of 3 exps. ± SD

Table 7 - The effect of preparing oestrogen receptor in the presence of 20mM Na MoO_4 on the specific binding to both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose columns. Immature rat uterine cytosol (1-2mg protein/ml) was prepared as described in Section 2.2.3.6. 10,000 cpm of both the H and H+C cytosols were applied in each case. 500mg columns of sepharose resin were used containing 300µg immobilised protein (Fraction C) and 1100µg immobilised protein (0.1M H_2SO_4 nuclear extract). Specific binding was calculated as described in Section 2.2.4.1.

The results from these studies are presented in Table 8.

From these results it can be seen that nuclear Fraction C is greatly enriched in binding activity compared to Fraction B. The more similar levels of binding observed in the two nuclear fractions previously may have been caused by contamination of nuclear Fraction B by small amounts of Fraction C.

3.2.2.5 Storage of the resins.

When stored in HE buffer containing 0.2% (w/v) NaN_3 at 4^oC, both nuclear Fraction C and the 0.1M H_2SO_4 nuclear extract/sepharose resins lost less than 10% of their binding activity over an 8-10 week period. Nuclear extract/sepharose resins were not normally stored for longer periods of time than this.

3.2.3 Discussion.

The results presented in this section are in broad agreement with those of Mainwaring et al. (1976) and Puca et al. (1974) who report a binding activity for androgen and oestrogen receptor complexes respectively which can be extracted from target cell nuclei using buffers containing 2M NaCl. Subsequently Puca et al. (1975) have reported that the binding activity can also be extracted from target cell nuclei using H_2SO_4. The data presented regarding the 0.1M H_2SO_4 extract of human myometrial nuclei are in agreement with this.

One possible criticism of this work is the conditions which are required to solubilise the binding activity from target cell nuclei. The integrity of the structure of proteins, in particular, after they have been exposed to 2M NaCl or 0.1M H_2SO_4 must be uncertain. Similar criticisms have been expressed of work carried out by Spelsberg, where high concentrations of denaturing agents such as guanidine hydrochloride have been used to either uncover or extract nuclear binding sites which can bind the progesterone receptor complex from chick

TABLE 8

Nuclear Fraction	cpm Specifically Bound (^{3}H-E_2 labelled oestrogen Receptor	f mol. Bound/mg Protein Immobilised
B	375 ± 120	18.5 ± 6
C	4800 ± 400	238 ± 20

Means of 5 Exps. ± SD

Table 8 - The data obtained when 500mg columns of sepharose resin containing 300μg of immobilised protein (both nuclear Fractions B and C) were assayed for the ability to specifically bind activated labelled oestrogen receptor. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 10,000 cpm of both H and H+C cytosols were applied in each case. Specific binding was calculated as described in Section 2.2.4.1.

oviduct. However, the best criterion by which to judge the integrity or nativeness of a biological structure is to assess whether or not the extracted biological molecule can still perform its intended in vivo function in vitro. In the case of the above studies by Puca et al. (1974, 1975), and Mainwaring et al. (1976) this is clearly demonstrated by the specific high affinity saturable binding of steroid receptor complexes to nuclear proteins which have been immobilised on sepharose in vitro. The same can also be said for the two nuclear fractions (i.e. nuclear Fraction C and the 0.1M H_2SO_4 nuclear extract) isolated from purified human myometrial nuclei with enhanced ability to specifically bind activated oestrogen receptor.

From this set of experiments it can be seen that all three nuclear fractions i.e. A, B and C, are able to bind activated labelled oestrogen receptor (see Table 3). The greatest binding activity was found in nuclear Fraction C. Subsequent experiments revealed that nuclear Fraction B possessed a much lower binding activity than nuclear Fraction C than appeared initially (see Table 8). The nature of the nuclear material remaining after 2M NaCl extraction made it impossible from these studies to be certain that the total binding activity of that fraction was being detected i.e. the bulk of Fraction A will not be coupled to the sepharose resin because of its insoluble nature. This was a limitation of the assay techniques used. The residual protein content of Fraction A (see Section 3.1.3) suggests more binding sites than those detected may be present in this fraction.

These results are in conflict with those of Ota et al. (1984), who report that the majority of binding sites for androgen receptor/ testosterone complexes reside in the material remaining after rat liver chromatin had been extracted with 2M NaCl which is equivalent to Fraction A. They also report binding sites for the androgen receptor complex in

the material solubilised from chromatin with 0.35M NaCl and 2M NaCl. The detection of binding sites associated with the nuclear material insoluble in 2M NaCl lends support to the involvement of the nuclear matrix in the binding of steroid receptor complexes as has been previously suggested (e.g. Barrack and Coffey, 1982).

Varying the conditions under which the binding activity was assayed revealed that the optimum KCl concentration for the interaction of labelled, activated oestrogen receptor with both the nuclear Fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins is in the range of 0.12 - 0.15M. Above this KCl concentration the observed binding activity decreases sharply (see Figure 12 and Tables 5 and 6). It is appropriate that maximal binding activity can be observed at an ionic strength which is approaching the physiological value. At high ionic strength (0.5M KCl) only low levels of binding activity could be observed and are assumed to represent non-specific background binding. It seems unlikely that specific interaction between steroid receptor proteins and nuclear components could take place under such conditions. 0.5M KCl also corresponds to a salt concentration which dissociates a large proportion of bound oestrogen receptor from intact nuclei (Clark and Peck, 1979).

However it would appear that the optimum ionic strength for the interaction of steroid receptor complexes with nuclear components _in vitro_ is not constant and varies depending on the system which is being studied. Mainwaring _et al_. (1976) omit KCl from their assay buffers but show that the inclusion of 0.5M KCl enables the specific high affinity, low capacity sites to be distinguished from non-specific, unsaturable binding. Puca _et al_. (1974, 1975) include KCl in their assay buffers to a final concentration of approximately 0.1M and as reported by Mainwaring _et al_. (1976) use 0.4M KCl to distinguish between specific high affinity binding and non-specific background binding. Kon and

Spelsberg (1982) report that the optimum specific binding of the progesterone receptor to hen oviduct nuclei is observed if 0.18M KCl is included in the binding assay buffer. Ruh and Spelsberg (1983) report that maximal binding of hen oviduct oestrogen receptor to purified hen oviduct chromatin occurs in buffers containing 0.1M KCl. When studying the binding of calf uterine oestrogen receptor to various target cell nuclei de Boer et al. (1984) include 0.15M KCl in the assay buffers. From these studies it can be seen that no set of standard, defined assay conditions are available to study the binding of steroid receptor complexes to nuclear components in vitro. It is, however, important that the assay conditions in vitro should at least resemble the conditions in which the interactions occur in vivo.

Puca et al. (1975) report an optimal DTT concentration of 1-2mM for the interaction of partially purified 4.5S oestrogen receptor with the mild acid extract/sepharose resin from calf uterine nuclei. In the present study it was observed that the presence (0.25mM) or absence of DTT in the eluting buffer had no affect on the observed interaction between labelled activated oestrogen receptor and both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins. These observations suggest that sulfhydral groups are not important for the interaction between receptor molecules and nuclear components immobilised on the sepharose resins. However, previous reports have shown that sulfhydral groups on the oestrogen receptor are important in the interaction with oestradiol (Jensen et al, 1967).

The presence or absence of EDTA in buffers used to both prepare the immature rat uterine cytosol and elute it through both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins seemed to have little effect on the observed binding activity (Tables 5 and 6).

Interestingly EDTA has been reported to inhibit the conversion of oestrogen receptor from the 4S $\rightarrow$ 5.3S form when mouse Leydig tumour

cytosol was heat activated (Sato *et al*. 1978a). It might be expected that inhibition of the activation would result in a decrease in the observed binding activity, but no such decrease was observed in the presence of EDTA.

Sodium molybdate has also been shown to inhibit the process of oestrogen receptor activation as measured by DNA binding activity (Pettersson *et al*., 1982). As can be seen from Table 7, preparation of immature rat uterine cytosol in the presence of 20mM sodium molybdate does not result in a decrease in the observed binding activity to both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins. This might suggest that the binding site on the receptor molecule responsible for the binding to the nuclear fractions is distinct from the DNA binding site on the oestrogen receptor molecule. It has been reported that approximately 20% of the receptor population in immature rat uterine cytosol is insensitive to inhibition of activation by 20mM molybdate (Hyder, 1983). It is possible that this small population of receptor molecules is responsible for the observed binding, but as no decrease whatsoever in the binding is observed in the presence of molybdate this explanation seems unlikely. A similar class of oestrogen receptor capable of being activated in the presence of molybdate has been reported in some breast tumours (Thomas *et al*., 1983).

Interestingly, Littlefield and Spelsberg (1985) report that molybdate stabilised progesterone receptor still binds to oviduct chromatin at a level of 40% of that seen with activated, fully functional receptor. However the binding to chromatin of this inactive progesterone receptor is unsaturable.

The best assay system as defined by the maximum amount of observed binding activity comprised oestrogen receptor from immature rat uterus interacting with human nuclear components immobilised on

sepharose. Several lines of evidence suggest that there is similarity between the immature rat uterine oestrogen receptor and human oestrogen receptor. The first of these concerns the cross reaction of both polyclonal and monoclonal antibodies raised against calf uterine oestrogen receptor with a variety of oestrogen receptor preparations from various sources. These included human breast tumour and uterine oestrogen receptor and rat uterine oestrogen receptor (Greene et al., 1980; Greene and Jenson, 1982). The cross-reactivity of these antibody preparations suggests that the oestrogen receptor molecules with which they interact must share a number of common features. The second line of evidence is that the oestrogen receptor from human and rat tissues interacts with DNA cellulose resin in comparable ways, suggesting that at least the DNA binding domain of these different receptors must share some common features. From these observations it does not seem unreasonable to use immature rat uterine oestrogen receptor to probe the extracts of human myometrial nuclei for specific binding sites.

One interesting observation was that only 2 out of approximately 20 oestrogen receptor positive breast tumour cytosols were capable of inducing binding to the nuclear Fraction C sepharose resin. The two cytosols which induced binding had reported oestrogen receptor levels of 234f.mol/mg protein and 361f.mol/mg protein. There was no obvious reason why these samples should have induced such a high level of binding except perhaps for a slightly better labelling pattern in the initial H and H+C incubations. It is interesting to speculate that the other receptor positive tumour cytosols may have contained similar levels of oestrogen receptor but also some inhibitory factor which prevented the binding of receptor to the nuclear Fraction C sepharose resin (Di Sorbo et al., 1980). The presence of such a factor which inhibits the binding of oestrogen receptor to DNA has been reported in human breast tumour cytosol (Hyder and Leake, 1983).

Previously Puca et al. (1974) have reported that both crude and partially purified calf uterine oestrogen receptor could be used to study the acceptor activity extracted from the calf uterine nuclei by 2M NaCl. In this study crude cytosol was used as a source of oestrogen receptor, with no attempt being made to purify the oestrogen receptor in any way.

The use of a crude cytosol preparation may complicate the assay system if the cytosol contains factors which either promote or inhibit the binding of receptor to components of the various nuclear extracts (Cake et al., 1978). It would be interesting to carry out experiments using purer receptor preparations, and observing any effect on the observed binding activity. Littlefield and Spelsberg (1985) report that purification of progesterone receptor prior to nuclear binding assays destroys specific saturable binding of the receptor suggesting that purification may have removed a factor which was essential for the previously observed specific, saturable binding.

The use of crude cytosol as a source of oestrogen receptor may also introduce complications in that other molecules may become labelled with oestradiol. The two types of molecule most likely to become labelled with oestradiol are the type II binding sites (Clark and Peck, 1979) and sex hormone binding globulin (SHBG). Two approaches were used to minimise the binding of label to these two classes of molecule. The first of these was to label cytosol with a concentration of ligand at which only oestrogen receptor molecules (type I sites) would approach saturation. The second involved assessing non-specific binding using an excess of cold DES, a synthetic molecule which has a higher affinity for the oestrogen receptor than oestradiol. DES does not bind to SHBG, hence if competition can be measured in the presence of cold DES, it is fairly certain the binding of label alone represents binding to the

oestrogen receptor and not SHBG.

The observed binding activity appeared to be very dependent on the presence of activated, labelled oestrogen receptor complex (see Figure 10 and Table 3). When ^{3}H-oestradiol alone was applied to the nuclear extract sepharose columns, very little, if any, binding was observed. This is in agreement with previous reports of Puca et al. (1974), Mainwaring et al. (1976), and more recently Ota et al. (1984), that when studying the binding of steroid receptor complexes to nuclear components in vitro, the ligand alone is incapable of inducing significant levels of binding. Further support that the presence of the activated, labelled, oestrogen receptor complex was required for binding came from the observation that prior elution of cold DES through the nuclear extract/sepharose columns could not block the binding of activated, labelled oestrogen receptor. This rules out the possibility of exchange between ^{3}H-E_2 applied in the cytosol and oestrogen receptor, extracted initially from the myometrial nuclei, and covalently coupled to the sepharose resin.

Comparing the levels of binding activity assayed in nuclear Fraction C (Tables 3 and 8) reveals that later preparations were enriched with the ability to bind activated labelled oestrogen receptor when compared to earlier ones. This can only be attributed to practice and experience in handling and preparing the nuclear fractions and also in performing the binding assay itself.

Comparing the binding activity exhibited by the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins, it appears that nuclear Fraction C is enriched in the ability to bind activated labelled oestrogen receptor (see Tables 5 and 6). This may suggest that the use of 2M NaCl is more efficient at solubilising the nuclear components responsible for the binding activity. The other obvious possibility is that 0.1M H_2SO_4 is equally efficient at solubilising the appropriate nuclear components but under these acid conditions a proportion of the extracted components are denatured which results in a decrease in the

3.3 Effects of Digestive Enzymes on Binding Activity

Both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/ sepharose resins were digested with various enzymes as described in Section 2.3.3. The ability of the digested nuclear extract/sepharose resins to bind activated ^3H-E_2 labelled oestrogen receptor was then determined as described in Section 2.3.4.

The data are presented in Tables 9 and 10.

As can be seen, only the proteolytic enzyme trypsin is capable of destroying the ability of both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins to bind activated ^3H-E_2 labelled oestrogen receptor. Digestion of the nuclear extract/sepharose resins with either DNase or RNase has no effect on the binding activity. It has been reported that nucleic acids must be structurally modified before they can be covalently attached to sepharose to any great extent (Poonian et al.,1971). Hence, only low levels of nucleic acid should become attached to the sepharose under the coupling conditions. This suggests the binding activity present on both nuclear extract/sepharose resins is proteinaceous.

However the reduction in binding activity caused by trypsin digestion is not as great as might be expected. To explore this problem further the nuclear extract/sepharose resins were digested with a variety of proteolytic enzymes and their ability to bind activated ^3H-E_2 labelled oestrogen receptor was determined. The results from these studies are presented in Tables 11 and 12.

As can be seen in Tables 11 and 12 papain, pronase and trypsin all destroy approximately 50% of the binding sites for activated ^3H-E_2 labelled oestrogen receptor present on both of the nuclear extract/ sepharose resins. The proteolytic enzyme protease S.aureus has a far less damaging effect on the binding activity.

TABLE 9

Enzyme used to Digest the Nuclear Extract/Sepharose Resin	% of Binding Activity Present in Control Binding Assay
Control (no digestion)	100%
DNase	92 ± 5%
RNase	92 ± 8%
Trypsin	55 ± 15%

Mean of triplicates ± SD

Table 9 - The effect of various digestive enzymes on the ability of the 0.1M H_2SO_4 nuclear extract/sepharose resin (900μg immobilised protein/ 500mg resin) to bind activated labelled oestrogen receptor. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of receptor. 10,000 cpm were added to each experiment. Before incubation the nuclear extract/sepharose resin was digested as described in Section 2.3.3. 10mg of resin was used for each binding assay.

TABLE 10

Enzyme used to Digest the Nuclear Extract/Sepharose Resin	% of Binding Activity Present in Control Binding Assay
Control (no digestion)	100%
DNase	94 ± 4%
RNase	91 ± 6%
Trypsin	51 ± 13%

Mean of triplicates ± SD

Table 10 - The effect of various digestive enzymes on the ability of the nuclear Fraction C/sepharose resin (600µg immobilised protein/500mg resin) to bind activated labelled oestrogen receptor. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 10,000 cpm were added to each experiment. Before incubation the nuclear extract/sepharose resin was digested as described in Section 2.3.3. 10mg of resin was used for each binding assay.

When compared to the results presented in Tables 9 and 10 it can be seen that trypsin destroys a slightly larger percentage of the available binding sites present on both nuclear extract/sepharose resins (Tables 11 and 12). In these studies the digestion of the nuclear extract/ sepharose resins was carried out at pH8.0 compared to pH7.4 in the earlier studies. pH8.0 represents the optimum pH of this enzyme and the slight increase in the observed potency of trypsin in destroying binding sites may reflect the slight difference in the conditions under which the digestions were carried out.

Again, the reduction in binding activity caused by these proteolytic enzymes does not approach 100%, as might have been expected under the conditions present in the digestion incubation (see Section 2.3.3).

3.3.1 Discussion.

The data presented in this section is again in broad agreement with data presented by Puca _et al_. (1974) and Mainwaring _et al_. (1976), who have shown that the binding activity they observe for calf uterine oestrogen receptor and rat ventral prostate androgen receptor respectively, is sensitive to proteolytic enzymes, but is unchanged by DNase or RNase digestion, indicating that the nuclear components responsible for the binding activity are protein in nature. However, the decrease in binding activity caused by proteolytic enzymes is approximately 90%, a figure somewhat larger than the figures reported in this study. There are several possible explanations for this discrepancy. One problem may be that the proteolytic enzymes cannot gain full access to the proteins immobilised on the sepharose or it may be that the proteins are immobilised on the sepharose 4B in a conformation which does not allow attack by the proteolytic enzymes. The observation that papain, pronase and trypsin reduce the binding activity by

TABLE 11

Proteolytic Enzyme used to Digest the Nuclear Extract/Sepharose Resin	% of Binding Activity Present in Control Binding Assay
Control (no digestion)	100%
Papain	49 ± 3%
Trypsin	48 ± 4%
Pronase	53 ± 2%
Protease *S.aureus*	75 ± 5%

Mean of Quadruplicates ± SD

Table 11 - The effects of various proteolytic enzymes on the ability of the 0.1M H_2SO_4 nuclear extract/sepharose resin. (1,000µg immobilised protein/500mg sepharose resin) to bind activated labelled oestrogen receptor. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 10,000 cpm were added to each experiment. Before incubation the nuclear extract/sepharose resin was digested as described in Section 2.3.3. 10mg of resin was used for each binding assay.

TABLE 12

Proteolytic Enzyme used to Digest the Nuclear Extract/Sepharose Resin	% of Binding Activity Present in Control Binding Assay
Control (no digestion)	100%
Papain	50 ± 6%
Trypsin	44 ± 3%
Pronase	57 ± 2%
Protease *S.aureus*	81 ± 3%

Mean of quadruplicates ± SD

Table 12 - The effects of various proteolytic enzymes on the ability of the nuclear Fraction C/sepharose resin (800µg immobilised protein/500mg sepharose resin) to bind activated labelled oestrogen receptor. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 10,000 cpm were added to each experiment. Before incubation the nuclear extract/sepharose resin was digested as described in Section 2.3.3. 10mg of resin was used for each binding assay.

approximately the same amount, but protease S.aureus reduces the binding to a lesser extent is interesting. Apart from pronase the other enzyme preparations represent a single enzyme type, all of which have a similar subunit molecular weight (20,000-25,000), so the lesser reduction in binding activity caused by protease S.aureus cannot be explained because it is too large to gain access to the immobilised proteins.

The digestion of the nuclear extract/sepharose resins by the various proteolytic enzymes was carried out as far as possible at the optimum pH of the enzyme in question. Comparing digestion of the nuclear extract/sepharose resins with trypsin at pH7.4 and 8.0, shows a slightly greater decrease in binding activity caused by digestion at pH8.0 which is the optimum pH for the enzyme. The effect of varying the pH on the three dimensional structure of the immobilised proteins should not be too great. The proteins are covalently attached to the sepharose, and as such should be held in a fairly rigid manner, which should not be altered to any great extent by the changes in pH, introduced during the digestion reactions.

One thing that it is important to bear in mind from these results is the role that DNA or RNA may play in the nuclear retention of steroid receptor complexes in vivo. Using this in vitro system it appears that the observed binding activity is protein in nature but it has been reported that DNase treatment of uterine nuclei releases bound oestrogen receptor (e.g. King and Gordon, 1972) and that RNase treatment of 'HeLa' cell nuclei causes a release of glucocorticoid receptor complexes (Rossini, 1984). Recent advances in the field of DNA/steroid receptor interactions have increased our knowledge of the role of DNA in the nuclear retention of steroid receptor complexes but as yet, no specific role for RNA in nuclear binding has become apparent, although Lin and Ohno (1983) have reported selective interaction of oestrogen receptor from

hen oviduct with poly A RNA and a similar interaction of mouse kidney androgen receptor with RNA (mRNA, tRNA and rRNA) (Lin and Ohno, 1981). Feldman et al. (1981) report that cytosol from MTW9 rat mammary tumour contains a high molecular weight inhibitor of oestrogen receptor binding to DNA. RNase treatment destroys the inhibitory activity, suggesting the involvment of an RNA molecule. A similar situation has been reported for the binding of both oestrogen and glucocorticoid receptors from MCF7 cells to DNA cellulose (Chong and Lippman, 1982). These observations suggest that steroid receptor - RNA interactions may play a role in gene regulation.

3.4 Saturation Analysis of the Nuclear Binding Activity

3.4.1 The 0.1M H_2SO_4 Nuclear Extract.

Initial saturation analysis was carried out by adding increasing amounts of labelled, activated oestrogen receptor complex and, in parallel, corresponding H+C labelled receptor to a 0.1M H_2SO_4 nuclear extract/sepharose column. From the difference the number of specifically bound cpm in each case is calculated. A typical saturation curve obtained from such a set of experiments is shown in Figure 13. This curve indicates that the binding of ^3H-E_2 labelled activated oestrogen receptor to the 0.1M H_2SO_4 nuclear extract/sepharose resin is saturable.

However this experimental technique was laborious and time consuming to perform and as a result, future saturation analyses were carried out using suspensions of protein linked sepharose as described in Section 2.4. These experiments were carried out using increasing amounts of ^3H-E_2 labelled oestrogen receptor in the presence of both HE/0.12M KCl and HE/0.5M KCl buffers. Binding which occurred in the presence of 0.5M KCl was assumed to be non-specific.

The data from such analyses were plotted out in two ways:- (a) as for conventional saturation analysis and (b) as described by Scatchard (1949).

Figure 14 shows a typical saturation curve obtained from such an analysis and Figure 15 shows a typical plot of the data when they were treated as described by Scatchard (1949). This indicates that specific binding of oestrogen receptor to the protein-sepharose matrix reflects a single class of high affinity binding sites. The parameters calculated from such a plot are shown in Table 13.

The estimated number of binding sites present was 2000 f mol/ mg immobilised protein.

The apparent Kd of the interaction of activated, labelled oestrogen receptor (see Table 13) indicates that the binding involved is very tight.

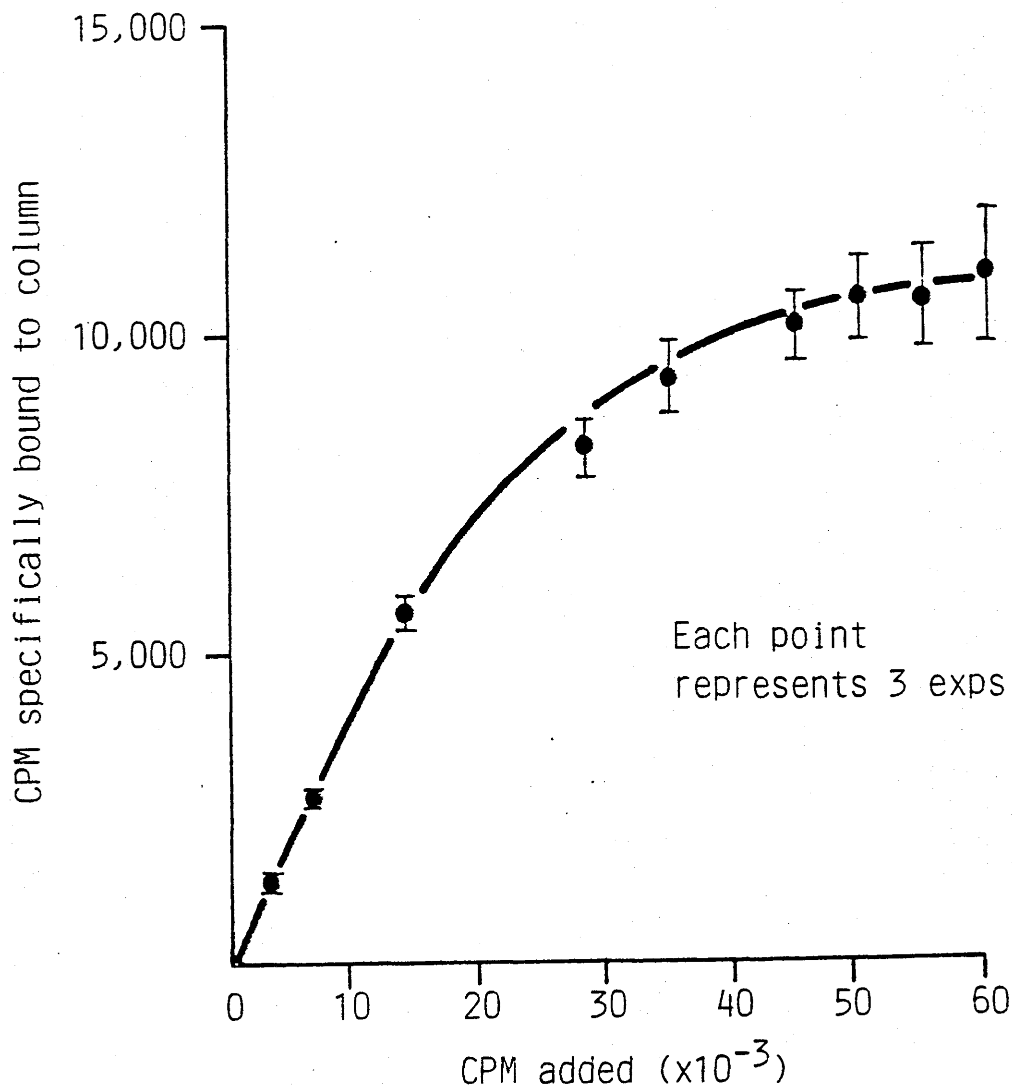


Figure 13 - The binding curve obtained when increasing numbers of cpm (both H and H+C) were applied to a 0.1M H_2SO_4 nuclear extract/sepharose column (1000µg immobilised protein/500mg resin). Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor as described in Sections 2.2.3.2 and 2.2.3.3. Specific binding was calculated as described in Section 2.2.4.1.

3.4.2 Nuclear Fraction C.

Saturation analysis of the binding of $^3H\text{-}E_2$ labelled, activated oestrogen receptor to nuclear Fraction C/sepharose resin was carried out as described in Section 2.4, again using both HE/0.12M KCl and HE/0.5M KCl as incubation buffers.

The pattern of binding of labelled, activated oestrogen receptor to this nuclear extract/sepharose resin was very similar to that shown in Figure 14, again suggesting the binding activity comprised a single class of high affinity saturable sites.

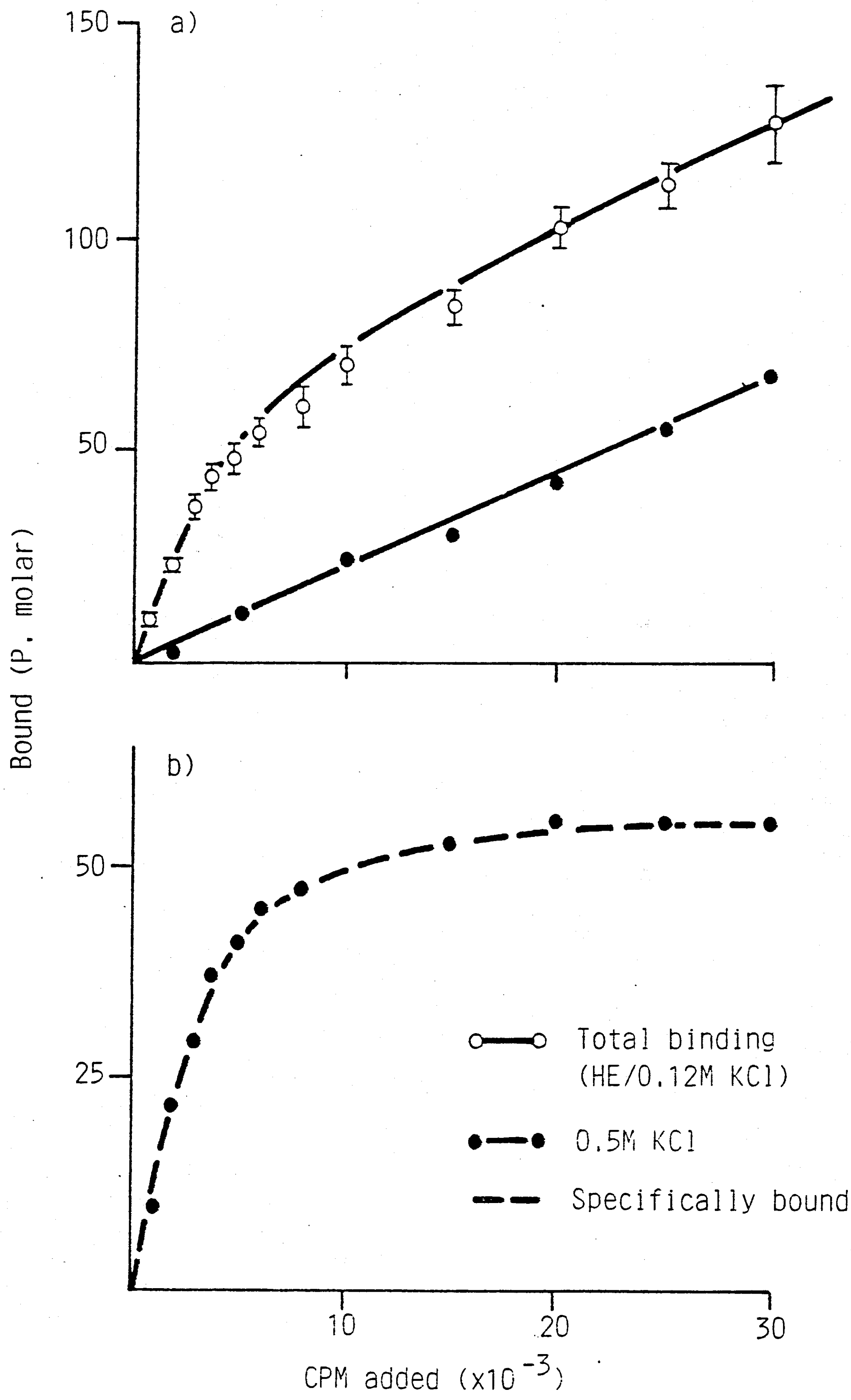
The parameters calculated from these experiments are also shown in Table 13.

These results when combined, show that both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins contain a single class of high affinity, saturable binding site for activated, labelled oestrogen receptor complex. The binding which is observed in the presence of 0.5M KCl indicates that both nuclear extract/sepharose resins contain non-specific binding sites for activated, labelled oestrogen receptor complex.

When compared to the saturating levels of binding of labelled, activated oestrogen receptor reported in Section 3.6 (Tables 23 and 24), it can be seen that when the binding assay is carried out in "suspension", the number of available binding sites is increased. From the studies in this section it appears that both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins contain comparable numbers of high affinity binding sites. The experiments reported in Section 3.6 show that nuclear Fraction C contains a greater number of binding sites for labelled, activated oestrogen receptor. This may suggest that nuclear Fraction C also contains numerous lower affinity binding sites not eliminated by the competition procedure used.

Figure 14(a) - The binding curve obtained when an increasing range of cpm (corresponding to an increasing amount of $^3H-E_2$ labelled oestrogen receptor) was added to 20mg amounts of the 0.1M H_2SO_4 nuclear extract/sepharose resin (850µg immobilised protein/500mg resin). Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of receptor as described in Sections 2.2.3.2 and 2.2.3.3. Non-specific binding was assessed in the presence of 0.5M KCl.

(b) Apparent saturation curve obtained from data in (a).


150
100
50
a)
b)
50
25
Bound (P. molar)
10
20
30
CPM added (x10 -3)
Total binding
(HE/0.12M KCl)
0.5M KCl
Specifically bound

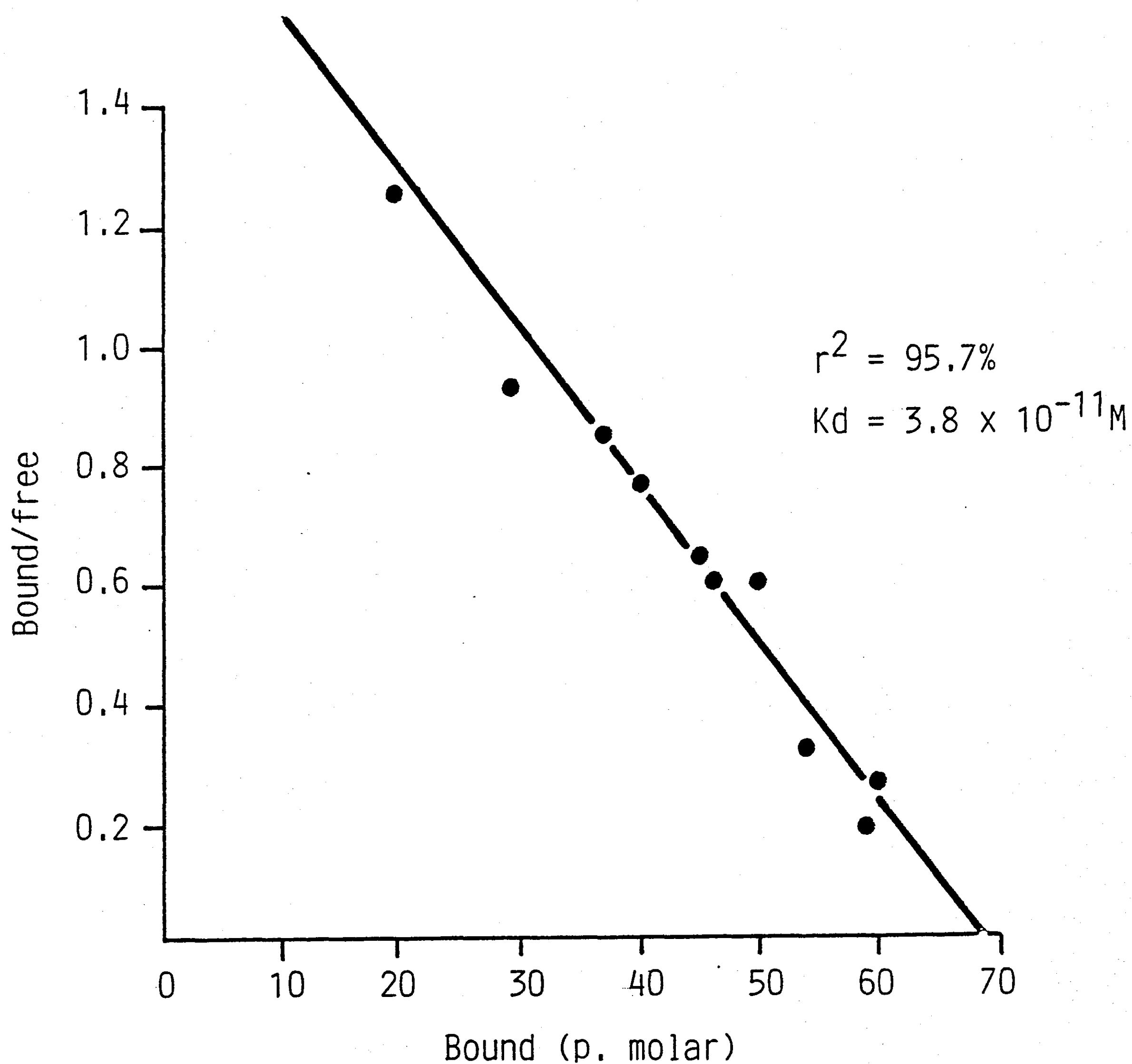


Figure 15 - A typical plot obtained when the data obtained from the saturation studies using the 0.1M H_2SO_4 nuclear extract/ sepharose resin (850µg immobilised protein/500mg resin) were analysed as described by Scatchard (1949). Analysis was carried out as described in Section 2.4.2.

The binding parameters for the saturable high affinity interaction of activated, labelled oestrogen receptor complex with these nuclear fractions have been calculated at $4^{o}C$, under physiological pH conditions and almost physiological ionic strength.

3.4.3 Discussion.

It is now generally accepted that under defined conditions of pH and ionic strength, it is possible to observe saturable binding of steroid receptor complexes to target cell nuclei, chromatin or isolated chromatin components (Spelsberg et al., 1976a).

Saturation analysis provides an insight into the characteristics of the binding sites at a concentration of steroid which falls within the physiological range. The data presented in Sections 3.4.1 and 3.4.2 show that both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins contain a limited number of specific, saturable binding sites for activated, labelled oestrogen receptor complex. Saturation analysis was carried out at an ionic strength which is known to extensively dissociate receptor complex from intact nuclei i.e. 0.4-0.6M KCl (Clark and Peck, 1979). It is well accepted that the nuclear binding of oestrogen receptor complexes can be divided into a form which is salt extractable and a form which is not extracted under these conditions (Clark and Peck, 1979). However it is not certain which of these two types of nuclear binding is physiologically important i.e. which results in specific gene activation. Hence, the assumption that the binding observed in the presence of 0.5M KCl represents non-specific binding may not be strictly correct as receptor can still be bound in the remaining nucleus under such conditions. However, the binding observed under these conditions was never saturable, and was of lower affinity than the specific saturable binding observed in 0.12M KCl. An attempt to analyse non-specific binding using "H" and "H+C" cytosols as described

TABLE 13

Nuclear Fraction Coupled to Sepharose 4B	Calculated Kd of Interaction with ^3H-E_2 Labelled Oestrogen Receptor
0.1M H_2SO_4 Extract	$Kd = 39 \pm 8x10^{-12}M$
850µg Immobilised Protein/500mg sepharose resin	Approximately $1.2x10^{12}$ Binding sites/mg immobilised protein (2000 f mol)
Nuclear Fraction C - 2M NaCl soluble material	$Kd = 44 \pm 9x10^{-12}M$
400µg Immobilised Protein/500mg sepharose resin	Approximately $1.0x10^{12}$ Binding sites/mg immobilised protein (1810 f mol)

Mean of 3 Exps. ± SD

Table 13 - The apparent dissociation constants for the interaction of activated ^3H-E_2 labelled oestrogen receptor complexes with the nuclear Fraction C (400µg immobilised protein/500mg resin) and the 0.1M H_2SO_4 nuclear extract (850µg immobilised protein/500mg resin)/sepharose resins at 4^oC. Analysis was carried out as described in Section 2.4.2. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor.

in Section 3.2.2, was made. Unfortunately, the results obtained were too erratic to enable any significant conclusions to be made. As a result, it was assumed that binding observed in the presence of 0.5M KCl was non-specific and did not represent binding of activated, labelled oestrogen receptor to specific saturable sites on both of the nuclear extract/sepharose resins.

The data presented are in broad agreement with those of Mainwaring et al. (1976), who show that the 2M NaCl extract from rat prostate nuclei binds labelled androgen receptor in a manner which can be resolved into two components. However only one of these was saturable. These high affinity, low capacity sites were shown to have an apparent Kd of 2.5×10^{-10}M which is comparable with the apparent Kd which can be calculated from the data in Figure 15. The second binding component observed by Mainwaring et al. (1976) comprised a large number of low affinity sites, which could be occupied by receptor complex in buffers containing 0.5M KCl. In contrast, the high affinity, low capacity sites could not be occupied by receptor complex in the presence of 0.5M KCl, in agreement with the data reported here.

The data are also in broad agreement with that of Puca et al. (1974), who show that the 2M NaCl extract from calf uterine nuclei binds oestrogen receptor complex with an apparent Kd of 3×10^{-9}M at 4°C. The presence of 0.4M KCl inhibited or strongly suppressed the observed binding activity. Subsequent work (Puca et al., 1975) on the interaction of a protein preparation solubilised by mild acid treatment of uterine nuclei with oestrogen receptor complex revealed an apparent Kd for the interaction at 4°C of 2×10^{-10}M. Again the presence of 0.4M KCl strongly inhibited the specific interaction between the two components. Studies which have examined the binding of oestrogen receptor complexes to intact nuclei have reported apparent Kd values which are in good agreement with those presented in this study. Higgins et al. (1973) report a Kd of

2-3x10^{-10}M for the interaction of oestrogen receptor complexes from immature rat uterus with uterine nuclei and Kon and Spelsberg (1982) report a Kd of 1.8x10^{-10}M for the interaction of the oestrogen receptor from hen oviduct with hen oviduct nuclei. Binding was confined to a single class of sites with 3000-5000 sites/nucleus. Similarly, De Boer et al. (1984) report that the binding of calf uterine oestrogen receptor to chicken target cell nuclei occurs with an apparent Kd of 0.4-1.0x10^{-9}M, which is again in good agreement with the data presented from this study. Similar high affinity binding bind sites (Kd=1x10^{-10}M) have been reported for androgen receptor complexes associated with the nuclear matrix of the prostate (Barrack, 1983).

Evidence to support the existence of different subclasses of nuclear binding sites for steroid receptor complexes has come mainly from work carried out by Spelsberg and his co-workers. Analysis of the saturable binding of the progesterone receptor to its "acceptor" led Spelsberg (1976) to conclude that there were in fact several classes of nuclear binding sites with differing affinities for the hormone receptor complex. The highest affinity class of binding sites display a Kd of 10^{-12}M and were fully saturated before any physiological changes were observed. The intermediate class of sites display a Kd of 5x10^{-10}M and are probably involved in fine genetic control. Occupation of this intermediate class of binding sites by receptor complexes correlated with changes in the activities of both RNA polymerases I and II. The lowest affinity binding sites display a Kd of 1x10^{-8}M and will only be partially filled by the concentration of steroid in the plasma.

Further evidence supporting the existence of different classes of binding sites for steroid receptors in chromatin has come from work where the binding of steroid receptor complexes to chromatin has been compared before and after the chromatin has been extracted with a range of concentrations of various chaotrophic agents. Using such

techniques Ruh et al. (1981) studied the binding of oestrogen receptor complex to calf uterine chromatin, and were able to resolve the binding of receptor into two components using an increasing range of guanidine thiocyanate concentrations (1-6M), and into 3 components using a range of guanidine hydrochloride concentrations (1-8M). These techniques rely on the ability of the chaotrophic agents to "uncover" binding sites, which appear to be "masked" by specific fractions of chromatin proteins, and as such gave support to the idea that in target and non-target chromatin, the high affinity, specific binding sites are "hidden" to differing extents, making them inaccessible to steroid receptor complexes.

A great deal of progress has been made recently regarding the interaction of various steroid receptor complexes with specific DNA sequences. However, the apparent Kd for the interaction of oestrogen receptor complexes with a variety of heterologous DNA molecules is only $4\text{-}6 \times 10^{-4}$M (Buller and O'Malley, 1976). The affinity of the interaction reported for steroid receptor complexes with specific DNA sequences is only 10-40 times greater than this, which still represents binding of insufficient affinity to explain the physiological effects of steroid hormones at the very low concentrations at which they occur in vivo. For the sake of comparison, the lac repressor protein binds to its specific DNA sequence in the lac operon region of the E.coli genome with an apparent Kd of approximately 10^{-13}M (Riggs et al., 1970), this being a classic example of a protein whose in vivo effects are realised through interaction with a specific DNA sequence.

When these observations are considered it seems unlikely that the interaction of steroid receptor complexes with specific DNA sequences can explain the in vivo effects of steroid hormones, thus it would seem likely that other nuclear components are involved in steroid hormone receptor recognition and binding. In view of the data presented in this

study, and data which have previously been published (e.g. Mainwaring et al., 1976; Puca et al., 1974; 1975), specific chromosomal proteins, such as those involved in the fractions used here, must be involved in the acceptor sites in vivo.

For many years Spelsberg has supported the idea that both DNA and protein have a role to play in the three dimensional structure of "acceptor" sites for steroid hormone receptor complexes in vivo, a view that is shared by Leake (1981). The strongest evidence to support this proposal so far comes from Spelsberg et al. (1984) who report that only a limited number of specific DNA sequences are present in the avian genome which can interact with the chromatin protein fraction they have previously shown to possess binding activity towards the chick oviduct progesterone receptor.

3.5 The Ability of Various Steroid Receptor Complexes to Bind to the Nuclear Extract/Sepharose Resins

3.5.1 Immature Rat Uterine Cytosol.

In order to probe both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins for binding sites for various steroid receptor complexes, immature rat uterine cytosol (1-2mg protein/ml) was labelled as described in Section 2.5. DCC stripping was carried out as described in Section 2.2.3.3. The specific activity of the ligand in question was taken into account when calculating the volume of cytosol to apply to the column, to ensure that similar amounts of protein bound ligand were applied in each case. 10,000 cpm of cytosol labelled with ^3H-E_2 was used as reference.

The labelling pattern obtained after the cytosols had been labelled with various ^3H-ligands ($5x10^{-9}M$) is shown in Table 14.

The immature rat uterine cytosol was also labelled with various ligands ($5x10^{-9}M$) plus a 200 fold excess of cold DES . The labelling patterns obtained are shown in Table 15.

As can be seen from Table 14, the levels of receptor molecules for androgens, glucocorticoids and progestins were much lower in immature rat uterine cytosol than the level of oestrogen receptor.

The pattern observed when labelling was carried out in the presence of a 200 fold excess of cold DES (see Table 15),indicates that these various ligands do not bind to the oestrogen receptor to any significant extent, and are presumably bound by the cellular receptor protein in question.

Interestingly, the anti-oestrogen tamoxifen, which expresses some of its anti-oestrogenic effects via the oestrogen receptor (Taylor et al., 1984) does not show the same labelling pattern as that induced by E_2 (see Table 14). This may be a reflection of the lower affinity that

TABLE 14

Ligand used to label cytosol ($5x10^{-9}M$)	cpm/10µl "H"	cpm/10µl "H+C"
E_2	1600 ± 200	150 ± 50
Dexamethasone	250 ± 40	70 ± 15
5 α DHT	180 ± 40	80 ± 25
Mibolerone	240 ± 70	80 ± 15
Tamoxifen	670 ± 130	300 ± 85
ORG 2058	250 ± 30	60 ± 10

Means of 3 Exps. ± SD

TABLE 14 - The pattern of labelling obtained when immature rat uterine cytosol (1-2mg protein/ml) was labelled with various ^3H-ligands ($5x10^{-9}M$). Competition was measured in the presence of a 200 fold excess of the same "cold" ligand except for E_2 where competition was assessed in the presence of a 200 fold excess of DES.

TABLE 15

Ligand used to label cytosol ($5x10^{-9}$M)	cpm/10µl "H"	cpm/10µl "H" + 200 fold excess DES
E_2	1600 ± 200	150 ± 50
Corticosterone	200 ± 40	185 ± 30
Testosterone	100 ± 20	95 ± 20
Mibolerone	220 ± 50	210 ± 50
5α DHT	190 ± 40	185 ± 20

Means of 3 Exps. ± SD

TABLE 15 - The pattern of labelling obtained when immature rat uterine cytosol (1-2mg protein/ml) was labelled with various ligands ($5x10^{-9}$M) alone or in the presence of a 200 fold excess of DES.

tamoxifen displays for the oestrogen receptor molecule, when compared to the natural ligand oestradiol (Sutherland and Whybourne, 1981).

The levels of specific binding induced by the various ligands to both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins are presented in Tables 16 and 17 respectively.

As shown in Tables 16 and 17 the greatest amount of binding is induced when the cytosol is labelled with $^3H\text{-}E_2$, i.e. labelled, activated oestrogen receptor is formed and is recognised by some component(s) of the nuclear extract/sepharose resins.

The other ligands are capable of inducing lower levels of binding, suggesting that there may be binding sites present on the nuclear extract/sepharose resins for the various other steroid hormone receptor complexes. The labelling pattern shown in Table 15 suggests that binding is not the result of the various other ligands interacting with the oestrogen receptor which then binds to the nuclear extract/sepharose resins.

As can be seen from Tables 16 and 17, nuclear Fraction C appears to show a 3 fold enrichment in binding sites for oestrogen receptor complexes when compared to the 0.1M H_2SO_4 nuclear extract. However, some masking of acceptor sites may occur in the 0.1M H_2SO_4 nuclear extract/sepharose resins since the protein content of the 0.1M H_2SO_4 nuclear extract used for these studies was 900μg immobilised protein/500mg resin compared with 400μg immobilised protein/500mg resin for the nuclear Fraction C/sepharose resin. Whatever the reasons, the fact that the nuclear Fraction C/sepharose resin contains more binding sites/unit of protein attached to the resin supports the existence of specific binding sites, i.e. the observed binding is not due to non-specific protein - protein interaction.

TABLE 16

^{3}H-ligand used to label cytosol (cpm applied)	f mol specifically bound/mg immobilised protein
E_2 (10,000)	66 ± 7
Tamoxifen (8800)	10 ± 2
5 α DHT (10,300)	14 ± 2
Mibolerone (7500)	13 ± 2
ORG 2058 (4500)	20 ± 2
Dexamethasone (7700)	11 ± 2

Means of 3 Exps. ± SD

TABLE 16 - The extent of binding to 0.1M H_2SO_4 nuclear extract/ sepharose resin (900μg immobilised protein/500mg resin) after labelling immature rat uterine cytosol (1-2mg protein/ml) with various ligands ($5x10^{-9}$M) alone or in the presence of a 200 fold excess of the same cold ligand except for E_2 where competition was assessed in the presence of a 200 fold excess of cold DES. Specific binding was calculated as described in Section 2.2.4.1.

TABLE 17

^{3}H-Ligand used to label the cytosol (cpm applied)	f mol specifically bound/mg immobilised protein
E_2 (10,000)	207 ± 20
Dexamethasone (7700)	0
5α DHT (10,300)	9 ± 2
Mibolerone (7500)	7 ± 2
Tamoxifen (8800)	30 ± 3
ORG 2058 (4500)	0

Mean of 3 Exps. ± SD

TABLE 17 - The extent of binding to a nuclear Fraction C/sepharose column (400μg immobilised protein/500mg resin) after labelling immature rat uterine cytosol (1-2mg protein/ml) with various ligands ($5x10^{-9}$M) alone or in the presence of a 200 fold excess of the same cold ligand except for E_2 where competition was assessed in the presence of a 200 fold excess of cold DES. Specific binding was calculated as described in Section 2.2.4.1.

The 0.1M H_2SO_4 nuclear extract/sepharose resin appears to be slightly enriched in specific binding sites for androgen receptor complexes when compared to nuclear Fraction C/sepharose resin, but the levels of specific binding in both cases are markedly lower than those induced by labelled activated oestrogen receptor complex.

Specific binding sites for both glucocorticoid receptor complexes and progesterone receptor complexes could be detected in low levels using the 0.1M H_2SO_4 nuclear extract/sepharose resin but no such binding sites could be observed when the nuclear Fraction C/sepharose resin was analysed.

Both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/ sepharose resins displayed specific binding sites for tamoxifen/oestrogen receptor complexes, with nuclear Fraction C showing a slight enrichment when compared to the 0.1M H_2SO_4 nuclear extract. The levels of binding to both nuclear extract/sepharose resins were much lower, approximately 15% of that induced by $^3H\text{-}E_2$ labelled, activated oestrogen receptor complex.

The labelling patterns in Table 14 show that the immature rat uterine cytosol is not a particularly good source of either androgen or glucocorticoid receptor. As a result further studies using female rat liver cytosol as a source of glucocorticoid receptor and ventral prostate cytosol as a source of androgen receptor were carried out to ensure that the low levels of binding sites for these receptor complexes observed in previous experiments were not due to poor receptor preparations.

3.5.2 Female Rat Liver Cytosol.

Female rat liver cytosol was prepared and labelled as described in Section 2.5.7.

The labelling pattern obtained is shown in Table 18. As can be seen the H+C incubation contains quite a high number of cpm, and this may be due to the greater protein concentration and complexity present in this cytosol.

TABLE 18

Source of Cytosol	Ligand used as label	cpm/10µl "H"	cpm/10µl "H+C"
Female Rat Liver (30-35mg protein/ml)	Dexamethasone	1800 ± 130	1150 ± 200
Ventral Prostate (5-6mg protein/ml)	5α DHT	250 ± 50	150 ± 30
	Mibolerone	280 ± 50	150 ± 30

Means of 3 Exps. ± SD

TABLE 18 - The pattern of labelling obtained when female rat liver cytosol was labelled with 5×10^{-9}M dexamethasone and ventral prostate cytosol was labelled with 5×10^{-9}M 5α DHT or mibolerone. Competition was measured in the presence of a 200 fold excess of the same cold ligand.

Cytosol (30-35mg protein/ml) was then applied to nuclear Fraction C/sepharose resin (300µg immobilised protein/500mg resin) and 0.1M H_2SO_4 nuclear extract/sepharose resin (1100µg protein/500mg resin). Again the specific activity of the ligand was taken into account when calculating the volume of cytosol to apply to the columns.

The levels of specific binding to both the nuclear extract/ sepharose resins are presented in Table 19.

As can be seen, using an improved source of glucocorticoid receptor enabled the detection of specific binding sites in the nuclear Fraction C/sepharose resin which had not been possible when immature rat uterus was used as a source or receptor.

Similarly, the use of rat liver cytosol as a source of glucocorticoid receptor resulted in an increase in the observed level of specific binding to the 0.1M H_2SO_4 nuclear extract/sepharose resin (see Table 19).

An interesting observation made during these experiments was that although the non-specific binding of dexamethasone to cytosol protein was much higher in liver than uterus (compare Tables 14 and 18), the non-specific binding to the columns was similarly low in both cases. This suggests that the observed specific binding to the nuclear extract/ sepharose columns represents binding of labelled, activated glucocorticoid receptor and that the label bound in the H+C incubation is bound to numerous lower affinity sites which do not bind to the nuclear proteins - again confirming the specificity of such interactions.

3.5.3 Ventral Prostate Cytosol.

Ventral prostate cytosol was prepared and labelled as described in Section 2.5.8.

TABLE 19

Ligand used to label female rat liver cytosol	f mol specifically bound/mg immobilised protein
Dexamethasone	
Nuclear Fraction C	48 ± 7
0.1M H_2SO_4 nuclear extract	29 ± 4

Means of 3 Exps. ± SD

TABLE 19 - The extent of binding to a nuclear Fraction C/sepharose column (300µg immobilised protein/500mg resin) and a 0.1M H_2SO_4 nuclear extract/sepharose column (1100µg immobilised protein/500mg resin) after labelling female rat liver cytosol (30-35mg protein/ml) with dexamethasone ($5x10^{-9}$M) alone and in the presence of a 200 fold excess of the same cold ligand. Specific binding was calculated as described in Section 2.2.4.1. 7700 cpm of both the H and H+C cytosols were applied in each case. The cytosol was prepared as described in Section 2.5.6.7.

The labelling pattern obtained is shown in Table 18. Virtually no difference in the labelling pattern could be observed by including 20mM sodium molybdate in the homogenisation buffer. The observed labelling pattern was slightly better when ^{3}H-mibolerone was used as ligand instead of 5αDHT. The inclusion of ORG 2058 had no effect on the observed labelling pattern, indicating that binding to the progesterone receptor was not responsible for the enhanced labelling pattern. However, the levels of binding present in the ventral prostate cytosol are very low,and it was expected that they would be higher. There appears no obvious reason for these consistent low binding levels.

Cytosol (5-6mg protein/ml) was then applied to both the nuclear Fraction C/sepharose resin (300µg/immobilised protein/500mg resin and the 0.1M H_2SO_4 nuclear extract/sepharose resin (1100µg immobilised/500mg resin). As previously, the specific activity of the ligand was taken into account before applying cytosols to the columns.

The observed levels of specific binding to both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins are presented in Tables 20 and 21 respectively.

As can be seen from Table 21 the highest levels of binding to the nuclear Fraction C/sepharose resin are observed when ^{3}H-mibolerone is used as ligand hence ^{3}H-mibolerone was used as ligand for future experiments.

As can be seen from Tables 20 and 21 the use of ventral prostate cytosol as a source of androgen receptor has enabled higher levels of binding sites for the androgen receptor complex to be observed in both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins compared to when immature rat uterus was used as a source of androgen receptor. Again from Tables 20 and 21 it can be seen that the inclusion of 20mM sodium molybdate in the homogenisation buffer has no great effect on the observed levels of binding to both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/ sepharose resins. This is a situation similar

TABLE 20

Ligand used to label cytosol	f mol specifically bound/mg immobilised protein
Mibolerone	
HED/0.12M KCl/10% Glycerol	36 ± 5
HED/0.12M KCl/10% Glycerol 20mM molybdate	38 ± 3

Means of 3 Exps. ± SD

TABLE 20 - The extent of binding to a 0.1M H_2SO_4 extract sepharose column (1100µg immobilised protein/500mg resin) after labelling rat ventral prostate cytosol (5-6mg protein/ml) ± 20mM sodium molybdate with mibolerone ($5x10^{-9}$M) alone and in the presence of a 200 fold excess of cold mibolerone. Specific binding was calculated as described in Section 2.2.4.1. 7500 cpm of both the H and H+C cytosols were applied in each case. Prostate cytosol was prepared as described in Section 2.5.7.8.

TABLE 21

Ligand used to label cytosol (cpm applied)	f mol specifically bound/mg immobilised protein
5 α DHT	
HED/0.12M KCl/10% Glycerol (10,300 cpm)	17 ± 4
Mibolerone	
HED/0.12M KCl/10% Glycerol	46 ± 6
HED/0.12M KCl/10% Glycerol/ 20mM molybdate (7500 cpm)	40 ± 5

Means of 3 Exps. ± SD

TABLE 21 - The extent of binding to a nuclear Fraction C/sepharose column (300μg immobilised protein/500mg resin) after labelling rat ventral prostate cytosol with either mibolerone ($5x10^{-9}$M) or 5 α DHT ($5x10^{-9}$M) alone or in the presence of a 200 fold excess of the same ligand. Specific binding was calculated as described in Section 2.2.4.1. Labelling with mibolerone was carried out ± 20mM sodium molybdate. Rat ventral prostate cytosol was prepared as described in Section 2.5.7.8.

to that reported for the oestrogen receptor from immature rat uterus (see Section 3.3.2.3).

3.5.4 Discussion.

The results from this set of experiments show that in addition to binding sites for labelled, activated oestrogen receptor both nuclear Fraction C and the 0.1M H_2SO_4 nuclear extract/sepharose resins contain binding sites for various other steroid receptor complexes.

When immature rat uterine cytosol was used as a source of androgen, glucocorticoid and progesterone receptor, only low levels of specific binding of each receptor complex to the 0.1M H_2SO_4 nuclear extract/sepharose resin could be detected (Table 16). In the case the nuclear Fraction C/ sepharose resin only specific binding sites for androgen receptor complexes could be detected (Table 17). The data presented in Table 14 show that lower levels of these other steroid receptor proteins are present in immature rat uterine cytosol that the level of oestrogen receptor. The presence of specific receptor molecules for the various steroids was established using a one point assay i.e. measuring competition in the presence of an excess of cold competitor - but only at one ligand concentration. The cytosol was not subjected to more elaborate analysis. However, as shown in Table 15, the distribution of labelled ligand in the presence and absence of excess DES indicates that the binding of the various ligands does not occur to the oestrogen receptor molecule.

To ensure that the low levels of receptor protein present in the immature rat uterine cytosol were not a limiting factor, female rat liver was used as a source of glucocorticoid receptor and rat prostate as a source of androgen receptor. In rat liver cytosol higher levels of labelled glucocorticoid receptor were accompanied by higher levels of non-specific binding but this latter class of binding protein was not retained by the nuclear protein/resin complexes. The use of glucocorticoid

receptor from this source enabled detection of specific binding sites for this steroid receptor complex on nuclear Fraction C/sepharose resin (Table 19) and a larger number of specific binding sites on the 0.1M H_2SO_4 nuclear extract/sepharose resin (Table 19). These results suggest that uterine cytosol as a source of receptor was limiting in receptor content.

The use of rat prostate cytosol as a source of androgen receptor enabled detection of an increased number of specific binding sites on both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C/sepharose resins (Tables 20 and 21). The labelling patterns obtained (Table 18), were rather low, barely greater than those obtained using immature rat uterine cytosol. Mainwaring and Randall (1984) report the inclusion of 10mM sodium molybdate in the homogenisation buffer, but the incorporation of molybdate into the homogenisation buffer in this study did not improve the labelling pattern. Table 21 shows that the androgen receptor labelled with ^{3}H-mibolerone induced higher levels of binding to the nuclear Fraction C/sepharose resin when compared to androgen receptor labelled with 5α DHT. One possible explanation for this is that mibolerone may cross-react with progesterone receptor resulting in binding of this receptor complex to the nuclear extract/sepharose resin. However, previous studies in our laboratory have shown that there is minimal cross-reaction of mibolerone with progesterone receptor under similar conditions.

As reported for immature rat uterine oestrogen receptor binding to the nuclear Fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins (see Section 3.2.2.3), the presence of sodium molybdate in the homogenisation buffer did not reduce the binding of labelled androgen receptor complexes to either of the nuclear extract/sepharose resins (Tables 20 and 21). As discussed earlier (Section 3.2.2.6), it is not possible to offer a

simple explanation for these observations.

In all of the above experiments the effect of the different protein concentrations in the various cytosols was kept to a minimum by applying the same amount (f mol) of protein bound ligand in each case. 10,000 cpm of immature rat uterine cytosol labelled with $5x10^{-9}$ ^{3}H-E_2 was used as a reference (approximately 150 f mol). It must also be noted that these experiments were performed under conditions where the specific binding sites for activated labelled oestrogen receptor were not saturated i.e. 10,000 cpm of ^{3}H-E_2 oestrogen receptor complex is not sufficient to occupy all of the available binding sites (see Section 3.4.1). In the cases of the other steroid receptor complexes, the proportion which is bound, of the cpm applied to the nuclear extract/sepharose resins, may suggest that the binding sites for these other receptor complexes are approaching saturation. This observation confirms that both the nuclear fraction/sepharose resins appear to contain a greater number of binding sites for oestrogen receptor complexes than for any of the other steroid receptor complexes. However, saturation or further analysis of the binding sites for androgen, glucocorticoid and progesterone receptor complexes were not carried out.

As suggested in Section 3.2.2.6 nuclear Fraction C appears to be enriched over the 0.1M H_2SO_4 nuclear extract in specific binding sites for oestrogen receptor complexes (compare Tables 16 and 17). Possible reasons for this enrichment have been discussed previously in Section 3.2.2.6. Both nuclear Fraction C and the 0.1M H_2SO_4 nuclear extract appear to contain roughly equal numbers of binding sites for androgen receptor complexes (compare Tables 20 and 21). In the case of binding sites for glucocorticoid receptor complexes, nuclear Fraction C appears to show a slight enrichment over the 0.1M H_2SO_4 nuclear extract. This seems a

little surprising as intially no binding sites for the glucocorticoid receptor complex could be detected in this nuclear fraction. However these experiments relied on immature rat uterus as a source of glucocorticoid receptor.

The only major difference observed between nuclear Fraction C and the 0.1M H_2SO_4 nuclear extract was that nuclear Fraction C was completely devoid of specific binding sites for the progesterone receptor complex. This observation suggests that such binding sites are preferentially solubilised by 0.1M H_2SO_4 when compared to 2M NaCl. A slightly different situation has been reported in hen oviduct chromatin, where Ruh and Spelsberg (1983) report that the acceptor activities for the oestrogen receptor complex and the progesterone receptor complex were "unmasked" by similar concentrations of guanidine hydrochloride.

The binding of tamoxifen-oestrogen receptor complexes to both the nuclear Fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins is much less than that observed by the corresponding amount of ^3H-E_2 labelled, activated oestrogen receptor (Tables 16 and 17). Nuclear Fraction C appears to be enriched in the ability to bind tamoxifen-oestrogen receptor complexes when compared to the 0.1M H_2SO_4 nuclear extract. There are several possible explanations for this observation. It is widely accepted that the active metabolite 4-hydroxytamoxifen has a similar affinity for the oestrogen receptor to that of oestradiol (Borgna and Rochefort, 1981) and can cause activation of the receptor as assessed by standard procedures (Mester <u>et al</u>., 1981). However, the lower affinity of tamoxifen for the oestrogen receptor and doubt over its ability to cause receptor activation may be the reason for the reduced level of binding, i.e. the levels of observed binding may be reduced because only a fraction of the receptor population has become activated,

resulting in a smaller number of the same binding sites as those occupied by $^{3}H-E_2$ labelled, activated oestrogen receptor being bound. Undoubtedly, the answer to this question is to repeat the experiments using tritiated 4-hydroxytamoxifen instead of tritiated tamoxifen.

The second possibility is that the tamoxifen oestrogen receptor complex binds to a completely different set of binding sites to those bound by $^{3}H-E_2$ labelled oestrogen receptor complexes. If this is the case, the number of binding sites present in the two nuclear extracts for anti-oestrogen oestrogen receptor complexes is substantially fewer than the number present for E_2 labelled oestrogen receptor complexes. The idea of non-identical chromatin binding sites for oestrogen and anti-oestrogen receptor complexes is not a new one (Baudendistal and Ruh, 1976; Massol et al., 1978; Lebeau et al., 1981; Singh et al., 1984) (see Section 1.4.3.10.1).

3.6 The Measurement of Competition between Various Steroid Receptor Complexes for Binding Sites on the Nuclear Extract/Sepharose Resins

3.6.1 Competition between Progesterone Receptor Complex and Oestrogen Receptor Complex.

These studies could only be carried out using the 0.1M H_2SO_4 nuclear extract/sepharose resin as no specific binding sites for the progesterone receptor could be detected on the nuclear Fraction C/sepharose resin (see Section 3.5.1).

Parallel columns of 0.1M H_2SO_4 nuclear extract/sepharose resin (800µg protein/500mg sepharose) were used. Immature rat uterine cytosol (1-2mg protein/ml) was prepared as described in Section 2.6.

The appropriate volume of cytosol, labelled with either ^3H-E_2 or ^3H-ORG 2058 was eluted through the columns alone or in the presence of cytosol labelled with either cold ORG 2058 or cold DES as described in Section 2.6. The experiments were carried out under conditions where the respective binding sites approach saturation. The observed binding in each case is shown in Table 22.

As can be seen, the ability of the 0.1M H_2SO_4 nuclear extract to bind activated labelled oestrogen receptor is greater than its ability to bind progesterone receptor complexes, confirming earlier observations (see Section 3.5.1).

Eluting labelled oestrogen receptor through the nuclear extract/sepharose resin in the presence of unlabelled,occupied progesterone receptor reduces the observed binding by approximately 30%. The low level of progesterone binding which was observed with the immature rat uterine cytosol meant large volumes of cytosol would have been required to obtain an excess of progesterone receptor complex in these assays, hence 1ml

TABLE 22

Sample applied to 0.1M H_2SO_4 nuclear extract/sepharose column	f mol bound/mg immobilised protein
^3H-E_2 oestrogen receptor alone	298 ± 26
^3H-E_2 oestrogen receptor + cold ORG 2058 progesterone receptor complex	210 ± 10
^3H-ORG 2058 progesterone receptor complex alone	67 ± 5
^3H-ORG 2058 progesterone receptor complex + cold DES oestrogen receptor complex	54 ± 10

Means of 3 Exps. ± SD

TABLE 22 - Assessment of competition between oestrogen receptor complexes and progesterone receptor complexes for binding sites present on the 0.1M H_2SO_4 nuclear extract/sepharose resin (800μg immobilised protein/500mg resin). Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of receptor (see Section 2.6). 50,000 cpm of ^3H-E_2 oestrogen receptor (740 f mol) was eluted through the columns alone or in the presence of 670 f mol (1ml of cytosol) cold progesterone receptor. 20,000 cpm of ^3H-ORG 2058 progesterone receptor (670 f mol) was eluted through the columns alone or in the presence of 800 f mol cold oestrogen receptor. Competition was assessed as described in Section 2.6.

(670 f mol) of cytosol was eluted through the column in the presence of the labelled oestrogen receptor. It may have been possible to reduce the observed binding further by increasing the amount of competing progesterone receptor complex.

Eluting ORG 2058 labelled progesterone receptor through the 0.1M H_2SO_4 nuclear extract/sepharose resin in the presence of unlabelled, occupied oestrogen receptor also reduced the observed binding, in this case by approximately 20%. In these experiments it was possible to have a slight excess of unlabelled, occupied oestrogen receptor present.

These combined results suggest that the binding sites present in the 0.1M H_2SO_4 nuclear extract for oestrogen receptor complex and progesterone receptor complex are not exclusive to each receptor and there is interaction of both receptors with both of the binding sites. However, the data obtained from eluting labelled progesterone receptor complex through the nuclear extract/ sepharose resin in the presence of excess cold oestrogen receptor suggest that binding sites which are unique to each receptor complex are also present.

3.6.2 Competition between Oestrogen and Androgen Receptor Complexes.

Parallel columns of 0.1M H_2SO_4 nuclear extract/sepharose resin (1000µg immobilised protein/500mg resin) and nuclear Fraction C/ sepharose resin (300µg immobilised protein/500mg resin) were used.

Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor and ventral prostate cytosol (5-6mg protein/ml) was used as a source of androgen receptor. Methodology was as described for oestrogen and progesterone receptor antagonism in Section 2.6 and Section 3.6.1. The experiments were carried out under conditions where the respective binding sites approach saturation.

TABLE 23

Sample applied to nuclear Fraction C/ sepharose column	f mol bound/mg immobilised protein
^{3}H-E_2 oestrogen receptor alone	700 ± 28
^{3}H-E_2 oestrogen receptor + cold mibolerone androgen receptor complex	480 ± 24
^{3}H-mibolerone androgen receptor alone	54 ± 4
^{3}H-mibolerone androgen receptor + cold DES oestrogen receptor complex	37 ± 3

Means of 3 Exps. ± SD

TABLE 23 - The assessment of competition between oestrogen receptor complexes and androgen receptor complexes for binding sites present on the nuclear Fraction C/sepharose resin (300µg immobilised protein/500mg resin). Immature rat uterine cytosol (1-2mg protein/ml) was used as a source oestrogen receptor. Rat ventral prostate cytosol (5-6mg protein/ml) was used as a source of androgen receptor. 50,000 cpm of ^{3}H-E_2 oestrogen receptor (740 f mol) was eluted through the columns alone or in the presence of 300 f mol cold androgen receptor. 15,000 cpm (300 f mol) of ^{3}H-mibolerone androgen receptor was eluted through the columns alone or in the presence of 1000 f mol cold oestrogen receptor. Competition was assessed as described in Section 2.6.

TABLE 24

Sample applied to 0.1M H_2SO_4 nuclear extract/sepharose column	f mol bound/mg immobilised protein
^{3}H-E_2 labelled oestrogen receptor alone	315 ± 20
^{3}H-E_2 labelled oestrogen receptor + cold mibolerone androgen receptor complex	189 ± 15
^{3}H-mibolerone labelled androgen receptor alone	42 ± 6
^{3}H-mibolerone labelled androgen receptor +cold DES labelled oestrogen receptor complex	26 ± 4

Means of 3 Exps. ± SD

TABLE 24 - The assessment of competition between oestrogen receptor and androgen receptor complexes for binding sites present on the 0.1M H_2SO_4 nuclear extract/sepharose resin (1100µg immobilised protein/500mg resin). Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. Rat ventral prostate cytosol (5-6mg protein/ml) was used as a source of androgen receptor. 50,000 cpm of ^{3}H-E_2 oestrogen receptor (740 f mol) was eluted through the columns alone or in the presence of 300 f mol cold androgen receptor. 15,000 cpm (300 f mol) of ^{3}H-mibolerone androgen receptor was eluted through the columns alone or in the presence of 1000 f mol cold oestrogen receptor. Competition was assessed as described in Section 2.6.

The observed binding in each case is shown in Tables 23 and 24.

Again as shown in Section 3.5.1, both the nuclear fraction/ sepharose resins possess a greater ability to bind the oestrogen receptor comple:

Table 24 shows that the binding of labelled activated oestrogen receptor is reduced by approximately 40% in the presence of unlabelled androgen receptor (300 f mol), suggesting there is competition between the two steroid receptor complexes for binding sites on the 0.1M H_2SO_4 nuclear extract/sepharose resin.

Table 23 shows that a similar situation occurs with columns containing nuclear Fraction C/sepharose resin. In this case the observed binding of labelled oestrogen receptor is reduced by approximately 31%.

For the same reasons mentioned in Section 3.6.1 for progesterone receptor, it was difficult to add a large amount of unlabelled androgen receptor to compete with the oestrogen receptor for binding sites.

When the reciprocal experiment was performed, Table 24 shows that competition is observed between labelled androgen receptor and excess (1000 f mol) cold oestrogen receptor for binding sites on the 0.1M H_2SO_4 nuclear extract/sepharose resin. The presence of an excess of cold oestrogen receptor in the binding assay reduces the observed binding by approximately 38%.

Table 23 shows that a similar situation occurs when competition between unlabelled oestrogen receptor and labelled androgen receptor for binding sites on the nuclear Fraction C/sepharose resin was assessed. In this case the observed binding was reduced by approximately 31%.

The results from this set of experiments suggest that there is competition between androgen and oestrogen receptor complexes for at least some of the sites on both the nuclear extract/sepharose resins.

3.6.3 Competition between E_2 labelled Oestrogen Receptor and Tamoxifen Labelled Oestrogen Receptor.

Parallel columns of the 0.1M H_2SO_4 nuclear extract/sepharose resin (800µg immobilised protein/500mg resin) and nuclear Fraction C/ sepharose resin (300µg immobilised protein/500mg resin) were used.

Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor.

The methodology was as described for oestrogen and progesterone receptor antagonism in Sections 2.6 and 3.6.1. The experiments were carried out under conditions where the respective binding sites approach saturation. The observed binding in each case is shown in Tables 25 and 26.

As has been previously observed (Section 3.5.1), the results in Tables 25 and 26 show that the tamoxifen-oestrogen receptor complex induces lower levels of binding to both the nuclear extract/sepharose columns than does E_2 labelled oestrogen receptor.

Table 26 shows that the binding of E_2 labelled activated oestrogen receptor to the 0.1M H_2SO_4 nuclear extract/sepharose resin is reduced by approxiamtely 41% in the presence of unlabelled tamoxifen receptor complex (670 f mol).

Table 25 shows that a similar situation occurs with columns containing nuclear Fraction C/sepharose resin. In this case the observed level of E_2 labelled oestrogen receptor binding is reduced by 21%.

When the reciprocal experiment was performed Table 26 shows that competition is observed between labelled tamoxifen receptor complex and unlabelled E_2 receptor complex (1030 f mol) for binding sites on the 0.1M H_2SO_4 nuclear extract/sepharose resin. The presence of an excess

TABLE 25

Sample applied to nuclear Fraction C/ sepharose column	f mol bound/mg immobilised protein
^3H-E_2 oestrogen receptor	728 ± 25
^3H-E_2 oestrogen receptor + cold tamoxifen oestrogen receptor	576 ± 80
^3H-tamoxifen oestrogen receptor	190 ± 28
^3H-tamoxifen oestrogen receptor + cold DES oestrogen receptor	156 ± 9

Means of 3 Exps. ± SD

TABLE 25 - Assessment of competition between E_2 labelled oestrogen receptor and tamoxifen labelled oestrogen receptor for binding sites present on the nuclear Fraction C/sepharose resin (300μg immobilised protein/500mg resin). Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 50,000 cpm (740 f mol) of ^3H-E_2 oestrogen receptor was eluted through the columns alone or in the presence of 670 f mol cold tamoxifen oestrogen receptor. 25,000 cpm (420 f mol) of ^3H-tamoxifen oestrogen receptor was eluted through the columns alone or in the presence of 1030 f mol cold DES oestrogen receptor. Competition was assessed as described in Section 2.6.

TABLE 26

Sample applied to 0.1M H_2SO_4 nuclear extract/sepharose column	f mol bound/mg immobilised protein
^3H-E_2 oestrogen receptor	304 ± 30
^3H-E_2 oestrogen receptor + cold tamoxifen oestrogen receptor	178 ± 6
^3H-tamoxifen oestrogen receptor	68 ± 10
^3H-tamoxifen oestrogen receptor + cold DES oestrogen receptor	59 ± 4

Means of 3 Exps. ± SD

TABLE 26 - Assessment of competition between E_2 labelled oestrogen receptor and tamoxifen labelled oestrogen receptor for binding sites on the 0.1M H_2SO_4 nuclear extract/sepharose resin (800µg immobilised protein/500mg resin). Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of oestrogen receptor. 50,000 cpm (740 f mol) ^3H-E_2 oestrogen receptor was eluted through the columns alone or in the presence of 670 f mol cold tamoxifen oestrogen receptor. 25,000 cpm (420 f mol) of ^3H-tamoxifen oestrogen receptor was eluted through the columns alone or in the presence of 1030 f mol cold DES oestrogen receptor. Competition was assessed as described in Section 2.6.

of cold DES labelled receptor reduces the observed binding by approximately 16%.

Table 25 shows that a similar situation occurs when competition between labelled tamoxifen receptor and cold DES receptor complexes was assessed for binding sites on the nuclear Fraction C/sepharose resin. In this case the observed binding was reduced by approximately 18%.

The results from these experiments suggest that competition between E_2 labelled oestrogen receptor and tamoxifen labelled oestrogen receptor does occur but the level of competition is not as high as that observed between oestrogen receptor complex and androgen receptor complex (see Tables 23 and 24).

Again the small reduction in tamoxifen oestrogen receptor binding in the presence of excess cold DES oestrogen receptor (sufficient to saturate the binding sites) indicates that although there are some common binding sites, many seem to be independent.

3.6.4 Discussion.

The results obtained from this set of experiments indicate that there is antagonism between labelled, activated oestrogen receptor and occupied progesterone receptor for some of the binding sites present on the 0.1M H_2SO_4 nuclear extract/sepharose resin. Nevertheless, the data obtained on the binding of labelled progesterone receptor complex in the presence of excess cold occupied oestrogen receptor (Table 22) suggests that there are also binding sites which are unique to each of the steroid receptor complexes. It was not possible to assess the relative affinities of the oestrogen and progesterone receptor complexes for the common binding sites, although the data in Table 22 (lower half) suggest that the progesterone receptor complex may have a higher affinity for the common binding sites.

When the binding of oestrogen and androgen receptor complexes to both the nuclear Fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins was examined the situation did not appear to be so simple. Again the data suggest there may be common binding sites for both steroid receptor complexes and also binding sites which are unique to each steroid receptor complex (Tables 23 and 24). However, the reduction in binding of labelled oestrogen receptor complex in the presence of androgen receptor complex is far greater than the number of binding sites which are occupied when labelled androgen receptor complex is eluted through the nuclear extract/sepharose columns alone. This suggests that not all of the competition measured under these conditions represents androgen receptor complex competing with oestrogen receptor for common binding sites. Some of the competition observed may be due to non-specific protein effects which are introduced by the relatively large volume of rat prostate cytosol which had to be used for these experiments. Another possibility to be considered in both the above sets of experiments is that competition is due not to binding of either androgen or progesterone receptor but is due to occupied endogenous oestrogen receptor complex present in the uterine and prostate cytosols. However, in both cases the low levels of endogenous oestradiol would not give rise to sufficiently large amounts of occupied oestrogen receptor complex to account for the observed level of competition. As such, it is difficult to assign the relative affinities of the androgen and oestrogen receptor complexes for the common binding sites.

These results are in conflict with Kon and Spelsberg (1982) who report no antagonism between oestrogen and progesterone receptor complexes for binding sites in hen oviduct nuclei. This is surprising since it has been shown that the kinetics of induction of conalbumin mRNA by both nuclear oestrogen and progesterone receptor complexes is very similar,

implying that a common binding site may be involved (Mulvihill and Palmiter, 1977; 1980). Higgins et al. (1973) have reported unique binding sites for both glucocorticoid and oestrogen receptor complexes in immature rat uterine nuclei. These studies, and those of Kon and Spelsberg (1982) examined the binding to intact nuclei, no attempt was made to analyse the binding of receptor complexes to nuclear components. The findings reported in this thesis when compared to these published observations may indicate that the three dimensional structure of the intact acceptor site in vivo may only be recognised by a particular class of steroid receptor complex. Once this structure has been disrupted during the extraction procedures involved in the present studies, a resultant loss in the absolute specificity of steroid receptor complex binding may occur.

As discussed in Section 3.7.1, a more likely explanation may involve the possibility that specific acceptor sites for steroid receptor complexes are normally "masked" and can be unmasked when the adjacent structural gene is to be activated by the appropriate steroid receptor complex. In this case, the question remains of how the unmasking of specific acceptor sites is initiated and how it is controlled and subsequently reversed.

The studies on the binding of $^{3}H-E_2$ labelled oestrogen receptor and ^{3}H-tamoxifen labelled oestrogen receptor to both nuclear extract/sepharose resins again suggest that some of the binding sites are common to both $^{3}H-E_2$ labelled and tamoxifen labelled oestrogen receptor complex but that there are binding sites which are unique to each receptor complex. Again it was not possible to assess the relative affinities of the E_2-oestrogen receptor complex and tamoxifen-oestrogen receptor complex for the common binding sites.

3.7 Analysis of the ability of Nuclear Fraction C/sepharose Resins from Various Immature Female Rat Tissues to specifically bind ^3H-E_2 labelled oestrogen receptor complex

Nuclei were purified from kidney, liver, spleen and uterus essentially as described in Section 2.2.2. Nuclear Fraction C was then prepared as described in Section 2.2.2.2 and coupled to CN-Br activated sepharose 4B as described in Section 2.2.2.5. The amount of immobilised protein was in the range of 400-900µg/500mg of sepharose resin.

Immature rat uterine cytosol (1-2mg protein/ml) was prepared as described in Sections 2.2.3.2 and 2.2.3.3 and eluted through the nuclear extract sepharose columns as described in Section 2.2.4.

The data are presented in Table 27.

As can be seen, nuclear Fraction C isolated from uterine nuclei has the greatest ability to specifically bind ^3H-E_2 labelled, activated oestrogen receptor complexes, although the equivalent nuclear fractions isolated from liver, kidney and spleen do possess the ability to specifically bind ^3H-E_2 labelled activated, oestrogen receptor complexes to a lesser extent. This indicates that the nuclear protein(s) responsible for the specific binding activity are not confined to "major" target tissues such as the uterus, and can be detected in tissues such as kidney and spleen which are considered not to be target tissues.

3.7.1 Discussion.

In order to study any binding activity which may have been present in other "non target" tissues of the immature female rat, it was necessary to purify nuclei from these various tissues. Generally, the non-target tissues which were examined were much "softer" than uterine tissue and hence nuclear purification was easier. The nuclei isolated from spleen and kidney in particular, were obtained in very good yields and appeared very "clean" under the phase contrast microscope.

TABLE 27

Immature female rat tissue used as a source of nuclear Fraction C	f mol specifically bound/mg immobilised protein
Uterus	186 $\pm$ 17
Liver	127 $\pm$ 12
Kidney	100 $\pm$ 19
Spleen	60 $\pm$ 17

Means of 3 exps. $\pm$ SD

Table 27 - The extent of specific binding of activated ^{3}H-E_2 labelled oestrogen receptor to nuclear Fraction C/sepharose resin prepared from various immature female rat tissues. The range of immobilised protein was 400-900µg/500mg resin. Immature rat uterine cytosol (1-2mg protein/ml) was used as a source of activated oestrogen receptor. 10,000 cpm of both H and H+C cytosols were applied in each case. Competition was calculated as described in Section 2.2.4.1.

Table 27 shows that the specific binding activity towards activated, labelled oestrogen receptor is present in nuclear Fraction C of all of the tissues analysed. However, the greatest amount of binding activity was extracted from uterine nuclei, with lesser amounts being extracted from liver, kidney and spleen nuclei. The detection of low levels of high affinity oestrogen receptor in both the kidney (Li et al., 1974) and liver (Aten et al., 1978) makes it possible to designate them as minor target tissues. The spleen must be considered as a non-target tissue.

There have been numerous reports that a variety of target cell nuclei for a variety of steroids contain markedly more acceptor sites than non-target cell nuclei using cell free binding assays (e.g. Mainwaring and Peterken, 1971; Spelsberg et al., 1972; Puca et al., 1975; Mainwaring et al., 1976; Pikler et al., 1976; De Boer et al., 1984). Many such reports did not state that non-target cell nuclei were completely devoid of acceptor sites, but that they contained fewer numbers of them. As such, the term "tissue specific" nuclear binding may be misleading and "enhanced" binding in target tissue nuclei may be more appropriate.

In contrast Higgins et al. (1973) report no difference in nuclear binding between the chromatin of target and non-target tissues. It may be possible that these observations are due to some of the methodology used during the analysis. Mild protease treatment of chromatin results in an increase in the number of nuclear binding sites availale (Spelsberg, 1982). Many of the conditions that enhance proteolysis such as the use of crude cytosol, temperatures above $4^{o}C$ and long periods of incubation were used in some of these studies. Hence, the chromatin from non-target tissues, which may have no natural steroid receptor binding sites may exhibit significant levels of binding when assayed under the above conditions.

The assay system used in these studies enables the detection of binding sites which are present in the various tissues of the immature rat. It does, however, give no idea of the role these binding sites have to play in the "non-target" tissues in vivo. It may be that in vivo these binding sites are "masked" and therefore concealed from activated oestrogen receptor, even if it were present in such cells. It has been reported that even in target tissues, masking of some acceptor sites occurs (Spelsberg, 1982; Spelsberg et al., 1983). The extraction procedure used to isolate nuclear Fraction C will extract the binding sites and presumably any masking proteins associated with them. Extraction will almost certainly disrupt any interaction which was present between them. Hence, in this in vitro system, the binding proteins are available for assay.

The question arises as to the biological role of such a masking phenomenon. One possible function is that it regulates which genes will respond to steroid receptor complex and at what time. If this were the case, it would help explain why different target tissues of the same organism with presumably the same type of receptor display markedly different responses to the same steroid with regard to gene expression, i.e. different genes seem to be regulated by the same steroid-receptor complex in different tissues. Recent evidence indicating that the steroid receptors in different tissues from different animals are antigentically similar (Greene et al., 1980; Greene and Jensen, 1982), minimises the tissue specificity of the receptors themselves. In short, if a gene is masked it is not available for activation by steroid receptor complex, but if unmasked is available for transcription in the presence of the appropriate steroid receptor complex. In non-target tissues which do not require the genes that respond to steroids, the acceptor sites of these genes will be masked (Spelsberg, 1982). However, since non-target tissues

are devoid of soluble receptor for the respective steroids, whether or not their chromatin contains acceptor sites for the steroid is, biologically speaking, of no consequence. During the development of a steroid target organ, quantitative and qualitative changes in the masking activity might be expected as different cell types appear or change in proportions. During oviduct development the number of available acceptor sites but not the total number of sites changes throughout the oestrogen induced development (Spelsberg, 1982). Thus, the extent of masking changes considerably. The masking phenomenon does not appear to be confined to the chick oviduct (Spelsberg, 1982) but has been reported for androgens in rat prostate (Klyzsejko-Stefanowicz, 1976), oestrogen and progesterone in sheep brain (Perry and Lopez, 1978) and oestrogen in rat and bovine uterus (Ruh et al., 1981).

3.8 Preliminary Protein Blotting Experiments

3.8.1 Introduction.

Having established that both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C possess a binding activity which is specific for the oestrogen receptor, the next objective should be to characterise the protein(s) responsible. Several attempts were made to do this for nuclear Fraction C using hydroxylapatite chromatography as described by Puca et al. (1974) and ion-exchange chromatography on the cation exchange resin Bio-Rex-70 as described by Mainwaring et al. (1976). However, after several attempts it proved impossible to recover any binding activity from either of these fractionation techniques. These techniques separate components on the basis of their overall charge, and thus it was hoped to show the nature of the binding components present in nuclear Fraction C using these techniques. The fact that the binding activity can be solubilised by mild acid treatment of nuclei at low temperature (as shown by the 0.1M H_2SO_4 nuclear extract) suggests that the components involved in binding are basic, but it would have been more satisfactory to confirm this using either or both of the above techniques.

In an alternative approach to characterising the protein(s) responsible for the specific nuclear binding activity, the techniques of protein blotting were employed.

Using these techniques, a crude extract of proteins is separated, usually by some means of electrophoresis. The pattern of separated protein is then transferred to a nitrocellulose filter in such a manner that the separated pattern is maintained. It is then possible to probe the nitrocellulose, in order to "pick out" or identify specific proteins which were present in the initial crude extract. This part of the technique relies on the availability of an appropriate probe.

The rationale behind these experiments was the separation of the nuclear extract using SDS PAGE, transfer of the proteins onto nitrocellulose filters, then probing the filters using labelled activated oestrogen receptor to identify proteins capable of binding the probe. In order to detect such a complex, antiserum against human myometrial oestrogen receptor in conjunction with ^{125}I labelled protein A was used.

3.8.2 Experiments using ^{125}I labelled E_2.

Experiments were performed essentially as described in Section 2.7.

Electrophoresis of nuclear proteins was carried out in duplicate. One half of the gel was stained as described in Section 2.7.2 to reveal the pattern of separated proteins. The separation obtained is shown in Figure 6. The pattern of separated proteins in the other half of the gel was transferred to nitrocellulose as described in Section 2.7.3. ^{125}I-E_2 labelled oestrogen receptor was prepared as described in Section 2.7.4, such that the cytosol contained between 500,000 and 600,000 cpm, all of which were subsequently applied to the nitrocellulose filter as described in Section 2.7.5.

The major problem encountered during these experiments was the amount of non-specific "background" binding which occurred to the nitrocellulose filter. One possible way in which the background could have been reduced, would have been the inclusion of 0.5% Tween-20 in the incubation buffer, but it was considered important to keep the assay conditions, as similar as possible to those present in previous binding assays. The resultant high background may have obscured "bands" corresponding to the binding of ^{125}I-E_2 labelled activated oestrogen receptor to specific protein fractions present on the nitrocellulose. Thus, it was not possible to observe "bands" corresponding to the binding of receptor to discreet areas of the nitrocellulose filters.

3.8.3 Experiments using rabbit antiserum raised against the human myometrial oestrogen receptor.

These experiments were carried out essentially as described in Section 2.7.6. Analysis of nuclear Fractions B and C and the 0.1M H_2SO_4 nuclear extract were carried out.

As described in Section 3.8.2, electrophoresis was carried out in duplicate. The portion of the polyacrylamide gel which was to be subjected to the "blotting" procedure was loaded with molecular weight markers (Pharmacia, low molecular weight range) which had been iodinated with Na ^{125}I as described for protein A in Section 2.7.6.1.

The patterns obtained on electrophoresis of the various nuclear fractions are shown in Figures 6 and 8. In an attempt to reduce the amount of non-specific binding to the nitrocellulose, which results in an increased background, the unreacted groups remaining on the nitrocellulose filter after transfer of the protein fractions were blocked by using BSA as described by Towbin et al. (1979). 3% BSA (w/v) was also included in buffers used for the various incubations in an attempt to keep the non-specific binding to a minimum.

Nuclear Fractions B and C

Previous experiments have shown that, when compared to nuclear Fraction B, nuclear Fraction C is greatly enriched in specific binding activity towards activated, occupied oestrogen receptor complexes, hence it should be interesting to compare both of these nuclear fractions using this approach.

Figure 16 shows an autoradiograph of a nitrocellulose filter which had been probed as described in Section 2.7.6.1. As can be seen several prominent bands are present.

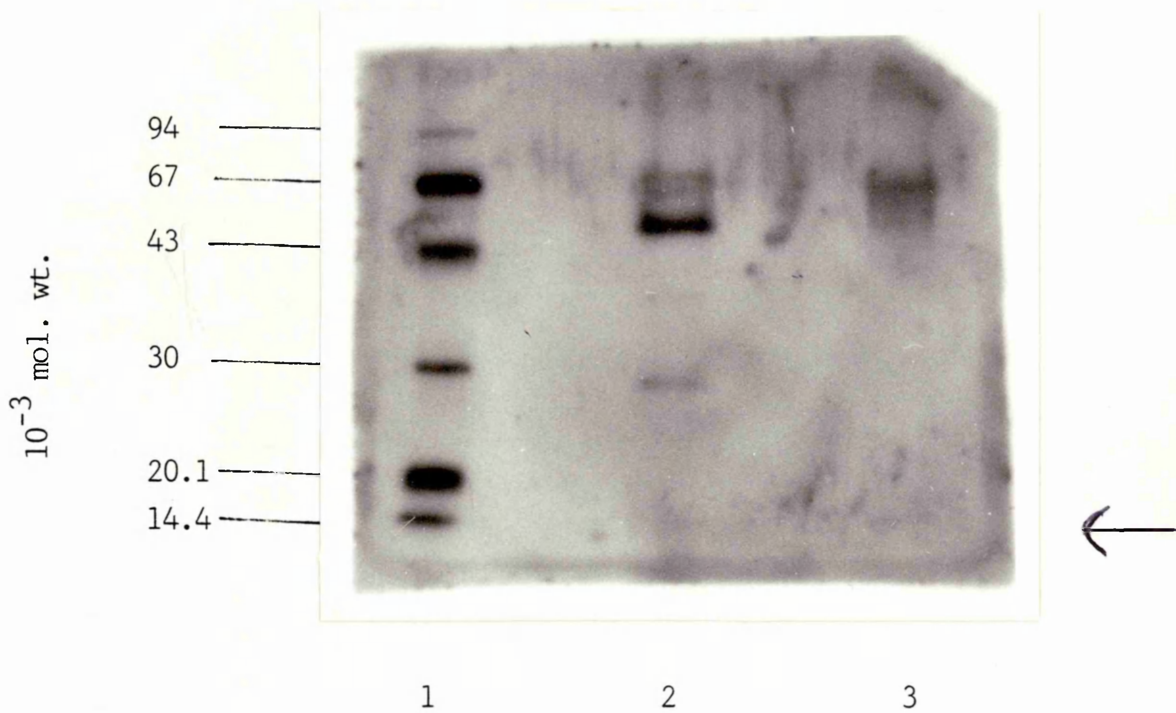


Figure 16 - Autoradiograph of nitrocellulose filter probed as described in Section 2.7.6.1 using rabbit antiserum raised against human myometrial oestrogen receptor in conjunction with ^{125}I-protein A. Electrophoresis and transfer of proteins to nitrocellulose were as described in Sections 2.7.2 and 2.7.3 respectively. Track 1 ^{125}I-molecular weight markers as described in Figure 6, Track 2 50µg nuclear Fraction B, Track 3 50µg nuclear Fraction C. Exposure time 5 days.

Fraction B contains bands at molecular weights of greater than 94,000, a doublet of bands around 67,000 and bands at approximately 55,000, approximately 38,000 and approximately 27,500. Fraction C contains two bands, one at a molecular weight of approximately 67,000 and another much fainter band at a molecular weight of approximately 15,000. The identity of these bands is uncertain, but several further experiments can be carried out to characterise them further. The bands present in this autoradiograph show that it is possible to pick out specific components of the initial crude nuclear extracts using this technique.

The next problem which must be tackled concerns the "origins" of the detected bands, i.e. do they correspond to proteins extracted initially from the myometrial nuclei and recognised by the antiserum, or do they correspond to proteins extracted from the myometrial nuclei which bind the added activated, occupied oestrogen receptor, which is then recognised by the antiserum.

To examine this problem, electrophoresis, transfer of proteins to nitrocellulose and blocking of unreacted groups using 0.5% Tween-20 was carried out as described in Section 2.7.3. The addition of activated, occupied receptor was omitted and the nitrocellulose filter probed using antiserum and ^{125}I-protein A as described in Section 2.7.6.1. The resultant autoradiograph is shown in Figure 17. As can be seen, all of the "bands" present in Lane B (Figure 16) are still present, suggesting they correspond to proteins extracted from the myometrial nuclei that are recognised by the antiserum in the absence of added oestrogen receptor. The band at molecular weight 67,000 present in Lane C (Figure 16) is still clearly visible, suggesting it too is a protein extracted from the myometrial nuclei which is recognised by the antiserum in the absence of oestrogen receptor. However the band of molecular weight approximately 15,000 is no longer visible (Figure 17) suggesting it might represent a

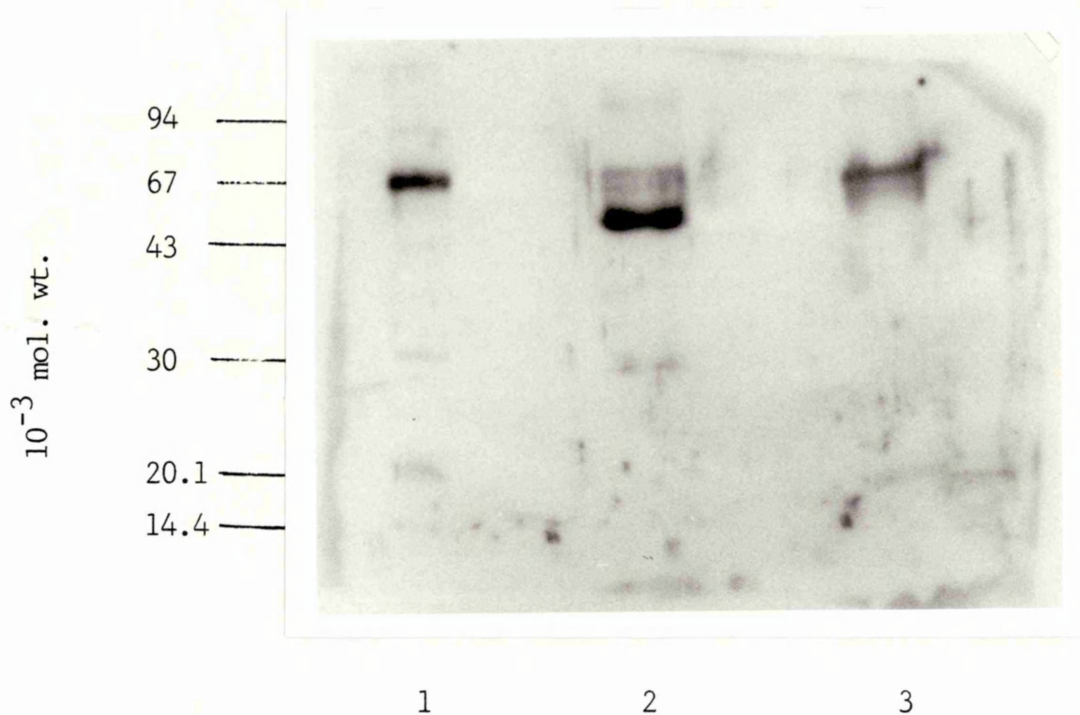


Figure 17 - Autoradiograph of a control nitrocellulose filter probed with anti-oestrogen receptor antiserum in conjunction with ^{125}I-labelled protein A as described in Section 2.7.6.1. Track 1 ^{125}I-molecular weight markers as described in Figure 6. Track 2 50µg nuclear Fraction B. Track 3 50µg nuclear Fraction C. Proteins were electrophoresed on a 12.5% SDS polyacrylamide gel as described in Section 2.7.2 and transferred to nitrocellulose as described in Section 2.7.3. Exposure time was 40 hours. Methodology was the same as described in Section 2.7.6.1 without the addition of oestrogen receptor.

protein recognised by the added activated, occupied oestrogen receptor which is then recognised by the antiserum in conjunction with ^{125}I protein A.

The next possibility to be eliminated is, do the observed bands correspond to proteins extracted from the myometrial nuclei which can bind IgG molecules which are present in serum, and are then recognised by the ^{125}I-protein A. To examine this possibility, antiserum was replaced by normal rabbit serum. All other procedures were carried out as described in Section 2.7.6.1. The resultant autoradiograph is shown in Figure 18. As can be seen, only one "band" is visible, which corresponds to that at a molecular weight of approximately 67,000(Fraction C)which can also be observed in the absence of activated occupied oestrogen receptor (see Figures 17 and 18). This suggests this "band" represents a nuclear protein capable of binding IgG molecules which are then recognised by the ^{125}I-protein A, i.e. the "band" is not induced by specific antibody molecules which recognise the oestrogen receptor but by IgG molecules in general.

The collective results from this set of experiments enable all of the observed bands except one (15,000 molecular weight band, Fraction C) to be explained. No other band involves the specific recognition of myometrial nuclear proteins, immobilised on the nitrocullulose filter, by activated, occupied oestrogen receptor, followed by subsequent identification of this complex by antiserum against the oestrogen receptor in conjunction with ^{125}I-protein A.

The antiserum has been shown to weakly cross-react with serum albumin. Preblocking of the antiserum with 3% BSA (see Section 2.7.6.1) should eliminate this problem but the observation that the 67,000 molecular weight std. (BSA) is recognised by the antiserum (see Figures 16 and 17) suggests that either the pre-blocking was not fully

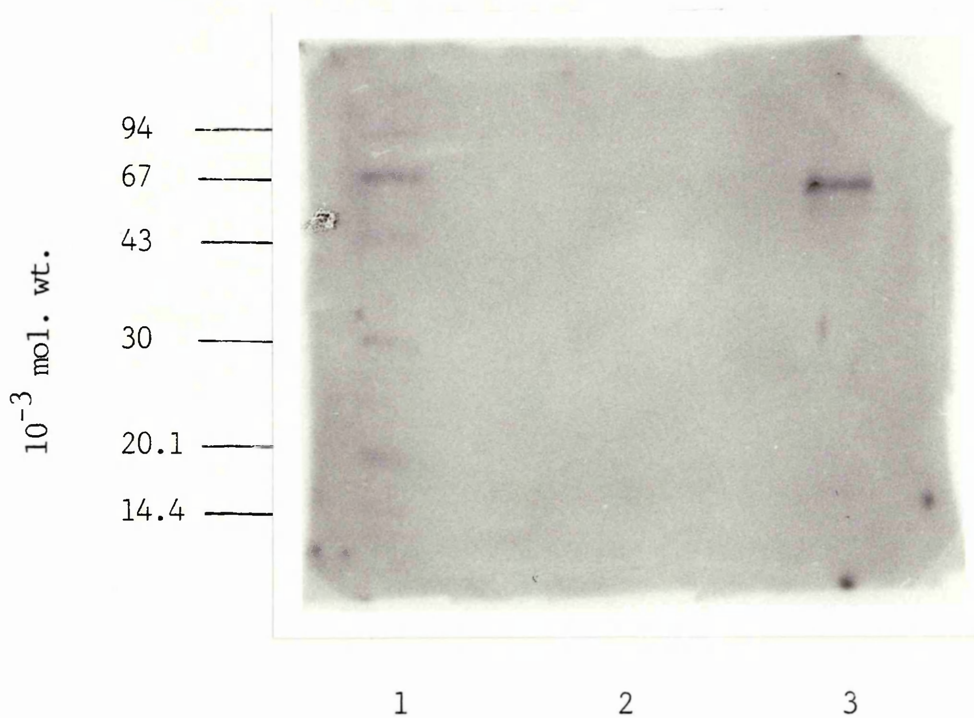


Figure 18 - Autoradiograph of control nitrocellulose filter. Track 1 ^{125}I-molecular weight markers as described in Figure 6. Track 2 50μg nuclear Fraction B, Track 3 50μg nuclear Fraction C. Proteins were electrophoresed and transferred to nitrocellulose as described in Sections 2.7.2 and 2.7.3 respectively. Methodology was as described for standard blot in Section 2.7.6.1 with antiserum being replaced by normal rabbit serum in conjunction with ^{125}I-protein A. Exposure time 40 hours.

effective or that an artifactual "band" at 67,000 is generated in the presence of antiserum/protein A following sample preparation in β-mercaptoethanol. The "band" at approximately 55,000 may also be an artifact of the sample preparation (Tasheva and Dessev, 1983).

The molecular weight of the mammalian oestrogen receptor has been estimated to fall in the range of 65,000-70,000(e.g. Sica and Bresciani, 1979; Sakai and Gorski, 1984). Hence the "bands" around 67,000 (Fraction B, Figures 16 and 17) could represent endogenous oestrogen receptor since much of this would be expected to be extracted into Fraction B. The other lower molecular weight bands seen in Fraction B are also difficult to explain. There is the possibility they represent proteolytic degradation products of the native oestrogen receptor, but this is unlikely as 10mM DFP was included in the buffer used for nuclear extraction, although proteolytic enzymes not inhibited by this reagent could be responsible for the degradation. They might also be physiological products of processing which still retain the antigenic site recognised by anti-oestrogen receptor antibody.

Overall, these experiments show a low molecular weight band (approximately 15,000) present in nuclear Fraction C, which may be capable of selectively binding activated, occupied oestrogen receptor. However the results are far from conclusive and more work must be carried out to substantiate these observations.

<u>0.1M H_2SO_4 Nuclear Extract</u>

The results from similar experiments on the 0.1M H_2SO_4 nuclear extract were complex as more "bands" could be seen in the control experiments than in the test experiment (data not shown).

The results from this set of experiments suggested that the presence of immature rat uterine cytosol had a blocking effect on the observed binding, as the greatest number of "bands" were observed in the

complete absence of cytosol. This observation is difficult to explain and contrasts with the similar experiments carried out with nuclear Fractions B and C. Further, the appearance of a mass of "bands" in a position corresponding to the core histone proteins was not observed when Fraction C was analysed. (Both the 0.1M H_2SO_4 nuclear extract and nuclear Fraction C contain the core histone proteins - see Figure 9). One possible explanation is that the two sets of experiments were carried out using different batches of antiserum, but the appearance of faint bands in the same region of the normal rabbit serum blot (data not shown), would tend to rule this out.

From these experiments it was thus not possible to designate any of the observed "bands" as being the result of interaction between a component of the 0.1M H_2SO_4 nuclear extract and activated, occupied oestrogen receptor.

3.8.4 Discussion.

The design of this set of experiments has involved an important assumption. This is, that following electrophoresis and transfer to a nitrocellulose membrane, the "acceptor" protein is still capable of being recognised by the activated, occupied oestrogen receptor complex. Preparation of samples for SDS PAGE involves the denaturation of proteins under analysis using the detergent SDS. The vast majority of protein transfer experiments have been used for the immuno-detection of specific antigens. In these experiments, an antiserum raised against the antigen in question is subsequently used to localise the antigen. The antibody/ antigen complex is then usually identified using radioactive or enzymatic means. The great specificity of this procedure enables the antigen to be detected in crude homogenates, which contain a vast protein population.

It is fairly well accepted that antibody-antigen interactions usually involve a region on the antigen molecule which corresponds to only a few amino acid residues. It thus appears that the antibody can often recognise this small region of the antigen even after the antigen has been denatured by SDS. It seems unlikely that the interaction between "acceptor" protein and activated occupied oestrogen receptor involves recognition of such a small area of the "acceptor" or receptor molecules. It may be necessary for larger areas of the "acceptor" protein molecule to "renature" before the receptor can interact with it. Several workers e.g. Sakai and Gorski (1984) have shown that some SDS-denatured polypeptides can be renatured on removal of the detergent, an operation that can be carried out more conveniently and effectively with blots. It follows from the preceding statements, that if the site on the "acceptor" protein is denatured beyond recognition by the receptor, then this technique will not help in the identification of chromatin proteins involved in binding activated, occupied oestrogen receptor. However, if some degree of renaturation occurs, it may be possible for the interaction to occur, but with lower affinity to that observed in vivo.

The results obtained from these studies do not allow any strong claims to be made, although they do offer an area to be pursued further. A very weak signal appears in Figure 16, track 3 (nuclear Fraction C) which is not induced in the absence of activated, occupied oestrogen receptor or in the presence of antiserum alone. This band may correspond to a chromatin protein acceptor activity for occupied oestrogen receptor.

The antiserum used in these studies was raised against human myometrial oestrogen receptor and had been shown to cross-react with both human and rat oestrogen receptor molecules. However, the experiments performed in this section indicate it also reacts with several other protein molecules (not necessarily breakdown products of oestrogen receptor)

extracted from human myometrial nuclei (see Figure 17). This meant any observed positive "reaction" had to be carefully scrutinised. .

When the anti-oestrogen receptor antiserum was used in protein blotting experiments involving the fraction of protein extracted from human myometrial nuclei with 0.1M H_2SO_4 the observed results were very difficult to interpret. The mass binding of antiserum in the absence of added oestrogen receptor to proteins immobilised on the nitrocellulose was most unexpected. The results suggest that extraction of protein with 0.1M H_2SO_4 (e.g. the core histones) has somehow made them immunologically reactive towards the anti-oestrogen receptor antiserum. This observation was not made when these proteins were extracted with 2M NaCl. The observation that immature rat uterine cytosol reduces the observed binding is even more difficult to explain, except that it obviously must contain some type of inhibitory molecule. No positive conclusions concerning the specific interaction of activated, occupied oestrogen receptor with nuclear proteins could be made from these experiments.
Protein blotting techniques have been used to study systems where protein-protein interactions take place e.g. Bowen et al. (1980) study histone - histone interaction using such techniques. Analysing protein ligand interactions by using protein blots is expected to develop further and provide a powerful means of studying macromolecular assemblies. To do so, conditions for more efficient renaturation of the protein should be explored e.g. more careful removal of denaturing agents and possibly regeneration of disulphide bridges in situ.

It has also been possible to use "blotting" techniques to study specific DNA-protein interactions (Bowen et al., 1980). If this "blotting" system enabled specific "acceptor" proteins to be identified it may then be possible to identify regions of genomic DNA which interact specifically with these "acceptor" proteins.

4. General Discussion

The modulation of gene expression by steroid hormones offers a number of useful model systems to study how the expression of a gene or set of genes can be positively regulated at the transcriptional level in animal cells.

It is generally thought that the effect of steroid hormones is mediated by hormone-specific intracellular proteins that associate with specific DNA or chromatin sites upon binding the hormone ligand (Mulvihill et al., 1982). It is assumed that this receptor-genome interaction is the primary event in the induction of transcription of genes which are programmed to respond to a given steroid hormone in a given differentiated target cell.

Mechanistic schemes for steroid hormone action highlight the influence of the nuclear steroid-receptor complex on gene expression, a feature common to steroid hormones involved both in cellular homeostasis and in growth processes. This concept has, for two decades, been considered the key feature of the molecular mechanism of steroid hormone action and stems from the rapid accumulation and retention of steroids within target cell nuclei after exposure to the steroid in question (Clark and Peck, 1979). Circumstantial evidence has thus popularised the oestrogen receptor complex (and all other steroid receptor complexes) as an intracellular regulator of gene expression. Its selective distribution throughout chromatin should be tissue specific, determined by "acceptor sites" and should influence expression of the relevant genes.

Steroid-receptor complexes act by interaction with or displacement of chromatin associated proteins to reveal initiation sites. This interaction with chromosomal proteins and/or nucleotide sequences is thought to create favourable promotor sites, by assisting in the unwinding of DNA, by promoting elongation factors or by inactivating termination factors.

The nature of the acceptor site or region is one of the many questions which, if answered, would increase our understanding of the mechanism of steroid hormone action at the molecular level. Rapidly advancing technology has accelerated certain aspects of our understanding of the mehanism of steroid action, but it has tended to occlude some of the problems. The nature of acceptor proteins has been abandoned by all but a few workers. The majority of work in this area has been carried out by Spelsberg and his co-workers (Spelsberg, 1982; Spelsberg et al., 1983). Many other groups have focussed their attention on the direct interaction of steroid-receptor complexes with the DNA of steroid inducible genes or the regions of DNA which are adjacent to these genes (e.g. Compton et al., 1983; Payvar et al., 1983; Cato et al., 1984; Compton et al., 1984; Maurer et al., 1985). However, as discussed previously, the relative affinity of these regions of DNA for the appropriate steroid-receptor complex is insufficient to explain the rapid way in which incoming steroid-receptor complexes are trapped within the nucleus. These observations support the involvement of other nuclear components in the nuclear binding of steroid-receptor complexes.

Data is presented in this thesis which supports the specific saturable interaction of oestrogen receptor complexes with nuclear components which can be extracted from human myometrial nuclei with either 2M NaCl or 0.1M H_2SO_4 (see Section 3.2). The observation that proteolytic enzymes can reduce the level of observed interaction suggests that chromosomal proteins may be responsible for the binding activity. The high affinity binding which is observed (Kd 10^{-11}) is of the correct order of magnitude to be physiologically significant and is certainly higher than the affinity of interaction observed between steroid-receptor complexes and the DNA from hormonally regulated genes. This data would

support the involvement of chromatin proteins in acceptor site function which has been suggested by several workers previously (Puca et al., 1974; 1975; Mainwaring et al., 1976; Spelsberg, 1982; Spelsberg et al., 1983). It has also been suggested that chromosomal proteins present at initiation sites lower the activation energy required for the opening of the DNA helix in forming a stable complex with the RNA polymerase. Such proteins would be expected to be major determinants in the location of initiation sites and perhaps be composed of a variety of proteins capable of destabilising DNA (Thomas and Patel, 1976). It is possible that interaction of a steroid hormone-receptor complex with such an area of chromatin protein would make the formation of a stable initiation complex even more favourable. Naturally, the three dimensional structure of "acceptor sites" in vivo will involve both the DNA and the acceptor proteins.

Steroid-receptor complexes may function at a number of levels to control gene expression. Multiple receptor binding domains of varying affinity may be involved. Such binding domains may be involved in determining priority effects, in maintenance of an overall structure conducive to transcription, more efficient utilisation of promoters or influencing DNA-protein interactions to facilitate higher rates of elongation within a gene or among genes of differing susceptibility. This would account for the large number of acceptor sites shown here and the spectrum of physiological response relative to numbers of occupied sites. However, the techniques used were not sufficiently sensitive to allow separation of acceptor sites with minor differences in affinity for oestrogen receptor.

The suggestion that the oestrogen receptor may be, at all times resident, in the nucleus (King and Greene, 1984; Welshons et al., 1984) increases the likelihood of binding sites of differing affinity

being present. Unoccupied receptor is readily extracted from the nucleus at low salt concentrations, while high salt is necessary to extract activated or transformed receptor. This might suggest that following oestrogen binding, the oestrogen-receptor complex may be relocated to an area of the genome which has a higher affinity for the receptor complex and is responsible for regulation of the expression of oestrogen inducible genes. Another possibility is that no relocation occurs, but the receptor is located in close proximity to the acceptor site at all times. Binding of ligand is necessary to induce expression of the adjacent gene. Of course, nuclear localisation of the oestrogen receptor is under debate (Szego and Pietras, 1985) and the preceding statements are pure speculation.

A very major problem to be overcome is the characterisation of the chromatin proteins which are involved in acceptor site function. These proteins are inherantly difficult to work with because of their hydrophobic nature and general insolubility. The attempts made to characterise such proteins so far have involved the use of high concentrations of denaturing agents. The use of such harsh regimes must risk irreversible loss of biological activity such that assay of the isolated proteins for acceptor activity will fail. Several attempts have been made to characterise such proteins (Puca et al., 1975; Mainwaring et al., 1976). However, the greatest efforts to narrowly define the chromatin protein fraction which demonstrates acceptor activity have been made by Spelsberg and his co-workers (Spelsberg et al., 1983). If the characterisation of a single protein or narrow group of proteins could be achieved in several other systems, it would surely swell support for the involvement of chromatin proteins in acceptor function.

In conclusion, the data presented in this thesis support the involvement of non-histone chromatin proteins in acceptor site function but further work, especially on the characterisation and isolation of the protein(s) responsible for the binding activity is required.

The data presented in this thesis contribute to the general concept of selective acceptor proteins. However, methodological problems complicate the final interpretation. These can be discussed as follows.

Nuclear Purification

The purity of a nuclear preparation can be assessed in a number of different ways. In the present study a combination of the DNA:Protein ratio of the final nuclear pellet and its appearance under the phase contrast microscope were used. Figure 5 shows an example of a final nuclear pellet under the phase contrast microscope. Careful examination of this figure reveals that the structures present are nuclei with prominent nucleoli visible in many of the nuclei. Many of the nuclei are also long and thin indicating their probable origin as the elongated muscle cells of the myometrium. Previous examination in our laboratory of such nuclei, using scanning and transmission electron microscopy, has indicated considerable structural integrity of the purified nuclei. For example, any possible contamination by endoplasmic reticulum is detected by this method. It is, however, unlikely that endoplasmic reticulum contamination will represent a major problem as the nuclei have been passed through 0.2% Triton X-100 which will 'strip off' both the outer nuclear membrane and the endoplasmic reticulum which is attached to it.

The major problem in using the DNA:Protein ratio as an indication of nuclear purity is that it is not constant from one tissue to another. Although the DNA content of somatic cell nuclei is the same, the amount of non histone chromatin protein they contain is not (Allfrey, 1971). Tissues which are metabolically active e.g. liver, contain greater amounts of non histone chromatin proteins, hence their DNA:Protein ratios are lower than those of metabolically relatively inactive tissues such as myometrium. The typical DNA:Protein ratio of

the final nuclear preparation obtained during these studies was 0.5 - 0.6 which is in broad agreement with the value of 0.5 for nuclei purified from chick oviduct (Spelsberg, 1976).

Another method by which nuclear purity can be assessed is by the determination of various enzyme activities. One approach would be to assay the purified nuclei for enzymes which are known to be nuclear in origin e.g. RNA polymerase (Widnell and Tata, 1964) but a more satisfactory method is the assay of enzyme activities which are known to be located elsewhere in the cell. Examples of these include cytochrome oxidase and glucose 6-phosphatase (Widnell and Tata, 1964). However, enzyme relocation, activation or destruction may modify the results obtained and all the systems available have built within them the hazards inherent in cell fractionation and differential centrifugation. The best idea of nuclear purity and integrity is achieved through the use of microscopic techniques in conjunction with some biochemical parameter.

Protein Assay

The hydrophobic nature and general insolubility of chromatin associated proteins poses problems for their analysis. Table 1 shows the typical protein content of the various nuclear protein fractions as analysed by the method of Bradford (1976). This method of analysis is not ideal for all of the protein fractions and must be considered a compromise, as no one assay method proved ideal for all the nuclear protein fractions. The presence of a reasonably high concentration of salt or denaturing agent is required to maintain most fractions in solution *in vitro*. Both nuclear fraction C and the 0.1M H_2SO_4 nuclear extract represent this class of protein.

Initial protein assays on the various nuclear fractions were carried out using the method of Lowry *et al*. (1951). This method proved reliable for nuclear fraction A and B but in the case of fraction C problems arose with precipitation of the Folin reagent in the presence of

the high salt. This problem did not arise on every occasion, but frequently enough to make protein estimation by this method unreliable. As an alternative the method of Bradford (1976) was used. This method was unreliable for estimating the protein content of Fraction A as the methods required to solubilise the proteins present in this fraction i.e. either alkali or detergent subsequently interfered with the assay. The Bradford micro-assay was used to estimate the protein content of Fraction B and the standard assay procedure was used to assay the protein content of Fraction C. No problems of reagent precipitation were encountered using this method on Fraction C. The protein content of the 0.1M H_2SO_4 nuclear extract was also assayed using the standard Bradford procedure.

However, when compared to the protein content of the intact nuclei, the recovery of protein into nuclear Fractions A, B and C was only approximately 40%, suggesting that there may be further problems with the Bradford protein assay. Figure 6 shows an SDS polyacrylamide gel of nuclear Fractions B and C. Closer inspection of the gel reveals that although the calculated amount of protein loaded in each track is the same (20ug) more protein has actually been loaded in track 3, which corresponds to nuclear Fraction C. This observation suggests that not all of the protein present in nuclear Fraction C is being detected using the Bradford method. It is possible that, on the addition of the Bradford reagent, the resulting dilution renders some of the protein insoluble, hence it is not detected. Figure 9 shows an SDS polyacrylamide gel of nuclear Fraction C and the 0.1M H_2SO_4 nuclear extract. Again the calculated amount of protein loaded in each track is the same (20ug), but more protein has actually been loaded in track 3 (the 0.1M H_2SO_4 nuclear extract). This suggests the problem outlined for Fraction C may be even more pronounced for the H_2SO_4 extract. An additional reason for the apparent low protein recovery may

be that further protein may be trapped and unassayable in Fraction A as the material remaining after the nuclei had been exposed to 2M NaCl was very 'sticky' and almost impossible to redissolve.

The above observations indicate the difficulties in accurately determining the protein content of nuclear Fractions A and C. In the case of nuclear Fraction B, the observation that both the method of Lowry et al. (1951) and that of Bradford (1976) gave comparable results indicates that it was possible to determine the protein content of this fraction with reasonable accuracy.

The fact that it was not possible to accurately determine the total protein content of all of the nuclear fractions also means that the actual amount of nuclear protein immobilised on the Sepharose 4B from each fraction must be uncertain. Since this is the case, the assignment of relative quantitative abilities to the nuclear Fractions A, B and C to specifically bind activated labelled oestrogen receptor is difficult as these were expressed per mg of immobilised nuclear protein. In the case of nuclear Fraction A the general insolubility problems which arose made accurate assessment of nuclear binding sites impossible. In the case of nuclear Fractions B and C it was possible to maintain the protein in solution to enable coupling to the Sepharose 4B to take place. However, as discussed previously Fraction C appears to contain more protein than the amount assayed. Hence, attempts to couple equal amounts of nuclear Fractions B and C to Sepharose (see Table 8) and then assay for binding activity towards activated labelled oestrogen receptor are subject to error. Nevertheless, Table 8 shows that Fraction B exhibits less than 10% of the binding activity shown by Fraction C. It seems unlikely that the protein content of Fraction C is tenfold greater than that of Fraction B (see Figure 6) and as such is probably correct that Fraction C is enriched in binding sites when compared to Fraction B. It must also be borne in mind that the aims of these investigations were to identify

specific binding sites for activated, labelled oestrogen receptor complexes. The data in Table 8 show that both nuclear Fractions B and C contain such binding sites. One obvious question is whether the same protein molecule(s) are involved in the binding activity shown by the different nuclear fractions or does each fraction contain unique molecules capable of specifically interacting with the activated, labelled oestrogen receptor? Hopefully, further investigation using, for example, antibody probes would provide an answer to this question.

In experiments which examined the binding of different steroid receptor complexes to the same batch of nuclear extract/sepharose resin, a knowledge of the exact immobilised protein content of the resin was of less importance. The main reason for this being that the appropriate nuclear extract/sepharose resin (either Fraction C or the 0.1M H_2SO_4 nuclear extract) remained constant throughout these experiments, whilst the nature of the steroid receptor complex was varied.

General Discussion

It is generally thought that the effect of steroid hormones is mediated by hormone specific intracellular proteins that associate with specific DNA or chromatin sites upon binding the hormone ligand (Mulvihill *et al*., 1982). It is assumed that this receptor-genome interaction is the primary event in the induction of transcription of genes which are programmed to respond to a given steroid hormone in a given differentiated target cell. This concept has been considered the key feature of the molecular mechanism of steroid hormone action and stems from the rapid accumulation and retention of steroids within target cell nuclei after exposure to the steroid in question (Clark and Peck, 1979). Circumstantial evidence has thus popularised the oestrogen receptor complex (and all other steroid receptor complexes) as an intracellular regulator of gene expression. Its selective distribution throughout chromatin should be tissue specific, determined by acceptor

sites and should influence expression of the relevant genes. The exact mechanism by which steroid receptor complexes alter the expression of specific genes is still unclear. Once in the nucleus, the steroid receptor complex must rapidly locate the DNA sequence(s) responsible for steroid induced gene activation. The affinity of interaction between steroid receptor complexes and DNA is too low to explain the rapid time course of these events hence it is likely that a much higher affinity interaction with a chromatin protein 'flag' is necessary to attract the steroid receptor complex to the correct DNA sequence and thus establish initiation sites.

Data is presented in this thesis which shows that the material extracted from purified human myometrial nuclei with 2M NaCl or 0.1M H_2SO_4 is capable of specifically binding activated, labelled oestrogen receptor from a variety of sources (see Table 2). The data presented in Figure 11 show that the majority of the observed binding is due to activated, labelled oestrogen receptor. Other cellular proteins capable of binding E_2 cannot induce the same level of binding to the nuclear extract sepharose resins. It was possible to observe the specific binding of activated, labelled oestrogen receptor complex to the nuclear Fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins at physiological pH and almost physiological ionic strength.

These data are in broad agreement with those of Mainwaring _et al_. (1976) and Puca _et al_. (1974; 1975) who report a salt-extract binding activity for androgen and oestrogen receptor complexes respectively. Subsequently Puca _et al_. (1975) reported that the binding activity could also be extracted from target cell nuclei using H_2SO_4. Their data regarding the 0.1M H_2SO_4 extract of human myometrial nuclei are in agreement with those described here.

Sodium molybdate has been shown to inhibit the process of oestrogen receptor activation as measured by DNA binding activity

(Pettersson et al., 1982). In this study, oestrogen receptor prepared in the presence of 20mM molybdate was still capable of being bound by both the nuclear Fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins. These observations suggest that molybdate does not interfere either directly, or via protein conformational changes with the binding site on the receptor recognised by both of these nuclear fractions. These data also suggest that the DNA binding site of the oestrogen receptor complex and the binding site which is active in the present studies are distinct.

Interestingly, Littlefield and Spelsberg (1985) report that molybdate stabilised progesterone receptor still binds to oviduct chromatin at a level of 40% of that seen with activated, fully functional receptor. However, the binding to chromatin of this inactive progesterone receptor is unsaturable - presumably because the subsequent interaction with DNA is inhibited.

The chemical nature of the 'acceptor' site, or region, is a question which must be answered before we understand the mechanism of steroid hormone action at the molecular level. Experiments with cloned genes, containing only DNA, will not give us the true physiological picture. It is generally considered that nuclear associated proteins ultimately control the acceptor function in either an active or passive way. Supporters of the passive theory (Mainwaring and Peterken, 1971) suggest that DNA is the acceptor whereas nuclear proteins restrict the binding sites available on DNA. The active theory (Spelsberg et al., 1971) attributes all acceptor activity to the nuclear proteins with little, if any, involvement of DNA. However, more recently Spelsberg et al. (1984) have shown that in the chick oviduct the acceptor sites for progesterone receptor comprise a specific set of chromatin proteins and a limited number of specific DNA sequences. Current data suggest that the binding of steroid receptor complexes to chromatin protein acceptor sites is characterised by specificity, saturability and high affinity

(Mainwaring et al., 1976; Puca et al., 1974; 1975). These features are not necessarily evident in the binding of receptor complexes to immobilised DNA (Puca et al., 1974).

Rapidly advancing technology has enabled many groups to focus their attention on the direct interaction of steroid receptor complexes with the DNA of steroid inducible genes or the regions of DNA which are adjacent to these genes (e.g. Compton et al., 1983; Payvar et al., 1983). However, as discussed previously, the relative affinity of these regions of DNA for the appropriate steroid receptor complex is insufficient to explain the rapid way in which incoming steroid receptor complexes are trapped within the nucleus. These observations support the involvement of other nuclear components in the nuclear binding of steroid receptor complexes.

The data presented in Section 3.3 of this thesis show that in this in vitro binding system the interaction of activated, labelled oestrogen receptor complex with chromatin associated proteins is being examined. Only proteolytic enzymes are capable of reducing the observed binding activity to any significant extent, DNase and RNase treatment had little effect on the observed binding activity (see Tables 9, 10, 11 and 12). However, it is likely that the environment presented to the oestrogen receptor complex in this in vitro binding system is very different from that found in vivo, especially in respect to the absence of DNA and RNA (Poonian et al., 1971). Previous work has shown that DNase treatment of uterine nuclei releases bound oestrogen receptor (e.g. King and Gordon, 1972) and RNase treatment of 'HeLa' cell nuclei causes a release of glucocorticoid receptor (Rossini, 1984). No specific role for RNA in nuclear binding has become apparent, although Lin and Ohno (1983) have reported selective interaction of oestrogen receptor from hen oviduct with poly A RNA and a similar interaction of mouse kidney androgen receptor with RNA (mRNA, tRNA and rRNA) (Lin and Ohno, 1981).

Feldman et al. (1981) report that cytosol from MTW9 rat mammary tumour contains a high molecular weight inhibitor of oestrogen receptor binding to DNA. RNase treatment destroys the inhibitory activity, suggesting the involvement of an RNA molecule. A similar situation has been reported for the binding of both oestrogen and glucocorticoid receptors from MCF7 cells to DNA cellulose (Chong and Lippman, 1982). These observations suggest that steroid receptor-RNA interactions may play a role in regulation of gene expression by steroid hormones.

By convention, non histone chromatin proteins are generally considered to be acidic in nature, although as discussed in Section 1.5.4.1.2 care must be exercised in the absolute classification of these proteins. Reports of both acidic and basic non histone chromatin proteins being involved in acceptor activity have been published. Puca et al. (1974; 1985) show that the acceptor activity for oestrogen receptor in calf uterine nuclei resides in the basic, but non histone chromatin protein fraction as do Mainwaring et al. (1976) for the acceptor activity towards androgen receptor in rat prostate nuclei. Conversely, the acceptor activity for progesterone receptor in the chick oviduct system has been shown quite clearly to reside in the acidic non histone chromatin protein fraction (Spelsberg, 1982; Spelsberg et al., 1983).

The detection of multiple binding sites in vitro suggests that more than one protein or protein fraction is involved in nuclear in vivo (Ruh et al., 1981). From the data presented in this study it is not clear what type of nuclear protein component is involved, although the observation that the binding activity can be solubilised with H_2SO_4, as is described by Puca et al. (1975) suggests that the nuclear proteins involved may be of basic overall charge.

It has also been suggested that chromosomal proteins present at initiation sites lower the activation energy required for the opening of

the DNA double helix in forming a stable complex with the RNA polymerase. Such proteins would be expected to be major determinants in the location of initiation sites and perhaps be composed of a variety of proteins capable of destabilising DNA (Thomas and Patel, 1976). It is possible that the interaction of a steroid hormone receptor compelx with such an area of chromatin protein would make the formation of a stable initiation complex even more favourable. Naturally, the three dimensional structure of 'acceptor sites' in vivo will involve both the DNA and the acceptor proteins.

Characteristics expected of an acceptor site are, (1) high affinity binding, (2) a limited number of binding sites must exist and (3) steroid specificity.

It is now generally accepted that under defined conditions of pH and ionic strength, it is possible to observe saturable binding of steroid receptor complexes to target cell nuclei, chromatin or isolated chromatin components in vitro (Spelsberg et al., 1976a). However, physiological ionic conditions are probably important for specificity.

The data presented in Section 3.4.1 and 3.4.2 show that both the 0.1M H_2SO_4 nuclear extract and nuclear fraction C/sepharose resins contain a limited number of specific saturable binding sites for activated labelled oestrogen receptor complex. Saturation analysis provides an insight into the characteristics of the binding sites at a concentration of steroid which falls within the physiological range. Saturation analysis was also carried out at an ionic strength which is known to extensively dissociate receptor complex from intact nuclei i.e. 0.4 - 0.6M KCl (Clark and Peck, 1979). It is well accepted that nuclear binding can be divided into a form which is salt extractable and a form which is not extracted under these conditions (Clark and Peck, 1979). It is not certain which of these two types of nuclear binding is physiologically important i.e. which results in specific gene

activation. Hence, the assumption that the binding observed in the presence of 0.5M KCl represents non-specific binding may not be strictly correct, as receptor can still be bound in the remaining nucleus under such conditions. However, the binding observed under these conditions was never saturable and was of lower affinity than the specific saturable binding observed in 0.12M KCl.

The apparent dissociation constants for the interaction of activated, labelled oestrogen receptor with both nuclear fraction C and the 0.1M H_2SO_4 nuclear extract/sepharose resin (table 13) are in reasonable agreement with values published concerning the interaction of oestrogen receptor complex with intact nuclei and sub nuclear components _in vitro_ (Higgins _et al_., 1973; Puca _et al_., 1974, 1975; Mainwaring _et al_., 1976; Kon and Spelberg, 1982; DeBoer _et al_., 1984). They are of the correct order of magnitude to be physiologically significant when compared to the low levels of circulating free steroid bound _in vivo_ and are certainly higher than the affinity of interaction observed between steroid receptor complexes and the free DNA from hormonally regulated genes.

A great deal of progress has been made recently regarding the interaction of various steroid receptor complexes with specific DNA sequences. However, the apparent Kd for the interaction of oestrogen receptor complexes with a variety of heterologous DNA molecules is only $4\text{-}6 \times 10^{-4}$M (Buller and O'Malley, 1976). The affinity of the interaction reported for steroid receptor complexes with specific DNA consensus sequences is only 10-40 times greater than this (Chambon _et al_., 1984) which still represents binding of insufficient affinity to explain the physiological effects of steroid hormones at the very low concentrations at which they occur _in vivo_. For the sake of comparison, the lac repressor protein binds to its specific DNA sequence in the lac operon region of the _E.coli_ genome with an apparent Kd of approximately

10^{-13}M (Riggs et al., 1970), this being a classic example of a protein whose in vivo effects are realised through interaction with a specific DNA sequence.

For many years Spelsberg has supported the idea that both DNA and protein have a role to play in the three dimensional structure of 'acceptor' sites for steroid hormone receptor complexes in vivo, a view that is shared by Leake (1981). The strongest evidence to support this proposal so far comes from Spelsberg et al. (1984) who report that only a limited number of specific DNA sequences in the avian genome which can interact with the chromatin protein fraction they have previously shown to possess binding activity towards the chick oviduct progesterone receptor.

Steroid receptor complexes may function at a number of levels to control gene expression. Multiple receptor binding domains of varying affinity may be involved. Such binding domains may be involved in determining priority effects, in maintenance of an overall structure conducive to transcription, more efficient utilisation of promoters or influencing DNA-protein interactions to facilitate higher rates of elongation within a gene or among genes of differing susceptibility. This would account for the large number of acceptor sites shown here and the spectrum of physiological response relative to the numbers of occupied sites. However, the techniques used were not sufficiently sensitive to allow separation of acceptor sites with minor differences in affinity for oestrogen receptor.

The data presented in Section 3.5 show that the protein fractions isolated from human myometrial nuclei using 2M NaCl and 0.1M H_2SO_4 contain specific binding activities towards steroid receptor complexes other than oestrogen receptor complex. The observation that both fractions do not contain specific binding sites for all the steroid receptor complexes assayed indicates that there is a selective extraction of binding sites by each extraction procedure.

The analysis of competition between steroid receptor complexes for binding sites on both the nuclear fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins (Section 3.6) suggests that although there appear to be unique binding sites for each steroid receptor complex there are also many common binding sites. This data is in conflict with that of Kon and Spelsberg (1982) who report no antagonism between oestrogen and progesterone receptor complexes for binding sites in hen oviduct nuclei although the kinetics of conalbumin mRNA induction by both nuclear oestrogen and progesterone receptor complexes is very similar implying that a common binding site may be involved (Mulvihill and Palmiter 1977; 1980). Higgins et al. (1983) have reported unique binding sites for both glucocorticoid and oestrogen receptor complexes in immature rat uterine nuclei. These studies, and those of Kon and Spelsberg (1982) examined the binding to intact nuclei, no attempt was made to analyse the binding of receptor complexes to nuclear components. The findings in this thesis when compared to those published observations may indicate that the three dimensional structure of the intact acceptor site in vivo may only be recognised by a particular class of steroid receptor complex. Once this structure has been disrupted during the extraction procedures involved in the present studies, a loss in the absolute specificity of steroid receptor complex binding may occur.

Von der Ahe et al. (1985) have shown that there is an overlapping pattern of binding of glucocorticoid and progesterone receptor complex to the chicken lysozyme promoter, since not all genes responsive to glucocorticoid are responsive to progesterone it may be that the absolute specificity of steroid hormone induced gene expression is provided by the presence of specific acceptor proteins in conjunction with a sequence of DNA in the 5' region of the hormonally regulated gene.

The data presented in Section 3.5 shows quite clearly that both the nuclear fraction C and 0.1M H_2SO_4 nuclear extract/sepharose

resins possess a greater number of binding sites for activated, labelled oestrogen receptor complexes than they do for anti-oestrogen/oestrogen receptor complexes. However this may be due to the inability of tamoxifen to cause complete activation of the oestrogen receptor population (Mester et al, 1981). To answer this question the experiments should be repeated using the activate metabolite 4-hydroxytamoxifen. The analysis of the antagonism between oestrogen receptor complexes and anti-oestrogen receptor complexes for binding sites in both the nuclear fraction C and 0.1M H_2SO_4 nuclear extract/sepharose resins again indicates that although there appears to be unique binding sites for both forms of the oestrogen receptor complex some common binding sites are present. The idea of non-identical nuclear binding sites for oestrogen and antioestrogen receptor complexes is not a new one (Baudendistal and Ruh, 1976; Massol et al., 1978; Lebeau et al., 1981; Singh et al., 1984) and may be the root of the different physiological responses induced by oestrogens and antioestrogen in some systems.

The observation that the nuclei isolated from various tissues (including non target tissues) of the immature female rat contained specific binding sites for activated, labelled oestrogen receptor complex indicates that the nuclear protein(s) responsible for the binding are not confined to target tissues (see Section 3.7). There have been a variety of reports that target cell nuclei for different steroids contain markedly more acceptor sites than non target cell nuclei, using cell free binding assays (e.g. Mainwaring and Peterken, 1971; Spelsberg et al., 1972; Puca et al., 1975; Mainwaring et al, 1976; Pikler et al., 1976; de Boer et al., 1984) and these greatly outnumber reports of no differences in nuclear binding between the chromatin of target and non target tissues (e.g. Higgins et al., 1973). The detection of these specific binding sites in non target tissues of the immature female rat gives no idea of the role of these binding sites have to play in vivo.

It may be that in vivo these binding sites are 'masked' (Spelsberg, 1982; Spelsberg et al., 1983) and therefore concealed from activated oestrogen receptor, even if it were present in such non target cells. The extraction procedure used to isolate nuclear fraction C will extract the binding sites and presumably any masking proteins associated with them. Extraction will almost certainly disrupt any interaction which was present between them and hence in this in vitro system the binding proteins are available for assay.

The biological role of such a masking phenomenon is uncertain. One possible function is that it regulates which genes will respond to steroid receptor complex and at what time. If so, it would help explain why different target tissues of the same organism display markedly different responses to the same steroid with regard to gene expression. Recent evidence indicating that the steroid receptors in different tissues from different animals are antigenically similar (Greene et al., 1980; Greene and Jensen, 1982) minimises the tissue specificity of the receptors themselves. In short, if a gene is masked it is not available for transcription but if unmasked is available for transcription in the present of the appropriate steroid receptor complex. The masking phenomenon does not appear to be confined to the chick oviduct (Spelsberg, 1982) but has been reported for androgens in the rat prostate (Klyzsejko-Stefanowicz, 1976) oestrogen and progesterone in the sheep brain (Perry and Lopez, 1978) and oestrogen in rat and bovine uterus (Ruh et al, 1981).

Without doubt, one of the major problems which still remains is that of isolating and characterising acceptor proteins. Early attempts to characterise such proteins lead Puca et al. (1975) to conclude the binding protein for oestrogen receptor complex in calf uterine nuclei was of basic overall charge and had a molecular weight of approximately 85,000, and Mainwaring et al. (1976) to conclude the binding protein for

androgen receptor in the rat prostate was a basic protein of molecular weight approximately 70,000. However, the majority of attempts to isolate and characterise acceptor proteins have been made by Spelsberg and his co-workers (Spelsberg, 1982; Spelsberg et al., 1983). Working with the avian oviduct system they conclude that the majority of acceptor activity can be assigned to a group of proteins with a molecular weight range of 14,000-18,000. Isoelectric focussing has shown that the group can be resolved into two major peaks, one of which focussed around pH5.0 and another which focussed in the range of pH6 to pH7.5. This suggests that these proteins belong to the acidic non histone protein fraction. Using protein blotting techniques, this study has attempted to identify the proteins present in the nuclear fractions which are responsible for the observed binding activity towards activated, labelled oestrogen receptor (see Section 3.8). Figure 16 shows what may be a very weak signal at a molecular weight of approximately 15,000, however, further work is required to substantiate these findings. The major problem encountered during these studies was that of non-specific background binding, which may have obscured other very weak signals.

If it were possible to identify specific 'acceptor' proteins using this approach it may then be possible to identify specific regions of genomic DNA which interact with the acceptor proteins, to complete the three dimensional structure of the acceptor site *in vivo*. the analysis of the specific interactions between the lac repressor protein and DNA contain the lac operator has been reported by Bowen et al. (1980) using similar techniques.

In conclusion, the data presented in this thesis support the involvement of non-histone chromatin proteins in acceptor site function, but further work, especially on the characterisation and isolation of the protein(s) involve is required to increase our understanding of the acceptor site *in vivo*.

The suggestion that the oestrogen receptor may be, at all times resident in the nucleus (King and Green, 1984; Welshons et al., 1984) increases the likelihood of binding sites of differing affinity being present. Unoccupied receptor is readily extracted from the nucleus at low salt concentrations, while high salt is required to extract activated receptor. This might suggest that following oestrogen binding, the receptor complex may be relocated to an area of the genome which has a higher affinity for the receptor complex and is responsible for regulation of the expression of oestrogen inducible genes. Another possibility is that no relocation occurs but the receptor is located in close proximity to the acceptor site at all times. Binding of the ligand is necessary to induce expression of the adjacent gene. Of course, nuclear localisation of the oestrogen receptor is under debate (Szego and Pietras, 1985) and the preceding statements are pure speculation.

5. References

Agutter B.S. & Birchall, K. (1979) Functional differences between mammalian nuclear protein matrices and pore-lamina complex laminae. Experimental Cell Research,. 124, 453-466.

Alberga, A., Massol, N., Raynaud, J.P. & Baulieu, E.E. (1971) Oestradiol binding of exceptionally high affinity by a non-histone chromatin protein fraction. Biochemistry, 10, 3835-3843.

Allfrey, V.G. (1971) Functional and metabolic aspects of DNA-associated proteins. In Histones and Nucleohistones, ed. Philips, D.M.P., Plenum Press, London and New York.

Anderson, J.N., Peck, E.R. Jnr. & Clark, J.H. (1975) Oestrogen-induced uterine responses and growth : relationship to receptor oestrogen binding by uterine nuclei. Endocrinology, 96, 160-167.

Andre, J. & Rochefort, H. (1975) In vitro binding of the oestrogen receptor to DNA : absence of saturation at equilibrium. FEBS Letters, 50, 319-323.

Andre, J., Pfeffer, A. & Rochefort, H. (1976) Inhibition of oestrogen receptor DNA interaction by intercalating drugs. Biochemistry, 15, 2964-2969.

Andrews, G.K., Dziadek, M. & Tomaski, T. (1982) Expression and methylation of the mouse α fetoprotein gene in embryonic, adult and neoplastic tissues. Journal of Biological Chemistry, 257, 5148-5153.

Aten, R.F., Weinberg, M.J. & Eisenfield, A.S. (1978) Oestrogen receptor in rat liver : translocation to the nucleus in vivo. Endocrinology, 102, 433-442.

Atger, M. & Milgrom, E. (1978) Interaction of glucocorticoid-receptor complexes with rat liver nuclei. Biochemica et Biophysica Acta, 539, 41-53.

Auricchio, F., Migliaccio, A & Rotondi, A. (1981) Inactivation of oestrogen receptor in vitro by nuclear dephosphorylation. Biochemical Journal, 194, 569-574.

Auricchio, F., Migliaccio, A., Castoria, G., Rotondi, A. & Lastoria, S. (1984) Direct evidence of in vitro phosphorylation/dephosphorylation of the oestradiol-17β receptor, role of Ca^{2+}-calmodulin in the activation of hormone binding sites. Journal of Steroid Biochemistry, 20, 31-35.

Axen, R., Porath, S. & Ernback, S. (1967) Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. Nature (London), 214, 1302-1304.

Baer, B. & Rhodes, M. (1983) Eukaryotic RNA polymerase II binds to nucleosome cores from transcribed genes. Nature (London), 301, 482-488.

Bailly, A., Sallas, N. & Milgrom, E. (1977) A low molecular weight inhibitor of steroid receptor activation. Journal of Biological Chemistry, 252, 858-863.

Bailly, A., Le Ferve, B., Savouret, J.F. & Milgrom, E. (1980) Activation and changes in sedimentation properties of steroid receptors. Journal of Biological Chemistry, 255 2729-2734.

Barker, K.L. (1971) Oestrogen induced synthesis of histones and a specific non-histone protein in the uterus. Biochemistry, 10, 284-291.

Barlow, J.W., Kraft, N., Stockigt, J.R. & Funder, J.W. (1979) Predominant high affinity binding of ^{3}H- dexamethasone in bovine tissues is not to classical glucocorticoid receptors. Endocrinology, 105, 827-834.

Barnett, C.A., Schmidt, T.J. & Litwack, G. (1980) Effects of calf intestinal alkaline phosphatase inhibitors and phosphorylated compounds on the rate of activation of glucocorticoid receptor complexes. Biochemistry, 19, 5446-5455.

Barrack, E.R. (1983) The nuclear matrix of the prostate contains acceptor sites for androgen receptors. Endocrinology, 113, 430-432.

Barrack, E.R., Hawkins, E.F., Allen, S.L., Hicks, L.L. & Coffey, D.S. (1977) Concepts related to salt resistant oestradiol receptors in rat uterine nuclei : nuclear matrix. Biochemical and Biophysical Research Communications, 79, 829-836.

Barrack, E.R. & Coffey, D.S. (1980) The specific binding of oestrogen and androgens to the nuclear matrix of sex hormone responsive tissues. Journal of Biological Chemistry, 255, 7265-7275.

Barrack, E.R. & Coffey, D.S. (1982) Biological properties of the nuclear matrix : steroid hormone binding. Recent Progress in Hormone Research, 38, 133-195.

Batteiger, B. , Newhall, W.J. (The 8th) & Jones, R.B. (1982) The use of Tween 20 as a blocking agent in the immunological detection of proteins transferred to nitrocellulose membranes. Journal of Immunological Methods, 55, 297-307.

Baudendistal, L.J. & Ruh, T.S. (1976) Anti-oestrogen action : differential nuclear retention and extractability of the oestrogen receptor. Steroids, 28, 223-237.

Bauer, E.A., Jeffrey, J.J. & Eisen, A.Z. (1971) Preparation of three vertebrate collagenases in pure form. Biochemical and Biophysical Research Communications, 44, 813-818.

Baxter, J.P., Rousseau, G.G., Rousseau, M.C., Garcea, R.L., Ito, J. & Tompkins, G.M. (1972) Specific cytoplasmic glucocorticoid hormone receptors in hepatoma tissue culture cells. Proceedings of the National Academy of Sciences (USA), 68, 932-937.

Berezney, R. & Bucholtz, L.A. (1981) Dynamic association of replicating DNA fragments with the nuclear matrix of regenerating liver. Experimental Cell Research, 132, 1-13.

Berezney, R. & Coffey, D.S. (1977) Nuclear matrix : isolation and characterisation of a framework structure from rat liver nuclei. Journal of Cell Biology, 73, 616-637.

Bienz, M. (1985) Transient and developmental activation of heat shock genes. Trends in Biochemical Sciences, 10, 157-161.

Birnbaumer, M., Schrader, W.T. & O'Malley, B.W. (1983) Assessment of structural similarities in chick oviduct progesterone receptor subunits by partial proteolysis of photoaffinity labelled proteins. Journal of Biological Chemistry, 258, 7331-7337.

Borgna, J.L. & Rochefort, H. (1980) High affinity binding to the oestrogen receptor of ^{3}H- 4-hydroxytamoxifen an active antioestrogen metabolite. Molecular Cellular Endocrinology, 20, 71-85.

Borgna, J.L. & Rochefort, H. (1981) Hydroxylated metabolites of tamoxifen are formed in vivo and bound to oestrogen receptor in target tissues. Journal of Biological Chemistry, 256, 859-868.

Bowen, B., Steinberg, J., Laemmli, U.K. & Weintraub, H. (1980) The detection of DNA-binding proteins by protein blotting. Nucleic Acids Research, 8, 1-20.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254.

Brambaugh, P.F. & Haussler, M.R. (1974) 1α, 25-dihydroxycholecalciferol receptors in intestine. II Temperature dependent transfer of the hormone to the chromatin via a specific cytosol receptor. Journal of Biological Chemistry, 249, 1258-1262.

Brandes, L.J., MacDonald, L.M. & Boydanovic, R.P. (1985) Evidence that the anti-oestrogen binding site is a histamine or histamine like receptor. Biochemical and Biophysical Research Communications, 126, 905-910.

Bruchovsky, N., Rennie, P.S. & Comeau, T. (1981) Partial purification of nuclear androgen receptor by micrococcal nuclease digestion of chromatin and hydrophobic interaction chromatography. European Journal of Biochemistry, 120, 399-405.

Buetti, E. & Diggelman, H. (1981) Cloned mouse mammary tumour virus DNA is biologically active in transfected mouse cells and its expression is stimulated by glucocrticoid hormones. Cell, 23, 335-345.

Bugany, H. & Beato, M. (1977) Binding of the partially purified glucocorticoid receptor of rat liver to chromatin and DNA. Molecular and Cellular Endocrinology, 7, 49-66.

Buller, R.E., Toft, D.O., Schrader, W.T. & O'Malley, B.W. (1975) Progesterone binding components of the chick oviduct - the kinetics of nuclear binding. Journal of Biological Chemistry, 250, 801-808.

Buller, R.E. & O'Malley, B.W. (1976) The biology and mechanism of steroid hormone receptor interaction with the eukaryotic nucleus. Biochemical Pharmacology, 25, 1-12.

Burton, K. (1956) A study of the conditions and mechanisms of diphenylamine reactions for the colorimetric estimation of deoxyribonucleic acid. Biochemical Journal, 62, 315-323.

Cake, M.H., Disorbo, D.M. & Litwack, G. (1978) Effect of pyridoxal 5' phosphate on the DNA binding site of activated hepatic glucocorticoid receptor. Journal of Biological Chemistry, 253, 4886-4891.

Capony, F. & Rochefort, H. (1978) High affinity binding of the anti-oestrogen ^{3}H-tamoxifen to the 8S oestradiol receptor. Molecular and Cellular Endocrinology, 11, 181-198.

Carlstedt-Duke, J., Okret, S., Wrange, O. & Gustafsson, J.A. (1982) Immunochemical analysis of the glucocorticoid receptor : identification of a third domain separate from the steroid-binding and DNA binding domains. Proceedings of the National Academy of Sciences (USA), 79, 4260-4264.

Cato, A.C.B., Geisse, S., Wenz, M., Westphal, H.M. & Beato, M. (1984) The nucleotide sequences recognised by the glucocorticoid receptor in the rabbit uteroglobin gene region are located far upstream from the initiation of transcription. EMBO Journal, 3, 2771-2778.

Catt, K.J., Harwood, J.P., Clayton, R.N., Davies, T.F., Chan, V., Katikineni, M., Nozu, K. & Dufau, M.L. (1980). Regulation of peptide hormone receptors and gonadal steroidogenisis. Recent Progress in Hormone Research, 36, 557-622.

Chambon, P., Dierich, A., Gaub, M-P., Jakowlev, S., Jongstra, J., Krust, A., Le Pennec, J-P., Oudet, P. & Reudelhuber, T. (1984) Promoter elements of genes coding for proteins and modulation of transcription by oestrogens and progesterone. Recent Progress in Hormone Research, 40, 1-42.

Chamness, G.C., Jennings, A.W. & McGuire, W.L. (1973) Oestrogen receptor binding is not confined to target nuclei. Nature (London),241, 458-460.

Chamness, G.C., Jennings, A.W. & McGuire, W.L. (1974) Oestrogen receptor binding to isolated nuclei. A nonsaturable process. Biochemistry (1974), 13, 327-331.

Chandler, V.L., Maler, B.A. & Yamamoto, K.M. (1983) DNA sequences bound specifically by glucocorticoid receptor in vitro render a heterologous promotor hormone responsive in vivo. Cell, 33, 489-499.

Chang, C.H., Rowley, D.R., Lobl, T.J. & Tindall, D.J. (1982) Purification and characterisation of androgen receptor from steer seminal vesicle. Biochemistry, 21, 4102-4109.

Chang, C.H., Rowley, D.R. & Tindall, D.J. (1983) Purification and characterisation of androgen receptor from rat ventral prostate. Biochemistry, 22, 6170-6175.

Chang-Ren, Y., Suley, D.H., Mester, J., Wolfson, A. & Baulieu, E.E. (1983) Temperature dependence of the dissociation rate constants for the non-activated (molybdate stabilised) and activated progesterone receptor-hormone complexes from the chick oviduct. Biochemica et Biophysica Acta, 755, 428-433.

Charney, P., Treisman, R., Mellon, P. Chao, M., Axel, R. & Maniatis, T. (1984) Differences in human α and β globin gene expression in mouse erythroleukaemia cells : the role of intragenic sequences. Cell, 38, 251-263.

Chen, T.J. & Leavitt, W. (1979) Nuclear progesterone receptor in hamster uterus : measurement by ^{3}H-progesterone exchange during the oestrous cycle. Endocrinology, 104, 1588-1597.

Childs, G.U. (1983) The use of multiple methods to validate immunocytochemical stains. Journal of Histochemistry and Cytochemistry, 31, 168-176.

Chiu, J.F., Hunt, M. & Hnilica, L.S. (1975) Tissue specific DNA-protein complexes during Azo dye hepatocarcinogenesis. Cancer Research, 35, 913-919.

Chong, M.T., & Lippman, M.E. (1982) Effects of RNA and RNase on the binding of oestrogen and glucocorticoid receptors from MCF-7 cells to DNA cellulose. Journal of Biological Chemistry, 257, 2996-3002.

Chytil, F. (1975) Immunochemical characterisation of chromosomal proteins. Methods in Enzymology, 40, 191-198.

Cidlowski, J.A. & Muldoon, T.G. (1974) Oestrogenic regulation of cytoplasmic receptor populations in oestrogen-responsive tissues in the rat. Endocrinology, 95, 1621-1629.

Clark, J.H., Anderson, J.N. & Peck, E.J.. (1973) Nuclear receptor : oestrogen complexes of rat uteri. In Receptors for reproductive hormones, eds. O'Malley, B.W. & Means, A.R., Plenum Press, New York, pp15-59.

Clark, J.H., Peck, E.H. & Anderson, J.N. (1974) Oestrogen receptors and antagonism of steroid hormone action. Nature (London), 251, 446-448.

Clark, J.H. & Peck, E.J. Jnr. (1976) Nuclear retention of receptor oestrogen complex and nuclear acceptor sites. Nature (London), 260, 635-637.

Clark, J.H., Paszko, F. & Peck, E.J. (1977) Nuclear binding and retention of the receptor oestrogen complex : relation to the agonistic and antagonistic properties of oestriol. Endocrinology, 100, 91-96.

Clark, J.H., Hardin, J.W., Upchurch, S. & Erikson, H. (1978a) Heterogeneity of eostrogen binding sites in the cytosol of rat uterus. Journal of Biological Chemistry, 253, 7630-7634.

Clark, J.H., Hardin, J.W., McCormack, S.A. & Padykula, H.A. (1978b) Mechanism of action of oestrogen antagonist : relationship to oestrogen receptor binding and hyperoestrogenisation. In Hormones, receptors and breast cancer, ed. McGuire, W.L., Raven, New York, pp107-133.

Clark, J.H., Hardin, J.W., Padykula, H.A. & Cardasis, C.A. (1978c) Role of oestrogen receptor binding and transcriptional activity in the stimulation of hyper-oestrogenisation and nuclear bodies. Proceedings of the National Academy of Sciences (USA), 75, 2781-2784.

Clark, J.H. & Peck, E.J. Jnr. (1979) Female sex steroids - receptors and function. Monographs on Endocrinology, 14, Springer Verlag, Berlin.

Clemens, L.E., Kleinsmith, L.J. (1972) Specific binding of the oestradiol receptor complex to DNA. Nature New Biology, 237, 204-206.

Cohen, M.E. & Hamilton, T.H. (1975) Effect of oestradiol-17β on the syntheis of specific uterine non histone chromosomal proteins. Proceedings of the National Academy of Sciences (USA), 72, 4346-4350.

Cohen, P. (1982) The role of protein phosphorylation in neural and hormonal control of cellular activity. Nature (London), 296, 613-620.

Cohen, S., Carpenter, G. & King, L. (1980) Epidermal growth factor-receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor enhanced phosphorylation activity. Journal of Biological Chemistry, 255, 4834-4842.

Colvard, D.S. & Wilson, E.M. (1981) Identification of an 8S androgen receptor promoting factor that converts the 4.5S form of the androgen receptor to 8S. Endocrinology, 109, 496-504.

Colvard, D.S. & Wilson, E.M. (1984) Androgen receptor binding to nuclear matrix in vitro and its inhibition by 8S androgen receptor promoting factor. Biochemistry, 23, 3479-3486.

Comings, D.E. & Wallock, A.S. (1978) DNA binding properties of nuclear matrix proteins. Journal of Cell Science, 34, 233-246.

Compton, J.G., Schrader, W.T. & O'Malley, B.W. (1983) DNA sequence preference of progesterone receptor. Proceedings of the National Academy of Sciences (USA), 80, 16-23.

Compton, J.G., Schrader, W.T. ' O'Malley, B.W. (1984) Progesterone receptor binding to DNA : studies by sedimentation velocity methods. Journal of Steroid Biochemistry, 20, 89-94.

Coty, W.A., Schrader, W.T. & O'Malley, B.W. (1979) Purification and characterisation of the chick oviduct progesterone receptor "A" subunit. Journal of Steroid Biochemistry, 10, 1-12.

Crawford, D., Cowan, S., Hyder, S., McMenamin, M., Smith, D. & Leake, R. (1984) New storage procedure for human tumour biopsies prior to oestrogen receptor measurement. Cancer Research, 44, 2348-2351.

Cuatrecassas, P. & Wilcheck, (1968) Single step purification of avidin from egg white by affinity chromatography on biocytin-sepharose columns. Biochemical and Biophysical Research Communications, 33, 235-239.

Cuatrecassas, P., Wilcheck, M. & Anfinsen, C.B. (1968) Selective enzyme purification by affinity chromatography. Proceedings of the National Academy of Sciences (USA), 61, 636-643.

Cuatrecassas, P. & Illiano, G. (1971) Purification of neuramidases from vibrio cholerae, Clostridium perfringens and influenza virus by affinity chromatography. Biochemical and Biophysical Research Communications, 44, 178-184.

Dallenbach-Hellweg, G. (1981) Histopathology of the endometrium (Springer-Verlag, Berlin).

Davies, P. & Thomas, P. (1984) Interaction of androgen receptor with DNA and chromatin. Journal of Steroid Biochemistry, 20, 57-65.

Dean, D.C., Knoll, B.J., Riser, M.E. & O'Malley, B.W. (1983) A 5' Flanking sequence essential for progesterone regulation of an ovalbumin fusion gene. Nature (London), 305, 551-554.

de Boer, W., De Vries, J., Mulder, E. & Van der Molen, H.J. (1978) Oestradiol-receptor complexes in sub-nuclear fractions of rat uterine tissue. Nucleic Acids Research, 5, 87-103.

de Boer, W., Snippe, L., Ab, G. & Gruber, M. (1984) Interaction of calf uterine oestrogen receptor with chicken target nuclei. Journal of Steroid Biochemistry, 20, 387-390.

de Brux, J., Mortel, R. & Gautray, J.P. (1981) The Endometrium, Plenum Press, London.

Dimitriadis, G.J. & Tata, J.R. (1982) Differential sensitisation to deoxyribonuclease I of Xenopus vitellogenin and albumin genes during primary and secondary activation by oestradiol. Biochemical Journal, 202, 491-497.

DiSorbo, D.M., Phelps, D.S. & Litwack, G. (1980) Chemical probes of amino acid residues affect the active sites of the glucocorticoid receptor. Endocrinology, 106, 922-929.

Dix, C.J. & Jordan, V.C. (1980) Subcellular effects of monohydroxy-tamoxifen in rat uterus steroid receptors and mitosis. Journal of Endocrinology, 85. 393-404.

Donnelly, B.J., Lakey, W.H. & McBlain, W.A. (1984) Androgen binding sites on the nuclear matrix of normal and hyperplastic human prostate. The Journal of Urology, 131, 806-811.

Dougherty, J.J., Puri, R.K. & Toft, D.O. (1982) Phosphorylation in vivo of chicken oviduct progesterone receptor. Journal of Biological Chemistry, 257, 14226-14230.

Dougherty,J.J., Puri, R.K. & Toft, D.O. (1984) Polypeptide components of two 8S forms of chicken oviduct progesterone receptor. Journal of Biological Chemistry, 259, 8004-8009.

Dworniczak, B.,Seidel, R. & Pongs, O. (1983) Puffing activities and binding of ecdysteroid to polytene chromosomes of Drosophila melanogaster. EMBO Journal, 2, 1323-1330.

Edwards, D.P., Chamness, G.C. & McGuire, W.L. (1979) Oestrogen and progesterone receptor proteins in breast cancer. Biochemica et Biophysica Acta, 560, 457-486.

Eisenfeld, A.J. & Axelrod, J. (1966) Effect of steroid hormones, ovariectomy, oestrogen pretreatment, sex and immaturity on the distribution of ^{3}H-oestradiol. Endocrinology, 79, 38-42.

Elkon, K.B., Jankowski, P.W. & Chu, J.L. (1984) Blotting intact immunoglobulins and other high molecular weight proteins after composite agarose - polyacrylamide gel electrophoresis. Analytical Biochemistry, 140, 208-213.

England, P.C., Skinner, L.G., Cottrell, K.M. & Sellwood, R.A. (1974) Serum oestradiol-17β in normal women. British Journal of Cancer, 29, 462-469.

Erikkson, H., Upchurch, S., Hardin, J.W., Peck, E.J., Jnr. & Clark, J.H. (1978) Heterogeneity of oetrogen receptors in the cytosol and nuclear fractions of the rat uterus. Biochemical and Biophysical Research Communications, 81, 1-7.

Evans, R.W. & Leavitt, W.W. (1980) Progesterone inhibition of uterine nuclear oestrogen receptor: dependence on RNA and protein synthesis. Proceedings of the National Academy of Sciences (USA), 77, 5856-5860.

Evans, R.W., Chen, T.J., Hendry, W.J. III, Leavitt, W.W. (1980) Progesterone regulation of oestrogen receptor in the hamster uterus during the oestrous cycle. Endocrinology, 107, 383-390.

Fabian, E., Lowell, T. & Steinson, L. (1981) Comparative binding affinities of tamoxifen, 4-hydroxytamoxifen and desmethyltamoxifen for oestrogen receptors isolated from human breast carcinoma: correlation with blood levels in patients with metastatic breast cancer. Biopharmacology Drug Dispos., 2, 381-390.

Fang, S. & Liao, S. (1971) Androgen receptors: steroid and tissue specific retention of a 17β hydroxy-5α-androstan-3-one protein complex by the cell nuclei of ventral prostate. Journal of Biological Chemistry, 246, 16-24.

Faye, J.C., Jozan, S., Redeuilh, B., Baulieu, E.E. & Baynard, F. (1983) Physicochemical and genetic evidence for specific anti-oestrogen binding sites. Proceedings of the National Academy of Sciences (USA), 80, 3158-3162.

Feldman, M., Kallos, J. & Hollander, V.P. (1981) RNA inhibits oestrogen receptor binding to DNA. Journal of Biological Chemistry, 256, 1145-1148.

Fishman, J.H., & Fishman, J. (1981) Differentiation of oestradiol receptors in rat uterine cytosol by sensitivity to tamoxifen. Biochemical and Biophysical Research Communications, 87, 550-558.

Fleming, H., Blumenthal, R. & Gurpide, E. (1983) Effects of cyclic nucleotides on oestradiol binding in human endometrium. Endocrinology, 111, 1671-1677.

Forster, D.N. & Gurney, T. (1976) Nuclear location of mammalian DNA polymerase activities. Journal of Biological Chemistry, 251, 7893-7898.

Franeschi, R.T. & Kim, K. (1979) Isolation of oestrogen receptor in complex with a discrete nuclear subfraction from hen oviduct. Journal of Biological Chemistry, 254, 3637-3646.

Fritton, H.P., Sippel, A.E. & Igo-Kemenes, T. (1983) Nuclease hypersensitive sites in the chromatin of the chicken lysozyme gene. Nucleic Acids Research, 11, 3467-3485.

Fuhner, A.M. & Fasman, G.D. (1979) Analysis of chromatin reconstitution. Biochemistry, 18, 659-668.

Garcia, M. Westley, B. & Rochefort, H. (1981) 5 bromodeoxyuridine specifically inhibits the synthesis of oestrogen induced proteins in MCF-7 cells. European Journal of Biochemistry, 116, 297-301.

Garel, A. & Axel, R. (1977) The structure of transcriptionally active ovalbumin genes in chromatin. Cold Spring Harbor Symposium of Quantitative Biology, 42, 701-708.

Gasc, J-M., Renoir, J-M., Radanyi, C., Joab, I., Touhimaa, P. & Baulieu, E.E. (1984) Progesterone receptor in chick oviduct: an immunohistochemical study with antibodies to distinct receptor components. Journal of Cell Biology, 99, 1193-1201.

Gerber-Huber, S., Felber, B.K., Weber, R. & Ryffil, G.U. (1981) Oestrogen induces tissue specific changes in the chromatin conformation of the vitellogenin genes in Xenopus. Nucleic Acids Research, 9, 2475-2494.

Gerber-Huber, S., Felber, B.K., Mary, F.E.B, Westley, B.R., Hosbach, H.A., Andres, A.C. & Ryffil, G.U. (1983) In constrast to other Xenopus genes the oestrogen inducible vitellogenin genes are expressed when totally methylated. Cell, 33, 43-51.

Gershoni, J.M. & Palade, G.E. (1982) Electrophoretic transfer of proteins from sodium dodecyl sulphate - polyacrylamide gels to a positively charged membrane filter. Analytical Biochemistry, 124, 396-405.

Gershoni, J.M. & Palade, G.E. (1983) Protein Blotting: principles and applications. Analytical Biochemistry, 131, 1-15.

Gershoni, J.M., Davies, F.E. & Palade, G.E. (1985) Protein blotting in uniform or gradient electic fields. Analytical Biochemistry, 144, 32-40.

Ghosh-Dastidar, P., Coty, W.A., Griest, R.E., Woo, D.D.L. & Fox, C.F. (1984) Progesterone receptor subunits are high affinity substrates for phosphorylation by EGF receptor. Proceedings of the National Academy of Sciences (USA), 81, 1654-1658.

Goidl, J.A., Cake, M.H., Dolan, K.P., Parchman, L.G. & Litwack, G. (1977) Activation of the rat liver glucocorticoid-receptor complex. Biochemistry, 16, 2125-2130.

Gorski, J., Toft, D., Shyamala, G., Smith, D. & Notides, A. (1968) Hormone receptors: studies on the interaction of oestrogen with the uterus. Recent Progress in Hormone Research, 24, 45-80.

Gorski, J., Gannon , F. (1976) Current models of steroid hormone action - a critique. Annual Review of Physiology, 38, 425-450.

Gorski, J., Welshons, W. & Sakai, D. (1984) Remodelling the oestrogen receptor model. Molecular and Cellular Endocrinology, 36, 11-15.

Goueli, S.A., Holtzman, J.L., & Ahmed, K. (1984) Phosphorylation of the androgen receptor by a nuclear cAMP independent protein kinase. Biochemical and Biophysical Research Communications, 123, 778-784.

Gower, D.B. (1979) Steroid Hormones, Croom Helm, London.

Gower, D.B. & Fotherby, K. (1975) Biosynthesis of the androgens and oestrogens. In Biochemistry of Steroid Hormones (Maken, H.L.J. ed) pp77-104. Blackwell Scientific Publications, Oxford.

Greene, G.L., Nolan, C., Engler, J.P. & Jensen, E.V. (1980) Monoclonal antibodies to human oestrogen receptor. Proceedings of the National Academy of Sciences (USA), 77, 5115-5119.

Greene, G.L. & Jensen, E.V. (1982) Monoclonal antibodies as probes for oestrogen receptor detection and characterisation. Journal of Steroid Biochemistry, 16, 353-359.

Gregory, M.R. & Notides, A.C. (1982) Characterisation of two uterine proteases and their actions on the oestrogen receptor. Biochemistry, 21, 6452-6458.

Grody, W.W., Compton, J.G., Schrader, W.T. & O'Malley, B.W. (1980) Inactivation of chick oviduct progesterone receptors. Journal of Steroid Biochemistry, 12, 115-120.

Grody, W.W., Schrader, W.T. & O'Malley, B.W. (1982) Activation, transformation and subunit structure of steroid hormone receptors. Endocrine Reviews, 3, 141-163.

Groenmeyer, A.F. & Pongs, S.D. (1980) Localisation of ecdysterone on polytene chromosomes of Drosophila melanogaster. Proceedings of the National Academy of Sciences (USA), 77, 2108-2112.

Gschwendt, M. & Kittstein, W. (1980) Transformation of the oestrogen receptor complex from chick oviduct in 2 steps. Molecular and Cellular Endocrinology, 20, 251-260.

Gschwendt, M. & Kittstein, W. (1983) Disactivation and inhibition of activation of the oestrogen receptor from chick oviduct by periodate-comparison with the effects of molybdate and o-phenathroline. Journal of Receptor Research, 3, 377-392.

Gustafsson, J.A., Carlstedt-Duke, J., Okret, S., Wikstrom, A-C., Wrange, O., Payvar, F. & Yamamoto, K. (1984). Structure and specific DNA binding of the rat liver glucocorticoid receptor. Journal of Steroid Biochemistry, 20, 1-4.

Hansen, P.E., Johnson, A., Schrader, W.R. & O'Malley, R.W. (1976) Kinetics of progesterone binding to the chick oviduct receptor protein. Journal of Steroid Biochemistry, 7, 723-732.

Hechter, O. & Halkenston, I. (1964) On the action of Mammalian hormones. In The Hormones vol. 5, 697, Academic Press, New York.

Hemminiki, K. (1976) Distribution of oestrogen receptors in subfractions of hen oviduct chromatin. Nucleic Acid Research, 3, 1499-1506.

Hemminiki, K. (1977) Differential distribution of oestrogen receptors in subfractions of oviduct chromatin. Acta Endocrinologica, 84, 215-224.

Hemminiki , K. Vaukhonen, M. (1977) Distribution of oestrogen receptors in hen oviduct chromatin fractions in the course of DNase II digestion. Biochemica et Biophysica Acta, 474, 109-116.

Higashi, K., Narayanan, K.S., Adams, H.R. & Busch, H. (1966) Utilisation of the citric acid procedure and zonal ultra-centrifugation for mass isolation of nuclear RNA from Walker 256 carinosarcoma. Cancer Research, 26, 1582-1590.

Higgins, S.J., Rousseau, G.G., Baxter, J.D. & Tomkins, G.M. (1973) The nature of nuclear acceptor sites for glucocorticoid and oestrogen/ receptor complexes. Journal of Biological Chemistry, 248, 5873-5879.

Hjelle, B.L., Philips, S.A. & Seeburg, P.M. (1982) Relative levels of methylation in human growth hormone and chorionic somatomammotropin genes in expressing and non-expressing tissues. Nucleic Acids Research, 10, 3459-3473.

Hollenberg, M.D. (1979) Hormone receptor interactions at the cell membrane. Pharmacological Review, 30, 393-410.

Horwitz, K.B. & McGuire, W.L. (1978) Nuclear oestrogen receptors - effect of inhibitors on processing and steady state levels. Journal of Biological Chemistry, 2223-2228.

Horwitz, S.B. & Moore,L.C. (1974) The nuclear permeability, intra-cellular distribution and diffusion of inulin in the amphibian oocyte. Journal of Cell Biology, 60, 405-415.

Houslay, M.D. & Heyworth, C.M. (1983) Insulin: in search of a mechanism. Trends in Biochemical Sciences, 8, 449-452.

Housley, P.R. & Pratt, W.B. (1983) A direct demonstration of glucocorticoid receptor phosphorylation by intact L-cells. Journal of Biological Chemistry, 258, 4630-4635.

Hughes, M.R., Compton, J.G., Schrader, W.T. & O'Malley, B.W. (1981) Interaction of the chick oviduct progesterone receptor with DNA. Biochemistry, 20, 2481-2486.

Hunt, B.F. & Vogelstein, B. (1981) Association of newly replicated DNA with the nuclear matrix of Physarium polycephalum. Nucleic Acids Research, 9, 349-363.

Hyder, S.M. (1983) Ph.D. Thesis, Glasgow University.

Hyder, S.M, & Leake, R.E. (1982) Stability of transformed oestrogen receptor from human endometrium and breast carcinoma. Biochemical Society Transactions, 10, 522-523.

Hyder, S. & Leake, R. (1983) Heterogeneity of low salt 4S oestrogen receptor from human breast cancer with respect to DNA binding. Journal of Steroid Biochemistry, 19 (supplement) 35.

Hynes, N., Oogen, A., Kennedy, N., Herrlich, P., Ponta, H. & Groner, B. (1983) Subfragments of the large terminal repeat cause glucocorticoid responsive expression of MMTV and of an adjacent gene. Proceedings of the National Academy of Sciences (USA), 80, 3637-3641.

Hyodo, M. & Ono, T. (1970) Degradation of DNA in isolated liver nuclei caused by mechanical forces and endogenous enzyme. Biochemica et Biophysica Acta, 213, 228-230.

Jackson, D.A., McCready, S.J. & Cook, P.R. (1981) RNA is synthesised at the nuclear cage. Nature (London), 292, 552-555.

Jackson, V. & Chalkley, R. (1974) The binding of oestradiol to the bovine endometrial nuclear membrane. Journal of Biological Chemistry, 249, 1615-1626.

Jensen, E.V. & Jacobson, H.I. (1962) Basic guides to the mechanism of oestrogen action. Recent Progress in Hormone Research, 18, 387-392.

Jensen, E.V., DeSombre, E.R., Hurst, D.J. & Jungblut, P.W. (1967) Sulphydral groups and oestradiol-receptor interaction. Science, 158, 385-387.

Jensen, E.V., Suzuki, T., Kawashuma, T., Stumph, W.E., Jungblut, P.W. & DeSombre, E.R. (1968) A two step mechanism for the interaction of oestradiol with rat uterus. Proceedings of the National Academy of Sciences (USA), 59, 632-638.

Jensen, E.V. & DeSombre, E.R. (1972) Annual Review of Biochemistry, 41, 203-230.

Johnson, M. & Everitt, B. (1980) Essential reproduction (Blackwell Scientific, Oxford, U.K.)

Jordan, V.C., Rowsby, L., Dix, C.J. & Prestwick, G. (1978) Dose related effects of non-steroidal antioestrogen and oestrogens on the measurement of cytoplasmic oestrogen receptors in the rat and mouse uterus. Journal of Endocrinology, 78, 71-81.

Jordan, V.C. & Dix, C.J. (1979) Effect of oestradiol benzoate, tamoxifen and monohydroxytamoxifen on immature rat uterine progesterone receptor synthesis and endometrial cell division. Journal of Steroid Biochemistry, 11, 285-291.

Jordan, V.C., Clark, E.R & Allen, K.E. (1981) Structure - activity relationships amongst non-steroidal antioestrogens. In Non-steroidal antioestrogens Eds. R.L. Sutherland and V.C. Jordan (Academic Press, Sydney) pp.31-57.

Jost, J.P., Geisser, M. & Seldran, M. (1985) Specific modulation of the transcription of cloned avian vitellogenin II gene by oestradiol-receptor complex in vitro. Proceedings of the National Academy of Sciences (USA), 82, 988-991.

Juliano, J.V. & Stancel, G.M. (1976) Oestrogen receptors in the rat uterus. Retention of hormone/receptor complexes. Biochemistry, 15, 916-920.

Kalini, M., Tsai, S.Y., Tsai, M.J., Clark, J.H. & O'Malley, B.W. (1976) Effect of oestrogen on gene expression in the chick oviduct. Journal of Biological Chemistry, 251, 516-523.

Kallos, J. & Hollander, V. (1978) Assessment of specificity of oestrogen receptor DNA interaction by a competitive assay. Nature (London), 272, 177-179.

Kallos, J., Fasy, T., Hollander, V. & Bick, M. (1978) Oestrogen receptor has enhanced affinity for bromodeoxyuridine substituted DNA. Proceedings of the National Academy of Sciences (USA), 75, 4896-4900.

Kallos, J., Fasy, T.M. & Hollander, V.P. (1981) Assessment of oestrogen receptor-histone interactions. Proceedings of the National Academy of Sciences (USA), 78, 2874-2878.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Westphal, H.M. & Beato, M. (1984) Characterisation of DNA sequences through which cadmium and glucocorticoid hormones induce human metallothionein-IIA gene. Nature (London) 308, 513-519.

Kassis, J.A. & Gorski, J. (1981) Oestrogen receptor replenishment: evidence for receptor recycling. Journal of Biological Chemistry, 256, 7378-7382.

Kassis, J.A. & Gorski, J. (1983) On the mechanism of oestrogen receptor replenishment: recycling resynthesis and/or processing. Molecular and Cellular Biochemistry, 52, 27-36.

Kassis, J.A., Sakai, D., Walent, J.H. & Gorski, J. (1984) Primary cultures of oestrogen responsive cells from rat uteri. Induction of progesterone receptor and a secreted protein. Endocrinology, 114, 1558-1566.

Kasuga, M., Karlsson, F.A. & Khan, C.R. (1982) Insulin stimulates the phosphorylation of the 95,000-dalton subunit of its own receptor. Science, 215, 185-187.

Katzenellenbogen, B.S. & Gorski, J. (1972) Oestrogen action in vitro - induction of the synthesis of a specific uterine protein. Journal of Biological Chemistry, 247, 1299-1305.

Katzenellenbogen, B.S. & Leake, R.E. (1974) Distribution of the oestrogen induced protein and total protein between endometrial and myometrial fractions of the immature and mature rat uterus. Journal of Endocrinology, 63, 439-446.

Katzenellenbogen, B.S. & Ferguson, E.R. (1975) Antioestrogen action in the uterus: biological ineffectiveness of nuclear bound oestradiol after antioestrogen. Endocrinology, 97, 1-12.

Katzenellenbogen, B.S. & Gorski, J. (1975) Oestrogen actions on synthesis of macromolecules in target cells. In Biochemical Action of Hormones vol. 3 187-243 (Ed. G. Litwack) Academic Press, New York.

Katzenellenbogen, B.S., Ferguson, E.R. & Lan, N.C. (1977) A comparative study of antioestrogen action: temporal patterns of antagonism of oestrogen stimulated uterine growth and effects on oestrogen receptor levels. Endocrinology, 100, 1252-1259.

Katzenellenbogen, B.S., Katzenellenbogen, J.A., Ferguson, E.R. & Kraisthammer, N. (1978) Antioestrogen interaction with uterine oestrogen receptors. Studies with a radiolabelled anti-oestrogen (CI-628). Journal of Biological Chemistry, 253, 697-707.

Katzenellenbogen, B.S., Bhakoo, H.S., Ferguson, E.R., Lan, L.C., Tata, T., Tsai, T.L. & Katzenellenbogen, J.A. (1979) Oestrogen and antioestrogen action in reproductive tissues and tumours. Recent Progress in Hormone Research, 35, 259-300.

Katzenellenbogen, B.S., Pavlick, E.J., Robertson, D.W. & Katzenellenbogen, J.A. (1981) Interaction of a high affinity antioestrogen (α- 4-pyrrolidinoethyoxyl phenyl-4-hydroxy-α' nitrostilbene CI-1628M) with uterine oestrogen receptors. Journal of Biological Chemistry, 256, 2908-2915.

Kaye, A.M. (1983) Sequential regulation of gene expression by oestrogen in the developing rat uterus. In Regulation of gene expression by Hormones (Ed. McKerns, F.W., Plenum Press, London).

Keene, J.L., Ruh, M.F. & Ruh, T.S. (1984) Interaction of the antioestrogen (^{3}H)-H 1285 with the two forms of molybdate stabilised calf uterine oestrogen receptor. Journal of Steroid Biochemistry, 21, 625-631.

King, R.J.B. & Gordon, J. (1967) The association of 6-7 ^{3}H -oestradiol with a nuclear protein. Journal of Endocrinology, 39, 533-542.

King, R.J.B., Gordon, J. & Steggles, A.W. (1969) The properties of a nuclear acidic protein fraction that binds oestradiol. Biochemical Journal, 114, 649-657.

King, R.J.B. & Gordon, J. (1972) Involvement of DNA in the acceptor mechanism for uterine oestradiol receptor. Nature New Biology, 240, 185-187.

King, R.J.B. (1984) Enlightment and confusion over steroid hormone receptors. Nature (London), 312, 701-702.

Klyzsejko-Stefanowicz, L., Chiu, J.F., Tsai, Y.H. & Hnilica, L.S. (1976) Acceptor proteins in rat androgenic tissue chromatin. Proceedings of the National Academy of Sciences (USA), 73, 1954-1958.

Kon, O.L. & Spelsberg, T.C. (1982) Nuclear binding of oestrogen/receptor complex: receptor specific nuclear acceptor sites. Endocrinology, 111, 1925-1935.

Kon, L., Webster, R.A. & Spelsberg, T.C. (1980) Isolation and characterisation of the oestrogen receptor in the hen oviduct. Evidence for two molecular species. Endocrinology, 107, 1182-1191.

Kono, T. (1983) Actions of insulin on glucose transport and cAMP phosphodiesterase in fat cells. Involvement of two distinct mechanisms. Recent Progress in Hormone Research, 39, 519-557.

Koreman, S.G. & Rao, B.R. (1968) Reversible disaggregation of the cytosol oestrogen binding protein of uterine cytosol. Proceedings of the National Academy of Sciences, 61, 1028-1038.

Kovaks, W.J., Griffin, J.E. & Wilson, J.A. (1983) Transformation of human androgen receptors to the DNA binding state. Endocrinology, 113, 1574-1581.

Kurl, R.N. & Jacob, S.T. (1984) Phosphorylation of purified glucocorticoid receptor from rat liver by an endogenous protein kinase. Biochemical and Biophysical Research Communications, 119, 700-705.

Kurtz , D.T. (1981) Hormonal inducibility of rat α2u globulin genes in transfected mouse cells. Nature (London), 291, 629-631.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature (London), 227, 680-685.

Lawson, G.M., Tsai, M-J. & O'Malley, B.W. (1980) Deoxyribonuclease I sensitivity of the non-transcribed sequences flanking the 5' and 3' ends of the ovomucoid gene and the ovalbumin and its related x and y genes in hen oviduct nuclei. Biochemistry, 19, 4403-4411.

Lawson, G.M., Knoll, B.J., March, C.J., Woo, S.L., Tsai, M-J. & O'Malley, B.W. (1982) Definition of the 5' and 3' boundaries of the chromatin domains containing the ovalbumin multigene family. Journal of Biological Chemistry, 257, 1501-1507.

Leach, K.L., Dahmer, M.K., Hammon, N.D., Sando, J.J. & Pratt, W.B. (1979) Molybdate inhibition of glucocorticoid receptor activation and transformation. Journal of Biological Chemistry, 254, 11884-11890.

Leake, R.E. (1981) Problems associated with dose response in steroid hormone activation of structural genes. Molecular and Cellular Endocrinology, 21, 1-13.

Lebeau, M.C., Massol, N. & Baulieu, E.E. (1981) Oestrogen receptor chromatin interactions: effect of antioestrogens. In Non-steroidal antioestrogens (Eds. R.L. Sutherland, V.C. Jordan) Academic Press, Sydney, pp249-260.

Lee, A.E. (1974) Effects of oestrogen antagonists on mitosis and ^{3}H-oestradiol binding in the mouse uterus. Journal of Endocrinology, 60, 167-174.

Lee, J. & Laycock, J. (1978) Essential endocrinology, Oxford University Press, Oxford.

Lee, S.H. (1982) Uterine epithelial and eosinophil oestrogen receptors in rats during the oestrous cycle. Histochemistry, 74, 443-452.

Lessey, B.A., Alexander, S. & Horwitz, K.B. (1983) The subunit structure of human breast cancer progesterone receptors: characterisation by chromatography and photo affinity labelling. Endocrinology, 112, 1269-1274.

Levinger, L. & Varshavsky, A. (1982) Selective arrangement of ubiquitinated and D1 protein - containing nucleosomes within the Drosophila genome. Cell, 28, 375-385.

Levy, B.W. & Baxter, J.D. (1976) Distribution of thyroid and glucocorticoid hormone receptors in transcriptionally active and inactive chromatin. Biochemical and Biophysical Research Communications, 68, 1045-1051.

Levy, C., Morbil, R., Fychenne, B., Dobil, P. & Baulieu, E.E. (1980) Unoccupied nuclear oestradiol receptor sites in normal human endometrium. Biochemical Journal, 185, 733-738.

Levy, S., Simpson, R.T. & Sober, H.A. (1972) Fractionation of chromatin components. Biochemistry, 11, 1547-1554.

Li, J.J., Talley, D.J., Li, S.A. & Villee, C.A. (1974) An oestrogen binding protein in the renal cytosol of intact, castrated and oestrogenised golden hamsters. Endocrinology, 95, 1134-1141.

Liao, S. (1975) Cellular receptors and mechanisms of action of steroid hormones. International Review of Cytology, 41, 87-172.

Liao, S., Liang, T., Fang, S., Castaneda, E. & Shao, T.S. (1973a) Steroid structure and androgenic activity. Specificities involved in the receptor binding and nuclear retention of various androgens. Journal of Biological Chemistry, 248, 6154-6162.

Liao, S., Liang, T. & Tymoczko, J.C. (1973b) Ribonucleoprotein binding of steroid receptor complexes. Nature New Biology, 241, 211-213.

Liang, T. & Liao, S. (1972) Interaction of oestradiol- and progesterone-receptors with nucleoprotein: heat labile acceptor factors. Biochemica et Biophysica Acta, 277, 590-594.

Lin, S.H. & Webb, T.E. (1977) Elevated concentration of a dexamethasone-receptor. Translocation inhibitor in Novikoff hepatoma cells. Cancer Research, 37, 1763-1767.

Lin, S.Y. & Ohno, S. (1981) The binding of androgen receptor to DNA and RNA. Biochemica et Biophysica Acta, 654, 181-186.

Lin, S.Y. & Ohno, S. (1983) Interactions of nuclear oestrogen receptor with DNA and RNA. Biochemica et Biophysica Acta, 740, 264-270.

Linki, D.M. (1982) Intranuclear transformation of the oestrogen receptor in adulthood: tissue and species similarities. Journal of Receptor Research, 2, 487-501.

Linki, D.M. & Siiteri, P.K. (1978) A re-examination of the interaction of oestradiol with target cell receptors. Journal of Steroid Biochemistry, 9, 1071-1078.

Little, M. Szendrop, P., Teran, C., Hugh, A. & Jungblut, P.N. (1975) Biosynthesis and transformation of microsomal and cytosol oestradiol receptors. Journal of Steroid Biochemistry, 6, 493-500.

Littlefield, B.A. & Spelsberg, T.C. (1985) Problems and artifacts in the identification of nuclear acceptor sites for the avian oviduct progesterone receptor. Endocrinology, 117, 412-414.

Logeat, F., Pamphile, R., Loosfelt, H., Jolivert, A., Fournier, A. & Milgrom, E. (1985) One step immunoaffinity purification of active progesterone receptor. Further evidence in favour of the existence of a single steroid binding subunit. Biochemistry, 24, 1029-1035.

Longcope, C., Widrich, W. & Sawin, C.T. (1972) The secretion of oestrone and oestradiol-17β by human testis. Steroids, 20, 439-448.

Loosfelt, H., Logeat, F., Hai, M.T.U. & Milgrom, E. (1984) The rabbit progesterone receptor. Evidence for a single steroid binding subunit and characterisation of receptor mRNA. Journal of Biological Chemistry, 259, 14196-14202.

Love, C.A., Cowan, S.K., Laing, L.M. & Leake, R.E. (1983) Stability of the human nuclear oestrogen receptor: influence of temperature and ionic strength. Journal of Endocrinology, 99, 423-433.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 193, 265-275.

McGhee, J.D., & Ginder, G.D. (1979) Specific DNA methylation sites in the vicinity of the chicken globin genes. Nature (London), 280, 419-420.

McGuire, W.L. & Julian, J.A. (1971) Comparison of macromolecular binding of oestradiol in hormone-dependent and hormone-independent rat mammary carcinoma. Cancer Research, 31, 1440-1445.

Mainwaring, W.I.P. & Peterken, B.M. (1971) A reconstituted cell free system for the specific transfer of steroid receptor complexes into nuclear chromatin isolated from rat ventral prostate gland. Biochemical Journal, 125, 285-295.

Mainwaring, W.I.P., Symes, E.K. & Higgins, S.J. (1976) Nuclear components responsible for the retention of steroid/receptor complexes, especially from the standpoint of the specificity of hormonal responses. Biochemical Journal, 156, 129-141.

Mainwaring, W.I.P. & Randall, V.A. (1984) Limitations in the use of tritiated methyltrienolone for the photoaffinity labelling of androgen receptor proteins. Journal of Steroid Biochemistry, 21, 209-216.

Mandel, J.L. & Chambon, P. (1979) DNA methylation: organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes. Nucleic Acids Research, 7, 2081-2103.

Martin, L. (1981) Effects of antioestrogen on cell proliferation in the rodent reproductive tract. In Non-steroidal antioestrogens Eds. R.L. Sutherland and V.C. Jordan (Academic Press, Sydney) pp 143-163.

Martin, P.M. & Sheridan, P.J. (1980) Intracellular distribution of oestrogen receptors: a function of preparation. Experimentia, 36, 620-622.

Martin, P.M. & Sheridan, P.J. (1982) Towards a new model for the mechanism of action of steroids. Journal of Steroid Biochemistry, 16, 215-229.

Massol, N., Lebeau, M.C. & Baulieu, E.E. (1978) Oestrogen receptor in hen oviduct chromatin digested by micrococcal nuclease. Nucleic Acids Research, 5, 723-738.

Maurer, R.A. (1985) Selective binding of oetrogen receptor to a region at least one kilobase upstream from the rat prolactin gene. DNA, 4, 1-9.

Mester, J. & Banken, E.E. (1972) Nuclear oestrogen receptor of chick liver. Biochemica et Biophysica Acta, 261, 236-244.

Mester, J. & Baulieu, E.E. (1975) Dynamics of oestrogen receptor distribution between the cytosol and nuclear fractions of the immature rat uterus after oestradiol administration. Biochemical Journal, 146, 617-623.

Mester, J., Binart, N., Catelli, M.G., Giejnet, C., Sutherland, R.L., Habnel, R., Puri, V., Seeley, D. & Baulieu, E.E. (1981) Mechanisms of oestrogen antagonism by tamoxifen and mono-hydroxytamoxifen in chick oviduct. In Non-steroidal antioestrogens Eds. R.L. Sutherland and V.C. Jordan (Academic Press, Sydney) pp177-194.

Meyers, C.Y. (1984) The role of hormone receptors in oestrogen activity is questionable. Trends in Biochemical Sciences, 9, 473.

Migliaccio, A. & Auricchio, F. (1981) Hormone binding of oestradiol receptor. Evidence for its regulation by cytoplasmic phosphorylation and nuclear dephosphorylation. Prevention of dephosphorylation by antioestrogens. Journal of Steroid Biochemistry, 15, 369-374.

Migliaccio, A., Lastoria, S., Moncharmont, B., Rotondi, A. & Auricchio, F. (1982) Phosphorylation of calf uterus oestradiol receptor by endogenous Ca^{2+} stimulated kinase activating the hormone binding of the receptor. Biochemical and Biophysical Research Communications, 109, 1002-1010.

Migliaccio, A., Rotondi, A. & Auricchio, F. (1984) Calmodulin-stimulated phosphorylation of oestradiol receptor on tyrosine. Proceedings of the National Academy of Sciences (USA), 81, 5921-5925.

Milgrom, E., Atger, M. & Baulieu, E.E. (1973) Studies on oestrogen entry into uterine cells and on oestradiol receptor complex attachment to the nucleus - is the entry of oestrogen into uterine cells a protein mediated process. Biochemica et Biophysica Acta, 320, 267-283.

Milgrom, E. & Atger, M. (1975) Receptor translocation inhibitor and apparent saturability of the nuclear acceptor. Journal of Steroid Biochemistry, 6, 487-492.

Miller, J.B. & Toft, D.O. (1978) Requirement of activation in the binding of progesterone receptor to ATP sepharose. Biochemistry, 17, 173-177.

Moore, D.D., Marks, A.R., Buckley, D.I., Kapler, G., Payvar, F. & Goodman, H.M. (1985) The first intron of the human growth hormone gene contains a binding site for glucocorticoid receptor. Proceedings of the National Academy of Sciences (USA), 82, 699-702.

Moudgil, U.K., Eessalu, T.E., Buchou, T., Renoir, J-M., Mester, J. & Baulieu, E.E. (1985) Transformation of chick oviduct progesterone receptor in vitro. Effects of hormone, salt, heat and ATP. Endocrinology, 116, 1267-1274.

Muldoon, T.G. & Cidlowski, J.A. (1980) Specific modification of rat uterine oestrogen receptor by pyridoxal 5' phosphate. Journal of Biological Chemistry, 255, 3100-3107.

Muller, R.E. & Wotiz, H.H. (1978) Oestrogen binding proteins in mouse and rat adrenal glands. Journal of Biological Chemistry, 253, 740-745.

Muller, R.E., Johnston, T.C. & Wotiz, H.H. (1979) Binding of oestradiol to purified uterine plasma membranes. Journal of Biological Chemistry, 254, 7895-7900.

Muller, R.E., Traish, A.M. & Wotiz, H.H. (1980) Effects of pyridoxal 5' phosphate on oestrogen receptor activation and nuclear binding. In Hormones and Cancer (Jacobelli, S., King, R.J.B., Lindner, H.R. & Lippman, M.E., Eds) Raven Press, New York, pp241-254.

Muller, R.E., Traish, A.M., Beebe, D.A. & Wotiz, H.H. (1982) Reversible inhibition of oestrogen receptor activation by molybdate. Journal of Biological Chemistry, 257, 1295-1300.

Muller, R.E, Traish, A.M. & Wotiz, H.H. (1983a) Oestrogen receptor activation precedes transformation. Journal of Biological Chemistry, 258, 9227-9236.

Muller, R.E., Mrabet, N.T., Traish, A.M. & Wotiz, H.H. (1983b) The role of arginyl residues in oestrogen receptor activation and transformation. Journal of Biological Chemistry, 258, 11582-11589.

Muller, R.E., Traish, A.M., Beebe, D.A., Bercel, E. & Wotiz, H.H. (1984) Oestriol and oestradiol interactions with the oestrogen receptor in vivo and in vitro. Journal of Steroid Biochemistry, 20, 1039-1046.

Muller, R.E., Traish, A.M., Hirota, T., Bercel, E. & Wotiz, H.H. (1985) Conversion of oestrogen receptor from a state with low affinity for oestradiol into a state with higher affinity does not require 4S-5S dimerisation. Endocrinology, 116, 337-345.

Mulvihill, E.R., & Palmiter, R.D. (1977) Relationship of nuclear oestrogen receptor levels to induction of ovalbumin and conalbumin mRNA in chick oviduct. Journal of Biological Chemistry, 252, 2060-2068.

Mulvihill, E.R. & Palmiter, R.D. (1980) Relationship of nuclear progesterone receptors to induction of ovalbumin and conalbumin mRNA in chick oviduct. Journal of Biological Chemistry, 255, 2085-2091.

Mulvihill, E.K., Le Pennec, J-P, & Chambon, P. (1982) Chicken oviduct progesterone receptor: location of specific regions of high affinity binding in cloned DNA fragments of hormone responsive genes. Cell, 24, 621-632.

Munck, A. & Foley, R. (1980) Activated and non-activated glucocorticoid-receptor complexes in rat thymus cells. Kinetics of formation and relation to steroid structure. Journal of Steroid Biochemistry, 12, 225-230.

Musliner, T.A. & Chader, G.J.. (1972) Oestradiol receptors of the rat uterus: interaction of the cytoplasmic oestrogen receptor with DNA in vitro. Biochemica et Biophysica Acta, 262, 256-263.

Nakhasi, H.L., Lynch, K.R., Dolan, K.P., Unterman, R., Antakley, T. & Feigelson,P. (1982) Modifiction in α 2u globulin gene structure, transcription and mRNA translation in hepatomas. Journal of Biological Chemistry, 257, 2726-2729.

Nalbandov, A.V. (1976) Reproductive Physiology of Mammals and Birds (W.H. Freeman and Co., San Francisco).

Nelson, D., Covault, J. & Chalkley, R. (1980) Segregation of rapidly acetylated histones into a chromatin fraction released from intact nuclei by the addition of micrococcal nuclease. Nucleic Acids Research, 8, 1745-1763.

Nielsen, P.J., Manchester, K.L., Towbin, H., Gordon, J. & Thomas, G. (1982) The phosphorylation of ribosomal protein S6 in rat tissues following cyclohexamide injection, in diabetes, and after denervation of the diaphragm.

Nishigori, H. & Toft, D. (1979) Chemical modification of the avian progesterone receptor by pyridoxal 5' phosphate. Journal of Biological Chemistry, 254, 9155-9161.

Nishigori, H. & Toft, D. (1980) Inhibition of progesterone receptor activation by sodium molybdate. Biochemistry, 19, 77-83.

Norris, J.S. & Kohler, P.D. (1983) Syrian hamster glucocorticoid receptors characteristics of binding of partially purified receptor to DNA. Journal of Biological Chemistry, 258, 2350-2356.

Noteboom, W.D. & Gorski, J. (1965) Sterospecific binding of oestrogens in rat uterus. Archives of Biochemistry and Biophysics, 111, 559-562.

Notides, A.C. & Neilsen, S. (1974) The molecular mechanism of the in vitro 4S to 5S transformation of the uterine oestrogen receptor. Journal of Biological Chemistry, 249, 1866-1873.

Notides, A.C. & Neilsen, S. (1975) A molecular and kinetic analysis of oestrogen receptor transformation. Journal of Steroid Biochemistry, 6, 483-486.

Notides, A.C., Lerner, N. & Hamilton, D.E. (1981) Positive cooperativity of the oestrogenreceptor. Proceedings of the National Academy of Sciences (USA), 78, 4926-4930.

O'Farrell, P.H. & O'Farrell, P.Z. (1977) Two dimensional polyacrylamide gel electrophoretic fractionation. Methods in Cell Biology, 14, 407-420.

O'Malley, B.W., Spelsberg, T.C., Schrader, W.T., Chytil, F. & Steggles, A.W. (1972) Mechanisms of interaction of a hormone receptor complex with the genome of a eukaryotic target cell. Nature (London), 235, 141-144.

O'Malley, B.N. & Means, A.R. (1974) Female steroid hormones and target cell nuclei. Science, 183, 610-613.

Ota, M., Sato, N. & Kyakumoto, S. (1984) Interaction of hepatic chromatin with androgen receptor complex. Experimental Clinical Endocrinology, 84, 159-166.

Panko, W.B. & McLeod, R.M. (1978) Uncharged nuclear receptors for oestrogen in breast cancers. Cancer Research, 38, 1948-1951.

Panko, W.B., Watson, C.S. & Clark, J.H. (1981) The presence of a second specific oestrogen binding site in human breast cancer. Journal of Steroid Biochemistry, 14, 1311-1316.

Panyim, S. & Chalkley, R. (1969) High resolution acrylamide gel electrophoresis of histones. Archives of Biochemistry and Biophysics, 130, 337-346.

Parker, C.S. & Topol, J. (1984) A Drosophila RNA polymerase II transcription factor binds to the regulatory site of an HSP 70 gene. Cell, 37, 273-283.

Pasqualini, J.R., Cosquer-Clavereul, C. & Gelby, C. (1983) Rapid modulation by progesterone and tamoxifen of oestradiol effects on nuclear histone acetylation. Biochemica et Biophysica Acta, 739, 137-140.

Pavlick, E.J., Rutledge, S., Eckert, R.L. & Katzenellenbogen, B.S. (1979) Localisation of oestrogen receptors in uterine cells. An appraisal of translocation. Experimental Cell Research, 123, 177-189.

Pavlick, E.J. & Katzenellenbogen, B.S. (1982) The intra-nuclear distribution of rat uterine oestrogen receptors determined after nuclease treatment and chromatin fractionation. Molecular and Cellular Endocrinology, 26, 201-216.

Payvar, F., Wrange, O., Carlstedt-Duke, J., Okret, S., Gustafsson, J.A. & Yamamoto, K.R. (1981) Purified glucocorticoid receptors bind selectively in vitro to a cloned DNA fragment whose transcription is regulated by glucocorticoids in vivo. Proceedings of the National Academy of Sciences (USA), 78, 6628-6632.

Payvar, F., Firestone, G.L., Ross, S.R., Chandler, V.L., Wrange, O., Carlstedt-Duke, J., Gustafsson, J.A. & Yamamoto, K.R. (1982) Multiple specific binding sites for purified glucocorticoid receptors on mammary tumour virus DNA. Journal of Cell Biochemistry 19, 241-247.

Payvar, F., De Franco, D., Firestone, G.L., Edgar, B., Wrange, O., Okret, S., Gustafsson, J.A. & Yamamoto, K.R. (1983) Sequence specific binding of glucocorticoid receptor to MMTV DNA at sites within and upstream of the transcribed region. Cell, 35, 381-392.

Pelham, H.R.B. (1982) A regulatory upstream promotor element in the Drosophila HSP 70 heat-shock gene. Cell, 30, 517-528.

Perrot-Applanet, M., Logeat, F., Groyer-Picard, M.T. & Milgrom, E. (1985) Immunocytochemical study of mammalian progesterone receptor using monoclonal antibodies. Endocrinology, 116, 1473-1484.

Perry, B.N. & Lopez, A. (1978) The binding of ^{3}H-oestradiol and progesterone/receptor complexes to hypothalamic chromatin of male and female sheep. Biochemical Journal, 176, 873-883.

Peterson, J.L. & McConkey, E.H. (1976) Non-histone chromosomal proteins from HeLa cells. A survey by high resolution two dimensional electrophoresis. Journal of Biological Chemistry, 251, 548-554.

Petrusz, P. (1983) Essential requirements for the validity of immunocytochemical staining procedures. Journal of Histochemistry and Cytochemistry, 31, 177-179.

Pettersson, K., Vanharanta, R., Soderholm, J., Puonnen, R. & Lovgren, T. (1982). Increase in the oestrogen binding capacity of breast cancer cytosols following limited proteolysis with trypsin. Journal of Steroid Biochemistry, 16, 369-372.

Picard, D. & Schaffner, W. (1984) A lymphocyte specific enhancer in the mouse immunoglobulin K gene. Nature (London), 307, 80-82.

Pietras, R.J. & Szego, C.M. (1977) Specific binding sites for oestrogen at the outer surfaces of isolated endometrial cells. Nature (London), 265, 69-72.

Pietras, R.J. & Szego, C.M. (1979) Metabolic and proliferative responses to oestrogen by hepatocytes selected for plasma membrane binding sites specific for oestradiol. Journal of Cell Physiology, 98, 145-160.

Pietras, R.J., Szego, C.M. (1984) Specific internalisation of oestrogen and binding to nuclear matrix in isolated uterine cells. Biochemical and Biophysical Research Communications, 123, 84-91.

Pike, J.W. (1982) Receptors for 1,25-dihydroxy-vitamin D_3 in chick pancreas: a partial physical and functional characterisation. Journal of Steroid Biochemistry, 16, 385-395.

Pikler, G.M., Webster, R.A & Spelsberg, T.C. (1976) Nuclear binding of progesterone in hen oviduct - binding to multiple sites in vitro. Biochemical Journal, 156, 399-408.

Plummer, D.T. (1978) In An introduction to practical Biochemistry (2nd edition) p241-242. McGraw-Hill Book Company (UK) Ltd.

Poonian, M.S., Schlabach, A.J. & Weissbach, A. (1971) Covalent attachment of nucleic acids to agarose for affinity chromatography. Biochemistry, 10, 424-427.

Pratt, K., Wierowski, R.H. & Bambara, R.A. (1984) Bovine oestrogen receptor binds chromatin at pre-existing nuclease hypersensitive sites. Molecular and Cellular Endocrinology, 35, 205-214.

Puca, G.A. & Bresciani, F. (1968) Receptor molecule for oestrogens from rat uterus. Nature (London), 218, 967-969.

Puca, G.A., Sica, V. & Nola, E. (1974) Identification of a high affinity nuclear acceptor site for eostrogen receptor of calf uterus. Proceedings of the National Academy of Sciences (USA), 71, 979-983.

Puca, G.A., Nola, E., Hibner, U., Cicala, G. & Sica, V. (1975) Interactions of the oestradiol receptor from calf uterus with its nuclear acceptor sites. Journal of Biological Chemistry, 250, 6452-6459.

Puca, G.A., Nola, E. & Sica, V. (1977) Oestrogen binding proteins of the calf uterus. Molecular and functional characterisation of the receptor transforming factor: a Ca^{2+} activated protease. Journal of Biological Chemistry, 252, 1358-1366.

Raam, S., Nemeth, E., Tamura, H., O'Brian, D.S. & Cohen, J.L. (1982) Immunohistochemical localisation of oestrogen receptors in human mammary carcinoma using antibodies to the receptor protein. European Journal of Cancer and Clinical Oncology, 18, 1-12.

Rao, G.S. (1981) Mode of entry of steroid and thyroid hormones into cells. Molecular and Cellular Endocrinology, 21, 97-108.

Renart, J., Reiser, J. & Stark, G.R. (1979) Transfer of proteins from gels to diazobenzyloxymethyl-paper and detection with antisera: A method for studying antibody specificity and antigen structure. Proceedings of the National Academy of Sciences (USA), 76, 3116-3120.

Renkawitz, R., Shutz, G., von der Ahe, D. & Beato, M. (1984) Sequences in the promoter region of the chicken lysozyme gene required for steroid regulation and receptor binding. Cell, 37, 503-510.

Rennie, P.S. (1979) Binding of androgen receptor to prostate chromatin requires intact linker DNA. Journal of Biological Chemistry, 254, 3947-3952.

Renoir, J.M., Buchan, T., Mester, J., Radanyi, C. & Baulieu, E-E. (1984) Oligomeric structure of molybdate stabilised non-transformed 8S progesterone receptor from chicken oviduct cytosol. Biochemistry, 23, 6016-6023.

Renoir, J.M. & Mester, J. (1984) Chick oviduct progesterone receptor: structure, immunology, function. Molecular and Cellular Endocrinology, 37, 1-13.

Reudelhuber, T. (1984) Upstream and downstream control of eukaryotic genes. Nature (London), 312, 700-701.

Richards, J.S., Ireland, J.J., Rao, M.C., Berneth, G.A., Midgley, A.F. & Reichert, L.E. (1976) Ovarian follicular development in the rat: hormone receptor regulation by oestradiol, follicle stimulating hormone and leutènising hormone. Endocrinology, 99, 1562-1570.

Riggs, A.D., Suzuki, H. & Bourgeois, S. (1970) Lac repressor-operator interactions I Equilibrium studies. Journal of Molecular Biology, 48, 67-83.

Robinson, S.I., Nelkin, B.D. & Vogelstein, B. (1982) The ovalbumin gene is associated with the nuclear matrix of chicken oviduct cells. Cell, 28, 99-106.

Robinson, S.I., Small, D., Idzerda, R., McKnight, G.S., Vogelstein. B. (1983) The association of transcriptionally active genes with the nuclear matrix of the chicken oviduct. Nucleic Acids Research, 11, 5113-5130.

Rochefort, H. & Capony, F. (1972) Binding properties of an antioestrogen to the oestradiol receptor of uterine cytosol. FEBS Letters, 20, 11-15.

Rochefort, H. & Borgna, J.L. (1981) Differences between oestrogen receptor activation by oestrogen and antioestrogen. Nature (London), 292, 257-259.

Rogers, J. & Wall, W. (1981) Immunoglobulin heavy chain genes: demethylation accompanies class switching. Proceedings of the National Academy of Sciences, 78, 7497-7501.

Ross, P. & Ruh, T.S. (1984) Binding of oestradiol receptor complex to reconstituted nucleo-acidic proteins from calf uterus. Biochemica et Biophysica Acta, 782, 18-25.

Rossini, G.P. (1984) RNA containing nuclear binding sites for glucocorticoid receptor complexes. Biochemical and Biophysical Research Communications, 123, 78-83.

Rousseau, G.G. (1984) Structure and regulation of the glucocorticoid hormone receptor. Molecular and Cellular Endocrinology, 38, 1-11.

Rousseau, G.G., Higgins, S.J., Baxter, J.P., Gelfend, D. & Tompkins, G.M. (1975) Binding of glucocorticoid receptors to DNA. Journal of Biological Chemistry, 250, 6015-6021.

Ruh, M.F., Brzyski, R.G., Strange, L. & Ruh, T.S. (1983) Oestrogen and antioestrogen binding to different forms of the molybdate stabilised oestrogen receptor. Endocrinology, 112, 2203-2205.

Ruh, M.F. & Ruh, T.S. (1984) Analysis of two forms of the molybdate-stabilized estrogen receptor. Endocrinology, 115, 1341-1349.

Ruh, T.S. & Baudendistal, L.J. (1977) Different nuclear binding sites for antioestrogen and oestrogen receptor complexes. Endocrinology, 100, 420-426.

Ruh, T.S., Ross, P., Wood, D.M. & Keene, J.L. (1981) The binding of ^{3}H-oestradiol/receptor complexes to calf uterine chromatin. Biochemical Journal, 200, 133-142.

Ruh, T.S. & Spelsberg, T.C. (1983) Acceptor sites for the oestrogen receptor in hen oviduct chromatin. Biochemical Journal, 210, 905-912.

Saffron, J., Loiser, B.K., Bohrsetl, S.A. & Faber, L.E. (1976) Binding of progesterone receptor by nuclear preparations of rabbit and guinea pig uterus. Journal of Biological Chemistry, 251, 5607-5613.

Sakai, D. & Gorski, J. (1984a) Reversible denaturation of the oestrogen receptor and estimation of polypeptide chain molecular weight. Endocrinology, 115, 2379-2383.

Sakai, D. & Gorski, J. (1984b) Oestrogen recptor transformation to a high affinity state without subunit-subunit interactions. Biochemistry, 23, 3541-3547.

Salacinski, P.R.P., McLean, C., Sykes, J.E.C., Clement-Jones, V.V. & Lowry, P.J. (1981) Iodination of proteins, glycoproteins and peptides using a solid phase oxidising agent, 2,3,4,6-tetrachloro-3α, 6α, diphenyl glycoluril (iodogen). Analytical Biochemistry, 117, 136-146.

Sander, S. (1968) The uptake of 17β-oestradiol in breast tissue of female rats. Acta Endocrinologica, 58, 49-56.

Sarosi, P., Schmidt, C.L., Essig, M., Steinetz, B.G. & Weiss, G. (1983) The effect of relaxin and progesterone on rat uterine contractions. American Journal of Obstetrics and Gynaecology, 145, 402-409.

Sato, B., Huseby, R.A. & Samuels, L.T. (1978a) Characterisation of oestrogen receptors in various mouse Leydig cell tumour lines. Cancer Research,38, 2842-2847.

Sato, B., Huseby, R.A. & Samuels, L.T. (1978b) Evidence of a small molecule in mouse Leydig cell tumours which inhibits the conversion of oestrogen receptor from 4S to 5S. Endocrinology, 102, 545-555.

Sato, B., Nishizawa, Y., Noma, K., Matsumoto, K. & Yamamura, Y. (1979) Oestrogen independent nuclear binding of receptor protein of rat uterine cytosol by removal of low molecular weight inhibitor. Endocrinology, 104, 1474-1479.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions. Annals New York Academy of Sciences, 51, 660-672.

Schallmann, K. & Pongs, O. (1982) Identification and characterisation of the ecdysterone receptor in Drosophila melanogaster by photo affinity labelling. Proceedings of the National Academy of Sciences (USA), 79, 6-10.

Scheidereit, C., Geisse, S., Westphal, H.M. & Beato, M. (1983) The glucocorticoid receptor binds to defined nucleotide sequences near the promoter of MMTV. Nature (London), 304, 749-752.

Schlissel, M. & Brown, D. (1984) The transcriptional regulation of the Xenopus 5S RNA genes in chromatin. The roles of active stable transcription complexes and histone H1. Cell, 37, 903-913.

Shoenberg, C.F. (1977) The contractile mechanism and ultrastructure of the myometrium. In Biology of the Uterus (ed. Wynn, R.) Plenum Press, New York.

Schoenberg, D.R. & Clark, J.H. (1981) Nuclear association states of rat uterine oestrogen receptors as probed by nuclease digestion. Biochemical Journal, 196, 423-432.

Schrader, W.T., O'Malley, B.W. & Kuhn, R.W. (1977) Progesterone binding components of chick oviduct: receptor B purified to apparent homogeneity from laying hens. Journal of Biological Chemistry, 252, 299-307.

Scott, R.W. & Frankel, F.R. (1980) Enrichment of oestradiol receptor complexes in a transcriptionally active fraction of chromatin from MCF 7 cells. Proceedings of the National Academy of Sciences (USA), 77, 1291-1295.

Sekula, B.C., Schmidt, T.J., Oxenham, E.A., DiSorbo, D.M. & Litwack, G. (1982) Dual effects of pyridoxal 5' phosphate on glucocorticoid receptor complexes. Biochemistry, 21, 2915-2922.

Senior, M.B. & Frankel, F.R. (1978) Evidence for two kinds of chromatin binding sites for the oestradiol/receptor complex. Cell 14, 857-863.

Shen, C.K.T. & Maniatis, T. (1980) Tissue specific methylation in a cluster of rabbit β like globin genes. Proceedings of the National Academy of Sciences (USA), 77, 6634-6638.

Sheridan, P.J., Anselmo, V.C., Buchanan, J.M. & Martin, P..M.(1979) Equilibrium: the intra cellular distribution of steroid receptors. Nature (London), 282, 579-582.

Sheridan, P.J., Buchanan, J.M., Anselmo, V.C. & Martin, P.M. (1984) Unbound progesterone receptors are in equilibrium between the nucleus and cytoplasm in cells of the rat uterus. Endocrinology, 108, 1533-1537.

Sherman, M.R., Moran, M.C., Tuazon, F.B. & Stevens, I.W. (1983) Structure, dissociation and proteolysis of mammalian steroid receptors. Multiplicity of glucocorticoid receptor form and proteolytic enzymes in rat liver and kidney cytosols. Journal of Biological Chemistry, 258, 10366-10377.

Shyamala, G. & Gorski, J. (1969) Oestrogen receptors in the rat uterus: studies on the interaction of cytosol and nuclear binding sites. Journal of Biological Chemistry, 244, 1097-1103.

Shyr, C.I. & Liao, S. (1978) A protein factor that inhibits binding and promotes release of androgen-receptor complex from nuclear chromatin. Proceedings of the National Academy of Sciences (USA), 75, 5969-5973.

Sica, V., & Bresciani, F. (1979) Oestrogen binding proteins of calf uterus. Purification to homogeneity of receptor from cytosol by affinity chromatography. Biochemistry, 18, 2369-2378.

Siiteri, P.K. (1978) Steroid hormones and endometrial cancer. Cancer Research, 38, 4360-4366.

Simmons, S.S., Martinez, H.M., Garcia, R.L., Baxter, J.D. & Tompkins, G.M. (1976) Interactions of glucocorticoid receptor-steroid complexes with acceptor sites. Journal of Biological Chemistry, 251, 334-343.

Singh, R.K., Ruh, M.F. & Ruh, T.S. (1984) Binding of ^{3}H-oestradiol and ^{3}H-H1285 receptor complexes to rabbit uterine chromatin. Biochemica et Biophysica Acta, 800, 33-40.

Singh, V.B., & Moudgil, V.K. (1984) Protein kinase activity of purified rat liver glucocorticoid receptor. Biochemical and Biophysical Research Communications, 125, 1067-1073.

Sluyser, M. (1983) Interactions of steroid hormone receptors with DNA. Trends in Biochemical Sciences, 8, 236-238.

Smith, R.G., Clarke, S.G., Zalta, E. & Taylor, R.N. (1979) Two oestrogen receptors in reproductive tissue. Journal of Steroid Biochemistry, 10, 31-35.

Smith, P. & Van Holt, C. (1981) Interaction of the activated cytoplasmic glucocorticoid hormone receptor complex with the nuclear envelope. Biochemistry, 20, 2900-2908.

Spelsberg, T.C. (1974) The role of nuclear acidic proteins in binding steroid hormones. In Acidic Proteins of the Nucleus, pp247-296, Academic Press, London, New York. (I.L. Cameron & J.R. Jeter Jr., Eds.)

Spelsberg, T.C. (1976) Nuclear binding of progesterone in chick oviduct multiple binding sites in vivo and transcriptional response. Biochemical Journal, 156, 391-398.

Spelsberg, T.C. (1982) Chemical characterisation of nuclear acceptors for the avian progesterone receptor. In Biochemical Actions of Hormones vol.9, pp141-204, Academic press, London (G. Litwack, Ed.)

Spelsberg, T.C., Steggles, A.W. & O'Malley, B.W. (1971) Progesterone binding components of chick oviduct - chromatin acceptor sites. Journal of Biological Chemistry, 246, 4188-4197.

Spelsberg, T.C., Steggles, A.W., Chytil, F. & O'Malley, B.W. (1972) Progesterone binding components of chick oviduct - exchange of progesterone binding capacity from target to non-target tissue chromatins. Journal of Biological Chemistry, 247, 1368-1374.

Spelsberg, T.C., Webster, R.A. & Pikler, G.M. (1975) Multiple binding sites for progesterone in the hen oviduct nucleus: evidence that acidic proteins represent the acceptors. In Chromosomal Proteins and their role in Gene Expression (Eds. G. Stein & L. Kleinsmith) Academic Press, New York, pp153-186.

Spelsberg, T.C. & Toft, D.O. (1976) In Receptors and Mechanism of Action of Steroid Hormones (J.R. Pasqualini Ed.) Raven Press, New York, pp261-309.

Spelsberg, T.C., Pikler, G.M. & Webster, R.A. (1976a) Progesterone binding to hen oviduct genome: specific versus non-specific binding. Science, 194, 197-198.

Spelsberg, T.C., Pikler, G.M. & Webster, R.A. (1976b) Chromosomal proteins regulate steroid binding to chromatin. Nature (London), 262, 65-67.

Spelsberg, T.C., Knowler, J.T., Boyd, P., Thrall, C. & Martindani,G. (1979) Support for chromatin acidic proteins as acceptors for progesterone in the chick oviduct. Journal of Biological Chemistry, 11, 373-379.

Spelsberg, T.C., Littlefield, B.A., Seelke, R., Martindani, G., Toyoda, H., Boyd-Leinen, P., Kon, O.L. & Thrall, C. (1983) Role of specific chromosomal proteins and DNA sequences in the nuclear binding sites for steroid receptors. Recent Progress in Hormone Research, 39, 463-517.

Spelsberg, T.C., Gosse, B.J., Littlefield, B.A., Toyoda, H. & Seelke, R. (1984) Reconstitution of nativelike nuclear acceptor sites of the avian oviduct progesterone receptor: evidence for involvement of specific chromatin proteins and specific DNA sequences. Biochemistry, 23, 5103-5113.

Steggles, A.W. & King, R.J.B. (1970) The use of protamine to study oestradiol binding in rat uterus. Biochemical Journal, 118, 695-701.

Stein, A. (1979) DNA folding by histones: The kinetics of chromatin core particle re-assembly and the interaction of nucleosomes with histones. Journal of Molecular Biology, 130, 103-134.

Stumph, W.E. (1968) Subcellular distribution of ^{3}H-oestradiol in rat uterus by quantitative autoradiography - a comparison between ^{3}H-oestradiol and ^{3}H-norethyodrel. Endocrinology, 83, 777-782.

Stumph, W.E., Baez, M., Beattie, W.G., Tsai, M-J. & O'Malley, B.W. (1983) Characterisation of DNA sequences at the 5' and 3' borders of the 100 kilobase ovalbumin gene domain. Biochemistry, 22, 306-315.

Sudo, K., Monsma, F.J. & Katzenellenbogen, B.S. (1983) Antioestrogen binding sites distinct from the oestrogen receptor: subcellular localisation, ligand specificity and distribution in tissues of the rat. Endocrinology, 112, 425-434.

Sutherland, R.L. (1981) Oestrogen antagonists in chick oviduct: activity of eight synthetic triphenylethylene derivatives and their interactions with cytoplasmic and nuclear oestrogen receptors. Endocrinology, 109, 2061-2068.

Sutherland, R.L., Mester, J. & Baulieu, E.E. (1977) Tamoxifen is a potent "pure" anti-oestrogen in chick oviduct. Nature (London), 267, 434-435.

Sutherland, R.L. & Whybourne, A.M. (1981) Binding of tamoxifen and its metabolites 4-hydroxytamoxifen and N-desmethyltamoxifen to oestrogen receptors from normal and neoplastic tissues. In Non-Steroidal Antioestrogens (Eds. Sutherland, R.L., Jordan, V.C.) Academic Press, Sydney, Australia, pp75-84.

Szego, C.M. (1965) Role of histamine in mediation of hormone action. Federation Proceedings, 24, 1343-1351.

Szego, C.M. (1984) Mechanisms of hormone action: parallels on receptor mediated signal propogation for steroid and peptide effectors. Life Sciences, 35, 2383-2396.

Szego, C.M. & Pietras, R.J. (1985) Subcellular distribution of oestrogen receptors. Nature (London), 317, 88.

Talwar, G.P., Segal, S.J., Evans, A. & Davidson, O.W. (1964) The binding of oestradiol in the rat uterus: a mechanism for expression of RNA synthesis. Proceedings of the National Academy of Sciences (USA), 52, 1059-1066.

Tasheva, B. & Dessev, G. (1983) Artifacts in sodium dodecyl sulfate polyacrylamide gel electrophoresis due to 2-mercaptoethanol. Analytical Biochemistry, 129, 98-102.

Taylor, C.M., Blanchard, B. & Zava, D.T. (1984) Oestrogen receptor mediated and cytotoxic effects of the antioestrogens tamoxifen and 4-hydroxytamoxifen, Cancer Research, 44, 1409-1414.

Tchernitichin, A. (1979) The role of eosinophil receptors in the non genomic response to oestrogen in the uterus. Journal of Steroid Biochemistry, 11, 417-424.

Teng, C-S. & Hamilton, T.H. (1969) Hormone induced synthesis of non histone acidic proteins which restore histone inhibited DNA dependent RNA synthesis. Proceedings of the National Academy of Sciences (USA), 63, 465-472.

Teng, C.T. & Teng, C.S. (1981) Changes in the quantities of HMG I in oviduct cellular fractions after oestrogen stimulation. Biochemical Journal, 198, 85-90.

Thanki, K., Beach, T. & Dickerman, H. (1978) Selective binding of mouse oestradiol receptor complexes to oligo (DT) cellulose. Journal of Biological Chemistry, 253, 7744-7750.

Thomas, N. & Bell, P.A. (1983) Preferential solubilisation of triton X-100 resistant nuclear glucocorticoid receptors by DNase I. Biochemical and Biophysical Research Communications, 111, 760-767.

Thomas, T., Leung, B.S., Yu, W.C.Y. & Kiang, D.T. (1983) Two classes of oestrogen receptors which differ in their activation mechanism. Biochemical and Biophysical Research Communications, 115, 685-691.

Thomas, T.L. & Patel, G.L. (1976) DNA unwinding component of the non histone chromatin proteins. Proceedings of the National Academy of Sciences (USA), 73, 4364-4368.

Thrall, C.L., Webster, R.A. & Spelsberg, T.C. (1978) Steroid receptor interaction with chromatin. In The Cell Nucleus vol.6 (Busch, H. Ed.) pp461-529, Academic Press, New York.

Thrower, S. Neithling, C., White, J.O. & Lin, G. (1981) The unoccupied nuclear oestradiol receptor in the rat uterus and hypothalamus during the oestrous cycle. Biochemical Journal, 194, 667-671.

Toft, D.O. (1972) The interaction of uterine oestrogen receptors with DNA. Journal of Steroid Biochemistry, 3, 515-522.

Toft, D.O. & Gorski, J. (1966) A receptor molecule for oestrogen: isolation from the rat uterus and preliminary characterisation. Proceedings of the National Academy of Sciences (USA), 55, 1574-1581.

Toft, D.O., Shyamala, G. & Gorski, J. (1967) A receptor molecule for oestrogens: studies using a cell free system. Proceedings of the National Academy of Sciences (USA), 57, 1740-1743.

Towbin, H., Staechelin, T. & Gordon, J.. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences (USA), 76, 4350-4354.

Traish, A.M., Muller, R.E. & Wotiz, H.H. (1979) Comparison of formation, activation and nuclear translocation of receptor/oestradiol complex at $0^{o}C$ and $37^{o}C$ in intact uterine cells. Journal of Biological Chemistry, 254, 6560-6563.

Traish, A.M., Muller, R.E. & Wotiz, H.H. (1980) Effects of pyridoxal 5' triphosphate on uterine oestrogen receptor. Inhibition of oestrogen receptor transformation.. Journal of Biological Chemistry, 255, 4068-4072.

Tsai, Y., Sanborn, B.M., Steinberger, A. & Steinberger, E. (1980) Sertoli cell chromatin acceptor sites for androgen receptor complexes. Journal of Steroid Biochemistry, 13, 711-718.

Tsai, Y.H. & Steinberger, A. (1982) Effect of sodium molybdate on the binding of androgen receptor complexes to germ cell and sertoli cell chromatin. Journal of Steroid Biochemistry, 17, 131-136.

Van den Broek, H.W.J., Nooden, L.D., Sevall, J.S. & Bonner, J. (1973) Isolation, purification and fractionation of non histone chromosomal proteins. Biochemistry, 12, 229-236.

Van der Ploeg, L.H.T. & Flavell, R.A. (1980) DNA methylation in the human γ δ β globin locus in erythroid and non erythroid tissues. Cell, 19, 947-958.

Vedeckis, W.V. (1983) Subunit dissociation as a possible mechanism of glucocorticoid receptor activation. Biochemistry, 22, 1975-1983.

Vedeckis, W.V., Schrader, W.T. & O'Malley, B.W. (1980) Progesterone binding components of chick oviduct: analysis of receptor structure by limited proteolysis. Biochemistry, 19, 343-349.

Von der Ahe, D., Janick, S., Scheidereit, C., Renkawitz, R., Schutz, G. & Beato, M. (1985) Glucocorticoid and progesterone receptors bind to the same sites in two hormonally regulated promoters. Nature (London), 313, 706-709.

Waalwijk, C. & Flavell, R.A. (1978) DNA methylation of a CCGG sequence in the large intro of the rabbit β globin gene: tissue specific variations. Nucleic Acids Research, 5, 4631-4641.

Walker, M.D., Edmund, T., Boulei, M.M. & Rutter, W.J. (1983) Cell specific expression controlled by the 5' flanking region of insulin and chyotrypsin genes. Nature (London), 306, 557-561.

Wecksler, W.R. & Norman, A.W. (1980) A kinetic and equilibrium binding study of 1α , 25 dihydroxy-vitamin D_3 with its cytosol receptor from chick intestinal mucosa. Journal of Biological Chemistry, 255, 3571-3574.

Weisbrod, S. & Weintraub, H. (1979) Isolation of a sub class of nuclear proteins responsible for conferring a DNase I-sensitive structure on globin chromatin. Proceedings of the National Academy of Sciences (USA), 76 630-634.

Weisbrod, S. & Weintraub, H. (1981) Isolation of actively transcribed nucleosomes using immobilised HMG14 and 17 and an analysis of α globin chromatin. Cell, 23, 391-400.

Weigil, N.L., Tash, J.S., Means, A.R., Schrader, W.T. & O'Malley, B.W. (1981) Phosphorylation of hen progesterone receptor by cAMP dependent protein kinase. Biochemical and Biophysical Research Communications, 102, 513-519.

Welshons, W.V., Lieberman, H.E. & Gorski, J. (1984) Nuclear localisation of unoccupied oestrogen receptors. Nature (London), 307, 747-749.

Westphal, U. (1971) Steroid Protein Interactions, Springer-Verlag, New York.

Westphal, U. (1980) How are steroids transported in the blood before they enter target cells? In Steroid Receptors and Hormone Dependent Neoplasia (Wittliff, J.L. & Dapont, O. Eds) Masson Publishing, USA, pp1-17.

White, J.O. & Lin, L. (1980) Unoccupied nuclear oestrogen receptors in the female rat hypothalamus. Biochemical Journal, 190, 833-837.

Widnell, C.C. & Tata, J.R. (1964) A procedure for the isolation of enzymically active rat liver nuclei. Biochemical Journal, 92, 313-319.

Wilhelm, J.A., Ansevin, A.T., Johnson, A.W. & Hnilica, L.S. (1972) Proteins of chromatin in genetic restriction. IV Comparison of histone and non-histone proteins of rat liver nucleolar and extranucleolar chromatin. Biochemica et Biophysica Acta, 272, 220-230.

Wilks, A.F., Cozens, P.J., Mattas, I.W. & Jost, J.P. (1982) Oestrogen induces a demethylation at the 5' end region of the chicken vitellogenin gene. Proceedings of the National Academy of Sciences (USA), 79, 4252-4255.

Williams, D.L. & Gorski, J. (1971) A new assessment of subcellular distribution of bound oestrogen in the uterus. Biochemical and Biophysical Research Communications, 45, 258-264.

Williams, D.L. & Gorski, J. (1972) Kinetic and equilibrium analysis of oestradiol in rat uterus.. A model of binding site distribution in uterine cells. Proceedings of the National Academy of Sciences (USA), 69, 3464-3468.

Williams, D.L. & Gorski, J. (1974) Equilibrium binding of oestradiol by uterine cell suspensions and whole uteri in vivo. Biochemistry, 13, 5537-5542.

Williams, J.L. & Tata, J.R. (1983) Simultaneous analysis of conformation and transcription of A and B groups of vitellogenin genes in male and female Xenopus during primary and secondary activation by oestrogen. Nucleic Acids Research, 11, 1151-1166.

Winneker, R.C. & Clarke, J.H. (1983) Oestrogenic stimulation of the antioestrogen specific binding site in rat uterus and liver. Endocrinology, 112, 1910-1915.

Wittliff, J.L. (1975) Steroid binding proteins in normal and neoplastic mammary cells. In Methods in Cancer Research (Busch, H. Ed.) vol.XI, pp293-354, Academic Press, New York.

Wright, S. Rosenthal, A., Flavell, R. & Grosveld, F. (1984) DNA sequences required for regulated expression of β globin genes in murine erythroleukaemia cells. Cell, 38, 265-273.

Wu, C. (1984) Two protein binding sites in chromatin implicated in the activation of heat shock genes. Nature (London), 309, 229-234.

Wu, C. (1985) Eukaryotic genes: Abstract - chromatin structure and regulation of heat shock genes. 5th Tenovus Symposium (Scotland).

Yamamoto, K.R. & Alberts, B.M. (1972) In vitro conversion of oestradiol-receptor to its nuclear form: dependence on hormone and DNA. Proceedings of the National Academy of Sciences (USA), 69, 2105-2110.

Yamamoto, K.R. & Alberts, B.M. (1974) On the specificity of the binding of the oestradiol receptor protein to DNA. Journal of Biological Chemistry, 249, 7076-7086.

Yamamoto, K.R. & Alberts, B.M. (1975) The interaction of oestradiol receptor protein with the genome. An argument for the existence of undetected specific sites. Cell, 4, 301-310.

Zasloff, M. & Felsenfield, G. (1976) The use of mercury substituted ribonucleoside triphosphates can lead to artifacts in the analysis of in vitro chromatin transcripts. Biochemical and Biophysical Research Communications, 75, 598-603.

Zava, D.T. & McGuire, W.L. (1977) Oestrogen receptor: unoccupied sites in nuclei of a breast tumour cell line. Journal of Biological Chemistry, 252, 3703-3708.