THE MOLECULAR MECHANISMS OF STEROID HORMONE ACTION

by

SALMAN M. HYDER, B.Sc.

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science

Department of Biochemistry,
University of Glasgow

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This thesis is dedicated to my family.

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ABBREVIATIONS

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

AV	adriamycin-vincristine
BSA	bovine serum albumin
^{o}C	degrees centigrade
cpm	counts per minute
CMF	cyclo phosphamide-methotrexate-fluorouracil
CNS	central nervous system
DCC	dextran coated charcoal
DES	diethylstilboestrol
DFP	diisopropyl fluorophosphate
DMBA	dimethylbenz(a)anthracene
DTT	dithiothreitol
E_2	oestradiol-17β
ER_c	cytoplasmic or soluble oestrogen receptor
ER_n	nuclear oestrogen receptor
FAC	fluorouracil-adriamycin-cyclophosphamide
$HDK._{15}$	Hepes buffer with DTT and 0.15M KCl
$HDK._4$	Hepes buffer with DTT and 0.4M KCl
$^3HE_2$	tritiated oestradiol-17β
Hepes	N-2-hydroxy-piperazine-N'-2-ethane sulphonic acid
PMSF	Phenyl-methyl-sulphonyl-fluoride
POPOP	1,4-di-{2-(5-phenyloxazolyl)}-benzene

PPO	2,5-diphenyloxazole
S	Svedberg units (10^{-13}s)
SDG	sucrose density gradient
SDGA	sucrose density gradient analysis
SHBG	sex hormone binding globulin
w/w	weight/weight

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SUMMARY

(a) Clinical Aspects

The relationship between the molecular forms of oestrogen receptor (4S and 8S forms) in human breast cancer and subsequent response to hormone therapy is controversial. The data presented in this thesis show that several factors can effect the final sucrose density gradient profile of soluble oestrogen receptor under low salt conditions. These include incubation time with steroid, temperature, ionic strength, extent of aggregation and intratumoural variation. It is further shown that buffer made 50% in glycerol can be used to preserve the molecular form of oestrogen receptor in human breast tumour biopsies prior to and subsequent to transportation. Receptor 8S form was preserved for up to 3 months under these conditions. Most tumour biopsies analyzed exhibited the presence of 8S form of the receptor either alone or in conjunction with the 4S form. Relatively few tumours exhibited predominantly the 4S form. Analysis of intratumoural sections revealed a loss of receptor concentration towards the centre of the tumour. The molecular forms found across a tumour usually remained constant. However, when both 8S and 4S forms of receptor were detected, the relative concentration of each form changed across the tumour. These results indicate that strict criteria, with respect to analysis of molecular forms of oestrogen receptor, must be observed if these are to be related to potential response of individual patients to endocrine therapy.

(b) Receptor activation/transformation

The mechanism of receptor activation/transformation was studied in immature rat uterus, human breast carcinoma and endometrial tissue. DNA-cellulose binding was characterized as an *in vitro* acceptor of activated receptor ($30^{\circ}C$, 30min). Up to 80% of the activated receptor from immature rat uterine tissue bound to DNA-cellulose in contrast to only $\sim$ 20% from human breast carcinoma. In the presence of protease inhibitors, human breast carcinoma ER still showed a lower level of DNA-cellulose binding

was significantly higher than control.

In the presence of sodium molybdate, an inhibitor of activation of immature rat uterine ER_c, ~ 20% of the receptor was found to be insensitive to this inhibitory effect. A proportion of receptor may be permantly activated.

When SDGA was conducted in low salt buffers, DNA (calf thymus) binding studies of ER_c indicated no requirement for heat activation. Both immature rat uterine 8S ER_c and human breast tumour 8S ER_c behaved similarly and sedimented to the bottom of the tube in conjunction with the DNA. However, variation in the affinity of different molecular forms of ER_c from human breast carcinoma for the calf thymus DNA was observed. The 4S form was found to bind with a lower efficiency than the 8S form (although in certain cases a large proportion of 4S form bound to DNA). In one case both 4S and 8S only partially bound to DNA. These results indicated that several factors, including proteolysis, dissociation of 8S complex, association of 4S complex, and involvement of various inhibitors could regulate the extent of 4S and 8S binding to DNA. This therefore indicates a possible equilibrium between the 8S and 4S forms. A 4S proteolyzed form of receptor which is unable to bind to DNA is postulated.

Sucrose density gradient (SDG) profiles obtained after receptor activation ($30^{\circ}C$ for 30min) showed the presence of only the 4S form in human breast carcinoma and endometrial cytosol when analyzed on high salt gradients at $4^{\circ}C$. In contrast, the immature rat uterine ER_c underwent a 4S 5S transformation in a time dependent manner, associated with the acquisition of DNA binding potential.

In the presence of protease inhibitors the human receptor still sedimented at 4S when analyzed on high salt gradients at $4^{\circ}C$, after prior

activation. Some tumour ER showed aggregation, resistant to 0.4M KCl conditions. Similar profiles were obtained in 0.15M KCl gradients. However, without previous warming and when extracted with buffers containing 0.15M KCl, the receptor sedimented at 6S at both 4°C and 20° C centrifugation in 0.15M KCl gradients. At elevated centrifugation run temperatures the presence of protease inhibitors (DFP, Leupeptin) were found to be essential to observe the 6S form. The data suggests that the formation of 6S complex in isotonic conditions is dependent on the concentration of the 8S complex present in low salt. The 6S complex was found to be sensitive to > 0.15M KCl and dissociated into the 4S form, even in the presence of DFP. It is therefore concluded that the possible reasons for low levels of DNA binding obtained with human tissue results from both proteolysis and aggregation of receptor on activation (30°, 30'). Proteolysis probably causes loss of the DNA binding site and aggregation masks this site. The 6S form observed with human receptor is similar in nature to the activated 5S form obtained from immature rat uterus. Thus, the 6S complex detected at 20°C, under defined conditions, may represent the activated form. It may be analogous to the progesterone receptor dimer.

Models are proposed for the interaction of the various sub-units involved in function of ER_c <u>in vivo</u>.

ADDENDUM

Recent evidence has been presented to indicate that steroid receptors are phosphoproteins:

Dougherty, J.J., Puri, R.K. & Toft, D.O. (1982) J.Biol.Chem. 257, 14226-14230.

Migliaccio, A., Lastoria, S., Moncharmont, B., Rotondi, A. & Auricchio, F. (1982) Biochem. Biophys. Res. Commun. 109, 1002-1010.

Housley, P.R. & Pratt, W.B. (1983) J.Biol.Chem. 258, 4630-4635.

1. INTRODUCTION

1.1 PART A STEROID-RECEPTOR INTERACTION

1.1.1 Control and Regulation Mechanisms in Higher Organisms

The orderly development and co-ordinate functioning of higher organisms implies the existence of sophisticated control mechanisms. In the human body, two control mechanisms (i) the central nervous system (CNS) and (ii) the endocrine system, meet this requirement. The electrical signals from the CNS are the mediators of the voluntary and involuntary actions. The chemical signals (hormones) released from the endocrine glands are required for the co-ordination of metabolism. Some interaction of the two control systems is now recognized.

The endocrine glands are ductless tissues which secrete the hormones directly into the blood stream. Whereas the response initiated by the CNS is usually instantaneous, responses initiated by hormones may be much slower, ranging from as little as a few seconds to as long as several days - the so-called short and long term responses.

The term 'hormone' is derived from a Greek word meaning 'I excite' or 'arouse', and was first used by Starling in 1905 (Starling, 1905) in connection with secretin, a chemical which enhances the production of pancreatic juice. This led to the definition of a hormone as 'any substance normally produced in specific cells of some part of the body and carried by the blood stream to distant parts, which it affects for the good of the body as a whole'(quoted in Lee and Laycock, 1978). However, this is a very general statement (Robison et al., 1971) and several chemicals such as the neurosecretions and the prostaglandins, which mimic hormones, cannot be regarded in this category. The neurosecretions do not enter the general circulation and the prostaglandins are not necessarily synthesized by particular

specialized glands and may, further, act directly on the tissues in which they are synthesized.

The endocrine glands, involved in the synthesis and release of hormones, are themselves under the influence of environmental fluctuations which may increase or decrease their activity. There is a close relationship between the neuronal and endocrine systems. The complex control mechanism of the endocrine system may involve both a neural control loop and a feedback chemical control, via the blood supply.

Once in the blood stream the hormone will contact many tissues and organs. However, response is only initiated in specific organs - termed the target organs, and is reflected by appropriate metabolic changes. These may include changes in enzyme activity (e.g. by phosphorylation and/or dephosphorylation), transport activity and longer term responses leading to modulation of growth and cell division. Target organs may be sensitive to either one or a combination of hormones. Target organs are characterized by the possession of a specific receptor for the relevant hormone. The tertiary or quaternary structure of the chemical receptors in the target tissue allows only the biological hormone to be recognized. For the polypeptide hormones e.g. insulin, these receptors appear to be located in the plasma membrane. For the steroid hormones they are located in the soluble fraction of the homogenate and, therefore, may, in the intact cell, be soluble or loosely attached to membrane structures within the cytoplasm (Leake, 1976). They may even be present in the nucleus (Martin and Sheridan, 1982).

Polypeptide hormone action is still not fully understood. Insulin, for example, binds to cell surface receptors (Cuatrecasas,

1974) resulting in the activation of an intracellular messenger system. However, intracellular binding sites have been reported (Goldfine and Smith, 1976). Both receptor degradation and recycling of cell surface receptors may be the cause of detection of intracellular binding (Kahn et al., 1981; King and Cuatrecasas, 1981). Alternatively it has been suggested that cell surface receptors may be involved in the initial short term effects of polypeptide hormones, initiated through a second messenger, whilst the long term effects (if any) may occur after hormone entry into the cell (Kolata, 1978). This thesis is concerned with the molecular mechanisms of steroid hormone action and further analysis will be confined to steroid hormones.

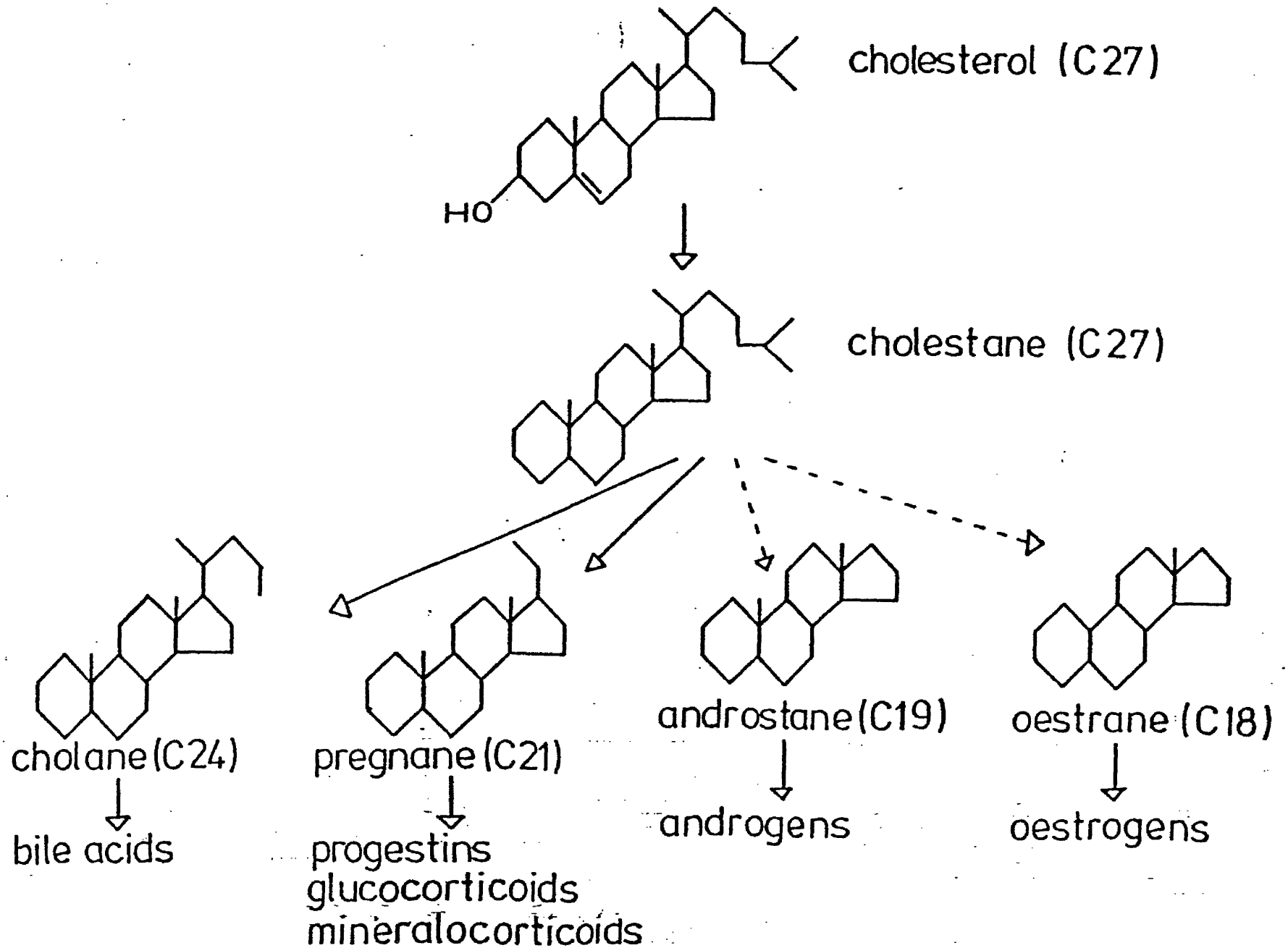
1.1.2 General Occurrence of Steroid Hormones in Higher Organisms

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. It seems surprising, however, that such simple chemical compounds can produce such diverse effects on both metabolic and behavioural patterns.

1.1.3 Steroid Structure

Steroids are relatively small hydrophobic molecules derived chemically from the parent compound cholesterol. The strong hydrophobic and therefore lipophilic nature of steroids is thought to assist in the diffusion of steroids across the cell membrane (see Section 1.1.5.1.2).


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

The two main organs synthesizing cholesterol are the liver and the intestine. The endocrine glands can, therefore, use plasma cholesterol for the synthesis of steroids. However, it has been shown that the adrenal cortex, ovaries, and other endocrine glands have the capacity to synthesize cholesterol from acetate. In the testis steroids are synthesized exclusively from acetate. In addition, Ramsey and Nicholas (1972) have shown that the brain also contributes to the body cholesterol pool but only to a very minor extent. The body cholesterol pool is therefore a balance of the extent of absorption from the diet plus the amount of de novo synthesis with excretion. Figure 1 shows the various dehydrogenation reactions involved in the production of the final steroid structure. The 27 carbon cholesterol is converted to pregnenolone, a 21 carbon compound, by a series of biosynthetic steps common to all mammalian steroidal hormones (Baird, 1972). Pregnenolone can then be converted to (i) 21 carbon atom progestins, glucocorticoids and mineralocorticoids, (ii) 19 carbon atom androgens and (iii) 18 carbon atom oestrogens. Three six membered rings and one five membered ring are common to all steroids (Figure 2). The steroid skeleton may be modified by various substitutions

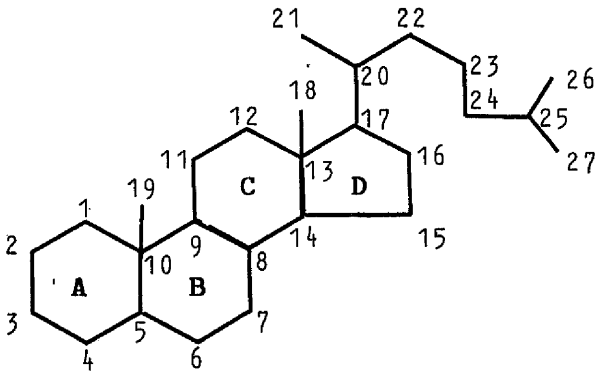


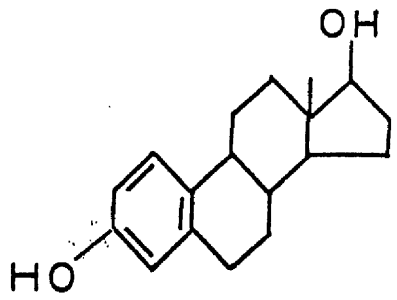
Figure 2. Numbering and lettering of steroids

such as double bonds, hydroxyl groups or ketone groups, either alone or in combination. The aldehyde group is less common and is only found in association with aldosterone. 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., 1973). The structure and nomenclature of steroids is fully described by Gower (1979). Interestingly, Young et al. (1977) have described steroidal oestrogens in plants, the physiological significance of which is unknown.

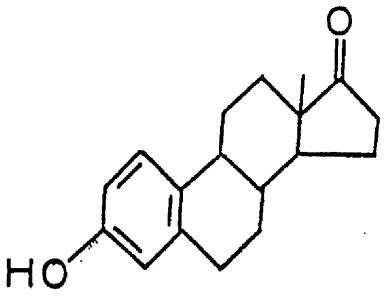
1.1.4 Oestrogens

1.1.4.1 Synthesis

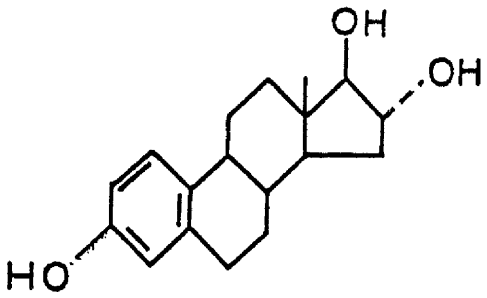
Oestrogens are involved primarily in the reproductive processes in females. Characteristically they have an A ring, bearing a phenolic group in position 3 of the steroid nucleus (Figure 3). Diethylstilboestrol (DES), included in Figure 3, is a potent synthetic non-steroidal oestrogen which binds to the oestrogen receptor with a higher affinity than the natural ligand oestradiol-17β . The three-dimensional structural similarity between DES and oestradiol-17β , however, is recognised (Duax et al., 1980). 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 synthesized from cholesterol in the ovary as a result of stimulation by gonadotrophins from the anterior pituitary. A second, minor, source of oestradiol-17β is the adrenal cortex. The ovaries of postmenopausal women synthesise little oestrogen. Oestrogen in postmenopausal women is synthesised mainly in the adrenal glands by the conversion of 4-androstene-3, 17-dione to oestrone (England et al., 1974; Gower and



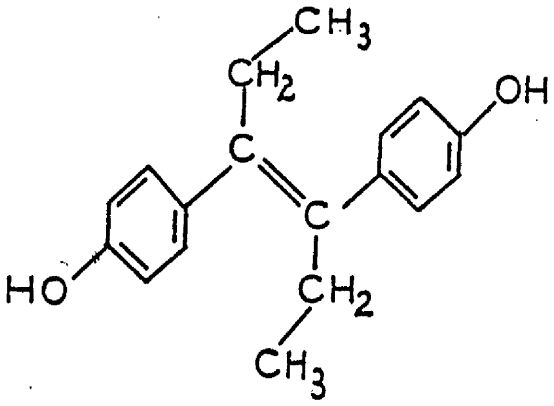
17-β oestradiol



oestrone



oestriol



diethylstilboestrol

Fotherby, 1975) and a similar conversion takes place in the peripheral tissues (Siiteri, 1978). During human pregnancy, the foetal-placental unit also produces substantial amounts of both oestradiol-17β and oestrone. The predominant oestrogen, however, is oestriol, especially during the last trimester (De Hertogh and Thomas, 1975). The pathways involved in oestrogen synthesis are shown in Figure 4.

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

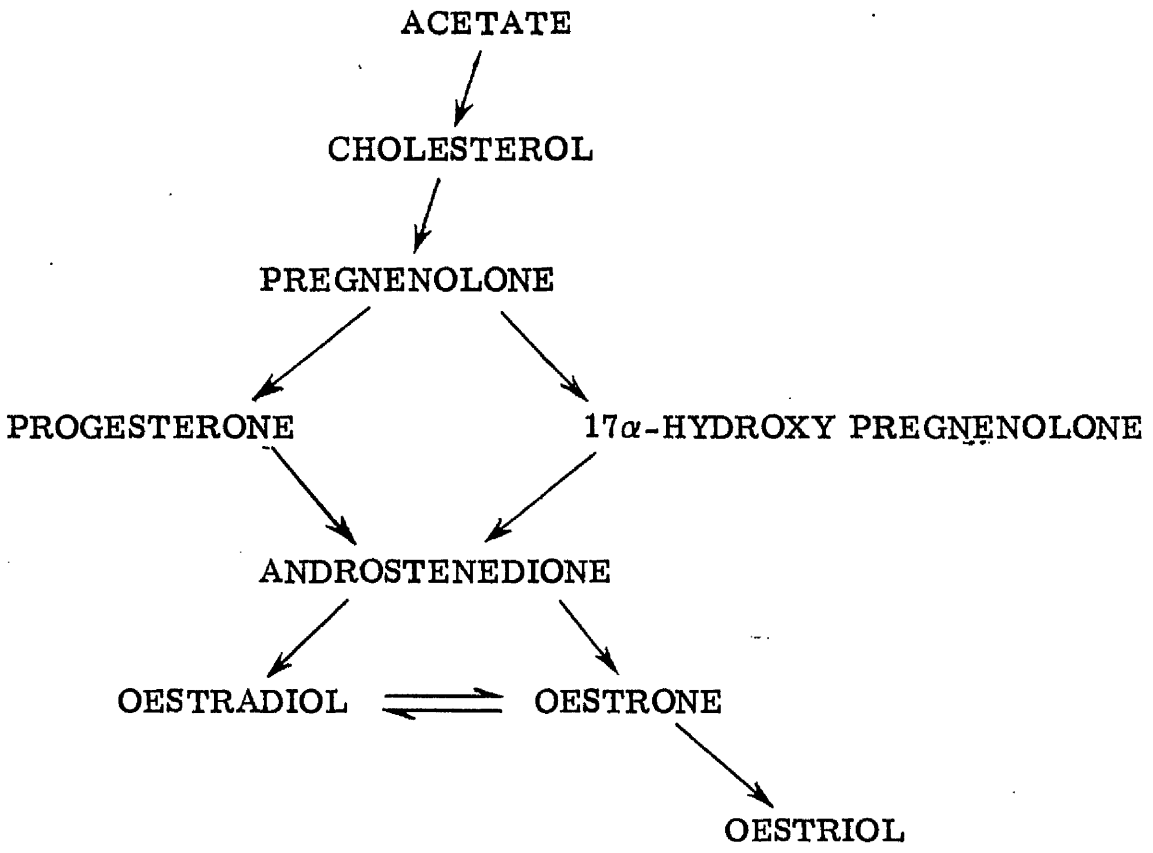
Once released into the circulation, the activity of the oestrogens has to be regulated. Consequently, oestradiol is metabolized to less active oestrone with which it forms an equilibrium. Oestrone can be further converted to oestriol. Other methods of inactivation include hydroxylation and methylation at C-2, oxidation at C-6 and hydroxylation at C-11. These conversions occur mainly in the liver, which is also responsible for the conjugation of oestrogens with glucoronic acid and sulphuric acid rendering the oestrogens more water soluble prior to excretion. The sulphate derivatives can be re-used after the removal of the sulphate group, as useful metabolic intermediates. For example dehydroepiandrosterone sulphate of foetal origin, is an important substrate in placental steroid synthesis. Oestrogens excreted in the urine include oestriol, oestrone and oestradiol-17β, as well as several unidentified oestrogens. Approximately 10% of total oestrogen excretion is by the faeces.

1.1.4.2 **Physiological Actions**

The principal actions of oestrogens include the following:-

(a) the development of female sex characteristics and female reproductive organs such as uterus, vagina and mammary glands. During

Figure 4. **Pathways involved in Oestradiol and Oestrone Synthesis** (adapted from Lee and Laycock, 1978).


ACETATE
CHOLESTEROL
PREGNENOLONE
PROGESTERONE
17α-HYDROXY PREGNENOLONE
ANDROSTENEDIONE
OESTRADIOL
OESTRONE
OESTRIOL

the proliferative phase of the menstrual cycle, oestrogens promote growth and development of the uterine endometrium. During pregnancy, both the glycogen and the actomyosin content of the myometrium are increased by oestrogens which also help in sensitizing the myometrium to the stimulatory actions of oxytocin, perhaps by making available the free Ca^{2+} ions (Lee and Laycock, 1978),

(b) the proliferation during pregnancy of the mammary ducts in preparation for lactation,

(c) in combination with progesterone, oestrogens are involved in the controlled development of the embryo,

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

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

The elucidation of the mechanisms by which the above-mentioned processes are brought about was greatly assisted when it became possible to designate 'oestrogen target tissues', as those 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).

1.1.4.3 **Development of the Concept of Receptors**

The physiological responses to oestrogens have been recognised for several years but 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 (Szego, 1957; Hechter and Halkerston, 1964; Bush, 1964). It was assumed that the distribution of receptors determined tissue specificity and that the nature of the hormone-receptor complex controlled the tissue response. Jensen and Jacobson (1962) demonstrated that with tritium labelled oestradiol-17β, rat uterus and vagina selectively bound and retained the steroid, unlike the non-target tissues. It was also demonstrated that oestradiol was retained in the unmetabolized form. Thus, it was proposed that target cells contain specific receptors which combine with oestrogen to form a complex. The sequence of events which constitutes the uterine response to oestrogens was thought to be receptor mediated. These studies were extended by Noteboom and Gorski (1965) who reported that the oestrogen receptor is stereo-specific and probably a protein. After *in vivo* administration of oestradiol-17β, Toft and Gorski (1966) detected the specific binding to an intracellular protein in the soluble uterine extract. Toft *et al.* (1967) identified it by sucrose density gradient analysis (SDGA) 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.

The proposal that uterotrophic action of oestradiol depends on its binding to specific receptors, was substantiated by experiments with specific binding inhibitors such as nafoxidine which was known to inhibit the characteristic uterotrophic response of oestrogens and was shown

to compete for the receptor (Lerner et al.,1958; Jensen, 1962; Callantine et al., 1966). Actinomycin D and puromycin also blocked the uterotrophic action (Ui and Mueller, 1963) but these did not decrease the uptake and binding of hormone (Jensen, 1965) suggesting that these inhibitors act at later stages in a sequence of biochemical events in which the hormone receptor binding is an early step. The finding that in vivo binding can also be achieved in vitro (Terenius, 1966) permitted rapid analysis of receptor properties.

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 (Leblond, 1951; 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 Jensen to propose a two step model for the interaction of oestrogen with the uterus (Jensen et al., 1967; see Section 1.1.5).

Other target organs 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 previously were considered as non-target tissues. These include the liver (Aten et al., 1978), kidney (Li et al., 1974), adrenal glands (Muller and Wotiz, 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).

1.1.4.4 **General Properties of Oestrogen Receptor**

The proteinaceous nature of oestrogen receptor was demonstrated by Toft and Gorski (1966). King (1968), using chromatographic purification, confirmed this observation. Oestrogen receptor was found to be sensitive to pronase but not to nucleases (Toft et al., 1967; Korenman, 1968). Mester et al. (1970) demonstrated the pH profile of oestrogen receptor and the optimum value was found to be pH 7. No binding was observed below pH 6 or above pH 9. Thus, ionic charges on the protein molecule influence receptor oestradiol interaction. The importance of sulphydryl groups for the binding of the oestrogen molecule has been demonstrated (Jensen et al., 1967; Terenius, 1967; Muldoon, 1971). The relevance of the sulphydryl group in oestrogen-receptor interaction in human breast cancer cytosol has been noted by McGuire and De La Garza (1973a) who further confirmed the heat lability of receptor. Receptor activation (Section 1.1.5.3) is also shown to depend on intact sulphydryl groups (Young et al., 1975; Nielsen and Notides, 1975; Kalimi and Love, 1980; Kalimi and Banerjee, 1981). Keightley et al. (1978), conversely, did not find inclusion of thiol reagents beneficial for oestrogen-receptor interaction. It has been suggested that steroid receptors are metalloproteins (Shyamala, 1975) and that activation causes an allosteric change, involving the altered availability of metal ions (Schmidt et al., 1981). The metal ion is thought to be located close to the DNA binding site. King et al. (1978) demonstrated that protein conformation is an integral part of receptor for determining its association with oestrogen. Lyophilisation has been shown not to alter the protein conformation (Koenders et al., 1978, 1980).

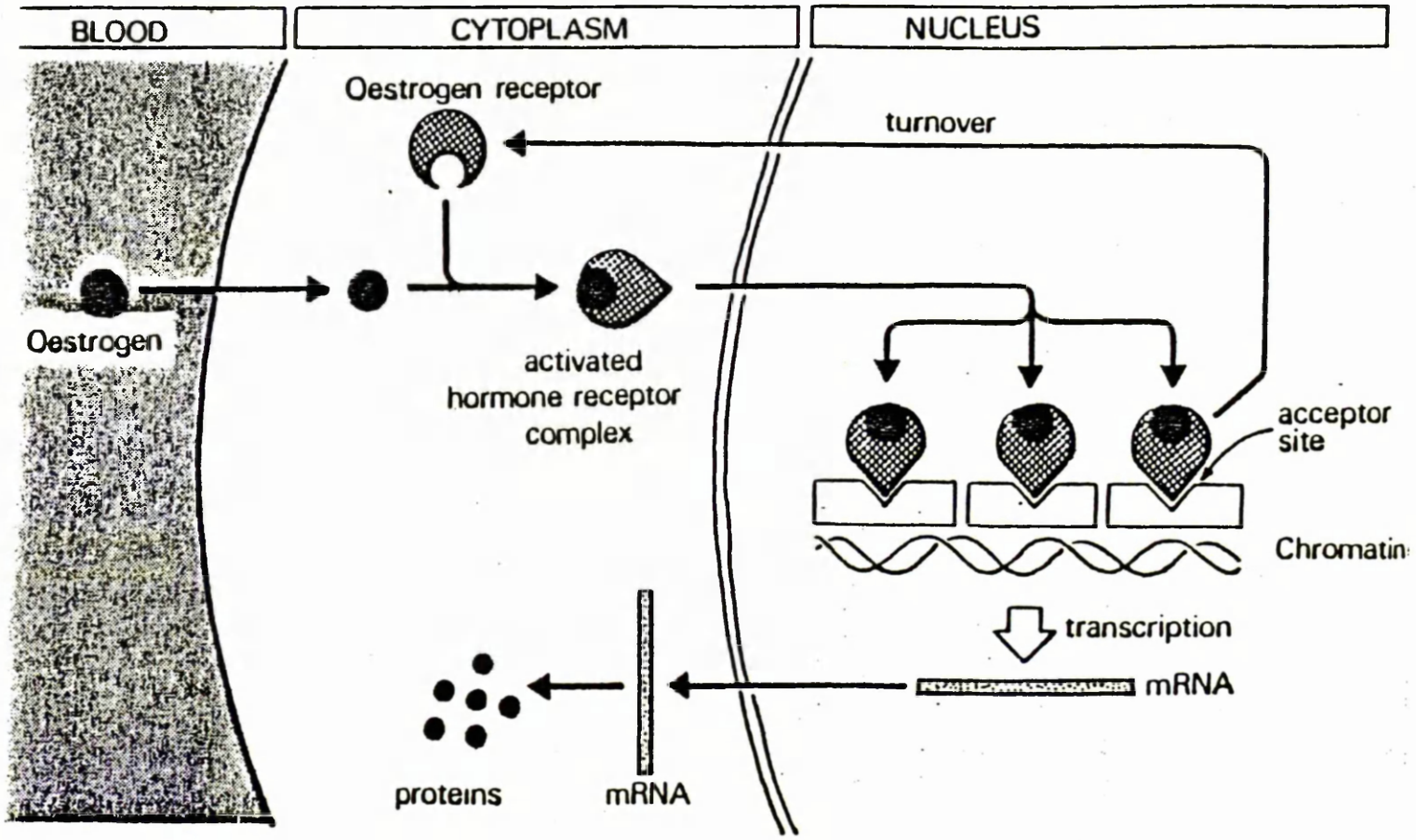
The appearance of a peak concentration of oestrogen receptor

during the rat oestrous cycle is controversial (Feherty et al., 1970; Lee and Jacobson, 1971; Kielhorn and Hughes, 1977; Fishman and Fishman, 1979). The picture has been further complicated by the report of White et al. (1978) who found no variation in the oestrogen receptor levels throughout the oestrous cycle. In the human endometrium, Soutter et al. (1979) have reported a peak value of nuclear oestrogen receptor at day 9 of the menstrual cycle. Bayard et al. (1978) and Pollow et al. (1981), have presented results similar to Soutter et al. (1979) and in addition have shown that cytoplasmic progesterone receptor concentration is high in the proliferative phase and declines in the early luteal phase. These studies have demonstrated that subcellular distributions of oestradiol-17β and progesterone receptor reflect the plasma concentration of the respective hormones. A change in the affinity of the receptor for oestradiol during the oestrous cycle has been reported, perhaps indicating some form of regulatory phenomena (Buchi and Keller, 1980).

1.1.5 Molecular Mechanisms of Steroid Hormone Action

Figure 5 illustrates the general concept involved in the mechanism of steroid hormone action. This general model was put forward in 1968 by two independent groups (Gorski et al.,1968; Jensen et al.,1968). After several years of intensive investigation, the general principles still hold true. However, modifications in the overall scheme have been suggested (Linkie and Siiteri,1978; Sheridan et al., 1979; Martin and Sheridan, 1982).

After its entry into a target cell, the steroid is bound by specific receptors. The possession of specific receptors defines a target tissue (Jensen and DeSombre, 1972; Leake, 1976). The steroid-receptor complex then undergoes some form of ill-understood transformation or activation


BLOOD
CYTOPLASM
NUCLEUS
Oestrogen receptor
turnover
Oestrogen
activated
hormone receptor
complex
acceptor
site
Chromatin
transcription
mRNA
proteins
mRNA

process (see Section 1.1.5.3). The receptor then translocates to the nucleus, binds to the chromatin and results in the modulation of transcription (Leake, 1981a) of certain specific messenger ribonucleic acid (mRNA) molecules (Aziz et al., 1979). This leads to the synthesis of certain specific proteins. Much work has been done on the kinetics of regulation of ovalbumin and conalbumin mRNA synthesis by oestrogen and progesterone in the chick oviduct (Mulvihill and Palmiter, 1980).

1.1.5.1 Mechanism of Steroid Entry into the Cell

1.1.5.1.1 Steroid Hormone Binding Proteins

Hormones are released into the blood stream by the endocrine glands. The steroids are then transported in the blood stream by sex hormone binding globulin (SHBG) and albumin (Clark and Peck, 1979) and other plasma proteins. The blood proteins bind steroid with varying affinity and the free hormone level determines the amount of steroid available to the tissue (Westphal, 1970). An additional function of these binding proteins seems to be the protection of the steroid from metabolism (Westphal, 1971, 1980).

1.1.5.1.2 Steroid Entry : Passive Diffusion or Facilitated Uptake?

It is generally believed that steroids enter target cells by passive diffusion (Higgins and Gehring, 1978) which explains steroid entry into both target and non-target cells (Jensen and Jacobson, 1962). However, the facilitated transport mechanism proposed by Milgrom et al. (1973a) cannot be ruled out. These authors demonstrated that by using sulphydryl blocking reagents, oestrogen uptake by target cells was inhibited. However, Peck et al. (1973a), using different sulphydryl blocking reagents from Milgrom's group, could not repeat the results. In addition, studies have been reported indicating involvement of a rate

limiting step in oestrogen uptake (Baulieu, 1973) but these have been contradicted by Williams and Gorski (1974) who provided evidence that rate limiting steps could result from artifacts.

Studies of Pietras and Szego (1977), using affinity chromatography, have demonstrated the existence of oestrogen binding sites on the surface of endometrial and liver cells . These sites were not found on non-target intestinal cells. Similar findings were reported by others (O'Malley and Means, 1974; Wittliff, 1975). Recently Pietras and Szego (1979) and Mueller et al. (1979) have demonstrated specific oestrogen binding sites associated with uterine plasma membranes. Enzyme digestion involved in the preparation of cells could result in an abnormal distribution of receptors or the binding sites could represent steroid metabolizing enzymes. Affinity of the binding was not always reported in these studies.

Interestingly, Terayama et al. (1976) have demonstrated that some plasma membrane steroid binding sites are lost during malignant transformation of cells, perhaps reflecting the de-differentiation process. A similar loss of oestrogen binding sites from plasma membrane of neoplastic breast tissue, in contrast to non-neoplastic tissue, is reported by Zanker et al. (1981) who further suggested that, in normal cells, receptor at the plasma membrane may be serving as the modulator of intracellular hormone levels, protecting the cell from excess exposure to hormone.

The studies of Nenci et al. (1980a, 1981), using fluorescent oestradiol-17β, have indicated involvement of plasma membranes in the initial uptake of oestradiol. However, these studies should be interpreted with caution due to the very high concentration of fluorescent oestradiol used (Chamness et al., 1980; Penney and Hawkins, 1982), and the low temperatures involved ($4^{\circ}C$) which are known to alter the cell

membrane.

It appears 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. Based on various studies, Rao (1981) has suggested that perhaps both passive and facilitated processes may be involved in steroid uptake.

1.1.5.2 **Cytoplasmic Binding of Steroid**

After its entry into the cell the steroid must be retained over several hours for responses to be effected (Anderson et al., 1972). The receptors are characterized by their high affinity (dissociation constant value of 10^{-10} - 10^{-9}M) low capacity and high specificity (Shyamala and Gorski, 1969; Giannopoulous and Gorski, 1971). Low affinity sites are also detected (dissociation constant 10^{-7} - 10^{-5}M) and probably represent non-specific sites (McGuire and Julian, 1971). King (1975) has indicated that the high affinity sites are the physiologically important ones for response. The possession of these highly specific sites is a prerequisite in defining a target tissue (Folca et al., 1961; Higgins et al., 1973a). It has been estimated that there are between 10,000 and 20,000 receptor molecules per cell in target tissues ($\sim 10^{-8}$M). However only 2000 of these are required for response (King and Mainwaring, 1974; Leake, 1981a). The remaining receptor is thought to concentrate steroid from the blood (Clark and Peck, 1979).

In addition to the high affinity receptors, another form of specific cytoplasmic receptor has been reported with a dissociation constant value of $\sim 10^{-8}$M (Clark et al., 1978; Eriksson et al., 1978; Smith et al., 1979; Clark and Markaverich, 1981). These latter sites are termed type II sites, in contrast to the classical type I sites. Type II sites do not translocate into the nucleus and are more abundant in the target

cells than are the type I (high affinity) sites. The type II sites have recently been demonstrated in human breast cancer (Panko et al., 1981), and such sites may have a role in concentrating steroid (Gorski and Gannon, 1976), or may represent a precursor or product of the type I site. Little et al. (1975) have reported an early 3.5S and a late 4.5S receptor entity isolated from microsomes. Maturation of receptor may be a prerequisite to its transformation and translocation. Such post-translational modifications are recognised (Blobel and Dobberstein, 1975). The nuclear type II sites detected by Eriksson et al. (1978), which are a distinct entity from cytoplasmic type II sites, may simply represent a processed form of nuclear type I receptors (Horwitz and McGuire, 1978a). Occupancy of the nuclear type I sites increases aldosterone-induced epithelial sodium transport but no further increase occurs when type II sites are occupied (Farman et al., 1978). This lends further support to the idea that type II sites are a non-functional form of the receptor.

Fishman and Fishman (1979) have reported yet another heterogeneous form of receptor binding site - one form which binds tamoxifen (an anti-oestrogen) and another which does not. Guilino and Pasqualini (1982), using foetal organs of the guinea pig, showed that tamoxifen bound both the oestrogen receptor, and another, distinct site.

Lysozymes (Szego, 1974) and microsomes (Little et al., 1975; Parikh, et al., 1980) have also been demonstrated to possess the oestrogen binding sites but the physiological significance of this is unknown.

1.1.5.2.1 **Application of Sucrose Density Gradients in the Analysis of Cytoplasmic Oestrogen Receptor**

A major advance in studying cytoplasmic proteins was made with the advent of the technique of sucrose density gradient. Initially, the

designated value for the oestrogen receptor was 9.5S (S represents Svedberg units, representing sedimentation coefficient) in low salt media (Toft and Gorski, 1966, Jensen et al.,1968). Later, values of 9S were reported (Shyamala and Gorski, 1969; Steggles and King, 1969) and following further studies the value of 8-9S was agreed upon (McGuire and Julian 1971; Shyamala and Nandi, 1972; Jensen et al.,1974). With the advent of radio-labelled marker proteins it has become possible to be more precise. Some variation remains with the 8S entity reflecting perhaps the various degrees of aggregation of receptor with itself or non-specific protein (Mueller et al.,1972; Stancel et al.,1973a). In high salt gradients (0.4M KCl) the results are less variable. Under these conditions receptor disaggregates to the 4S form. The 4S form has been assigned a molecular weight of between 60,000 and 80,000 daltons (Notides and Nielsen, 1974; Yamamoto, 1974). Under isotonic conditions (0.15M KCl) Reti and Erdos (1971) reported the detection of the 6S form of oestrogen receptor whereas Yamamoto and Alberts (1974) and Notides and Nielsen (1974) both reported detecting only the 4S form.

Analysis of mature rat uterine cytosol in hypotonic conditions yields a second peak at approximately 4S, in addition to the 8S form (King and Mainwaring, 1974). In the immature rat uterine cytosol this peak is not always present or is present at very low concentrations. Uriel et al. (1976) postulated that the 4S peak obtained may be due to α-fetoprotein contamination, which is known to exist in very young rats in high concentration and binds oestradiol-17β with high affinity. However, α-fetoprotein is not present in adult rats, although Laberbara and Linkie (1978) could still detect it at 20 days post partum. Using diethylstilboestrol, which has a very low affinity for α-fetoprotein, the 4S form can still be detected in adult rat uterus (Section 3.1.5).

Both 4S and 8S forms of receptor have also been described in the lactating mammary gland (Muldoon, 1978) and human breast cancer (Wittliff and Savlov, 1978).

Interestingly, a calcium-derived 4S oestrogen receptor has been reported in low ionic strength in the cytosol fraction obtained from calf uterus (Molinari et al.,1977; Puca et al., 1977). Other reports have suggested that mild trypsinization of the 8S form of the oestrogen receptor can also result in its conversion into the 4S form which now fails to bind to chromatin or DNA (Andre and Rochefort, 1975). However, this 4S form still binds to crude uterine nuclei (Rochefort and Baulieu, 1973). A protease mediated 8S → 4S conversion has also been observed in human breast cancer cytosol (Schneider and Dao, 1977). Park and Wittliff (1980) have shown that the 4S receptor from human breast tumour cytosol also lacks DNA binding properties. The significance of the non-aggregating type of 4S receptor is not known. Sica and Bresciani (1979) have isolated to apparent homogeneity, the calf uterine oestrogen cytosol receptor. A single band of molecular weight 70,000 was observed on polyacrylamide gels. However, this probably does not represent the native form but the proteolyzed 4S form (Murayama et al., 1980a).

In conclusion, SDGA has led to the discovery of the presence of two or more different 4S populations of oestrogen receptor, (i) a fraction which on lowering ionic strength can aggregate with either other oestrogen receptors or with other proteins (Murayama et al., 1980b), and (ii) a fraction which has lost this aggregation property. It may be significant that it is only when the 8S form is present that transfer of oestrogen-receptor complex to the nucleus occurs (Lukola et al., 1980).

1.1.5.2.2 **Specificity of Oestrogen Receptor**

The oestrogen receptor is specific for biological oestrogens.

It binds oestradiol-17β with very high affinity (Kd 10^{-10}-10^{-9}M) but oestradiol-17α very weakly. It has a very high affinity for certain synthetic non-steroidal oestrogenic compounds such as diethylstilboestriol (DES) and selected triphenylethylenes (Duax et al., 1980). These non-steroidal oestrogenic compounds compete effectively with oestradiol-17β for the steroid binding site (Puca and Bresciani, 1968, 1969). Both oestriol and oestrone bind receptor but with a lower affinity than oestradiol-17β. These studies have led to the elucidation of the type of configuration required by the receptor for specific binding. By using various competitive steroids, Hahnel and Twaddle (1974) suggested that steroids must have an aromatic A ring, ethanolic hydroxyl group at C-3 and oxygen function in ring D (Figure 3). Powell-Jones et al.(1975) then studied the actual binding of oestradiol-17β to the receptor. They concluded that two sites exist on the carbon skeleton : a highly specific one at the C-3 hydroxyl group which is involved in the initial attachment, and a less specific site at the β-hydroxyl group of carbon-17. These studies led to the synthesis of various antioestrogens such as tamoxifen, and nafoxidine.

1.1.5.3 Steroid Receptor Activation/Transformation

The two terms, activation and transformation of oestrogen receptor have been used interchangeably in the literature and this has led to much confusion. It would therefore be useful to clarify each one. All steroid receptors, including vitamin D_3 (Brumbaugh and Haussler, 1974) show a temperature dependent activation step which gives the receptor an increased affinity for binding to DNA, chromatin and other polyanions (Section 1.1.5.5; see also Toft, 1972; Buller et al., 1975; Parchman and Litwack, 1977; Miller and Toft, 1978). The accompanied transformation

process, however, seems to be unique to the oestrogen receptor and involves a 4S to a 5S conversion (Notides and Nielsen, 1975). Other steroid receptors do not undergo this transition prior to binding to chromatin. Other physical changes in non-oestrogenic steroid receptors, such as the isoelectric point of the glucocorticoid receptors have been noted and are claimed to distinguish between the activated and non-activated receptor (Katzenellenbogen, 1980).

1.1.5.3.1 **Activation/Transformation and the Two Step Model**

A major advance in the study of steroid receptor interaction was made when a relationship between the cytosol and nuclear oestrogen receptor complex was detected. The first indication of this phenomenon came from the observations of differences between the two complexes on sucrose density gradients (Toft and Gorski, 1966). Jensen et al. (1969) incubated uterine nuclei with oestrogen in the presence of cytosol and found a 5S receptor complex which was indistiguishable from that found in the whole uteri. They concluded that the complex was made in the nucleus. Subsequently it was discovered that the 5S hormone-receptor complex can also be obtained in the absence of nuclei (Gschwendt and Hamilton, 1972) by warming the cytosol in the presence of oestradiol-17β. (Sato et al. (1979) suggest that transformation can occur at low temperatures and that oestradiol is not an absolute requirement). It was also shown that the transformed receptor, a term used by Jensen et al. (1973), led to the stimulation of RNA polymerase in uterine tissue (Mohla et al., 1971; Jensen et al., 1972). The conclusion, therefore, was that the alteration in oestrogen receptor may be a prerequisite to translocation into the nucleus.

Gschwendt and Hamilton (1972) demonstrated that the transformation reaction was temperature dependent. The process was found to be

pH and ionic strength dependent i.e. accelerated with increasing pH (6.5 → 8.5), and increasing ionic strength (0 - 0.4M KCl). The transformation process was reduced by the presence of divalent ions and EDTA. Oestrone was first found to be ineffective in inducing transformation (Jensen, et al., 1971a). However, it was later shown to be effective at higher concentrations (Rochefort et al., 1972). Oestrone, in a single dose, has subsequently been found to initiate only the early genomic responses in immature rat uterus reflecting the shorter nuclear retention time of the oestrone-receptor complex (Clark and Peck, 1979). Thus these findings strengthened the two step model of Jensen et al. (1968) and Gorski et al. (1968) indicating that the extranuclear 8S form undergoes temperature dependent transformation and is then translocated to produce the 5S nuclear complex. Greene et al. (1977, 1980) have now shown the similarity between the nuclear and cytosolic receptor, using immunological methods. Salt disaggregated receptor (8S receptor is converted to 4S in 0.4M KCl) can also be transformed to the 5S form and Stancel et al. (1973a) have proposed the 4S form as the physiological form of receptor. Under low salt conditions it either self aggregates or aggregates with other specific or non-specific uterine proteins to form the 8S complex. Recently a 4S → 8S converting protein has been characterized and this protein is thought to be three times as abundant as the native 4S receptor (Murayama et al., 1980b). This protein fails to act on proteolyzed 4S oestrogen receptor.

Most experimental findings are consistent with the two step model, but do not prove it. Munck and Foley (1979), for example, have been able to detect non-activated receptor complexes in thymus cells at 37°C after very short intervals of exposure to hormone. The initial exclusive presence of such a form was rapidly succeeded by the appearance of the

activated species. The studies of Weichman and Notides (1977, 1979) on receptor activation as a function of temperature, receptor concentration and ionic strength further lend support to the two step model. Gorski and Gannon (1976) have stressed, however, that subfractionation of cells may lead to artifactual redistribution of receptors. Nevertheless, autoradiographic analysis of receptor distribution in tissues prepared under conditions of minimal cellular damage, do demonstrate a temporal sequence of specific steroid binding which progresses from the cytoplasm into the nucleus (Stumpf and Sarr, 1977). In addition, using cell suspensions, Pavlik et al. (1979) and Munck and Foley (1979) have shown that the rate of nuclear accumulation of oestrogen receptor complex is measurably slower than the rate of uptake into cells. The studies of Nenci (1981) using fluorescent oestradiol would also favour such a view.

1.1.5.3.2 **Inhibition of Activation/Transformation**

Some results are difficult to reconcile with the two step model. In contrast to the temperature dependent transformation in the uterine cytosol, which takes place only if the receptor is complexed with an oestrogen, the alteration (8S $\longrightarrow$ 5S) that accompanies ammonium sulphate precipitation proceeds rapidly in the cold and does not require oestrogen (Traish et al., 1979). These authors have shown that, in the intact uterine cells both at $0^{\circ}C$ and $37^{\circ}C$, the activated 5S complex can be formed in the absence of oestradiol. The rate of formation remains temperature dependent. When the uncomplexed receptor of calf uterine cytosol is precipitated with ammonium sulphate and radioactive oestradiol-17β then added to the redissolved precipitate, the 5S complex is found (DeSombre et al., 1972). Nielsen and Notides (1975) and Sato et al. (1978a) were unable to obtain similar results. The latter report suggests that the dialysis procedure employed by DeSombre et al.

(1972) to remove ammonium sulphate may have resulted in the transformation process, indicating involvement of a low molecular weight inhibitor(s) of the 5S formation.

Evidence for low molecular weight inhibitors of activation in rat uteri, and mouse Leydig cell tumours have been reported (Sato et al., 1978a, b; 1979). The rat liver glucocorticoid receptor complex has a similar inhibitor (Goidl et al.,1977). Dialysis was found to increase the nuclear binding of the oestrogen receptor (Sato et al., 1979) but the 5S complex was only observed if the oestradiol-17β was added prior to SDGA in high salt. Thus these authors have questioned the necessity of 5S formation prior to nuclear binding. However it should be pointed out that (i) these studies were conducted in low salt, perhaps resulting in spurious binding of receptors to nuclei (which is used as a measure of activation), (ii) the SDGA in high salt in the absence of oestradiol-17β may well have resulted in the dissociation of the 5S complex back into the 4S form and (iii) the extracted nuclear receptor sedimenting at 4S rather than 5S could have been a consequence of the very low concentration of receptor being loaded onto the gradient. Nevertheless, 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 presented an interesting report showing that electrolysis of the rat uterus resulted in a marked increase in nuclear binding of steroid receptor, a result attributed to removal of an inhibitor. Shyamala and Yar-Fen (1977) have also provided evidence for a dialyzable substance which inhibits activation in the rat mammary gland.

In contrast to the low molecular weight inhibitors of activation, low molecular weight inhibitors of DNA or chromatin binding are also described (Cake et al.,1978; Isohashi et al.,1980). 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; Liu and Webb, 1977; Simons, 1977; Atger and Milgrom, 1978).

Recently it has been reported that, in vitro, ATP can activate both the glucocorticoid (Moudgil and John, 1980) and oestrogen receptor (Moudgil and Essalau, 1980). Further, it has been suggested that inactivation of oestrogen receptor in the nucleus involves dephosphorylation. (Auricchio et al.,1982). This would imply that activated receptor is phosphorylated. However, experiments of Nishigori and Toft (1980) and Sando et al. (1979a, b) suggest that activated receptor is dephosphorylated and, purified progesterone receptor subunits do not show any phosphorylated amino acids (Schrader et al.,1977; Coty et al., 1979). Although the nature of the activated receptor, with regard to phosphorylation is in doubt, the phosphorylation process is required for the actual steroid binding (Sando et al.,1979a, b; Auricchio et al.,1982). Sodium molybdate is thought to stabilize receptors (Anderson et al., 1980; Noma et al.,1980; Krozowski and Murphy, 1981; Ratajazak et al.,1981) by inhibition of either phosphatase(s), a direct interaction with receptor phosphate groups or interaction with sulphydryl groups.

Sodium molybdate has been found to inhibit the process of activation and transformation (Leach et al.,1979; Nishigori and Toft, 1980; Shyamala and Leonard, 1980; Kalimi and Banerjee, 1981; Muller et al., 1982; Notides et al.,1982). The inability of receptor to disaggregate

from the 8S form in the presence of sodium molybdate, is thought to be the reason for its failure to activate (Grody et al.,1980; Redeuilh et al.,1981; Muller et al.,1982). On the other hand, molybdate could retard the dissociation of the inhibitory factors by binding directly to them. There is still controversy over whether the molybdate effect of preventing activation/transformation is reversible or not (Shyamala and Leonard, 1980; Muller et al.,1982; Notides et al.,1982). It is further reported that molybdate retards the 4S $\longrightarrow$ 5S transformation of the anti-oestrogen-receptor complex (Rochefort and Borgna, 1981). Other, less commonly used inhibitors of the activation/transformation process are discussed by Grody et al.(1982).

1.1.5.3.3 **The Mechanism of Activation/Transformation of Oestrogen Receptor**

Calcium activated proteolysis as a means of receptor activation (Puca et al.,1972) is now largely discounted because such proteolysis of receptor generally leads to the loss of DNA binding properties (Andre and Rochefort, 1973).

A second mechanism proposed, confines receptor activation to a simple conformational change within the native molecule, leading to the exposure of certain acidophilic groups (Bailly et al.,1980; Rochefort et al.,1980). Using kinetic studies in low salt conditions, Bailly et al. (1980) could only demonstrate a first order plot for receptor activation suggesting that other molecules are not involved in activation. These authors were using high salt gradients for the separation of 4S and 5S forms. This was followed by DNA binding studies to measure the extent of activation. Both the 4S and 5S forms bound equally well and hence this led to the proposal that activation and transformation are two

independent events. However, in conducting the DNA binding studies, the high salt buffers, in which the 4S and 5S forms are present, have to be diluted to low salt. Such a dilution can lead to a rapid formation of the 5S form (Notides, 1978), most probably as a result of removal of inhibitors on the gradient. Bailly et al. (1980) claim that this was taken into consideration. The problem remains unresolved.

In keeping with the simple conformational change, Rochefort et al. (1980) found that both the native soluble receptor and the micrococcal nuclease extracted nuclear receptor showed similar properties in terms of their densities. It is not clear however, whether enzymatic treatment leads to some form of modification within the receptor structure.

The most popular model for receptor transformation (4S $\longrightarrow$ 5S) is that involving the addition of another subunit(s) to the native oestrogen receptor. This could result from either dimerization of the 4S receptor (Nielsen and Notides, 1975; Notides and Nielsen, 1975) or the addition of other protein(s) (Yamamoto, 1974). The immature rat uterine oestrogen receptor has been the principal model of investigation. The transformation process has also been documented in various other tissues including the hypothalamus (Fox, 1977) and the pituitary (Linkie, 1977). However, this process may not occur in human breast tissue (Fazekas and MacFarlane, 1980).

Kinetic analysis of 4S ($\sim$80,000 daltons) to 5S ($\sim$ 130,000 daltons) transformation (Notides and Nielsen, 1974; Notides et al., 1975) has shown the process to obey second order rate kinetics, independent of initial 4S concentration, in high salt buffers. This suggests a dimerization process with a similar or a dissimilar macromolecule present in approximately the same concentration as the 4S receptor. In low salt

buffers, in contrast to the report of Bailly et al. (1980), a complex second order reaction was observed, possibly suggesting the dissociation of an inhibitor(s) from the receptor molecule prior to the binding of the second subunit(s). The involvement of a second polypeptide(s) was also suggested by the studies of Yamamoto (Yamamoto, 1974; Yamamoto and Alberts, 1972, 1974). Although studying the same tissue as Notides and Nielsen (1974), Yamamoto reported a molecular weight change from 60,000 daltons for the 4S molecule to 105,000 daltons for the activated 5S form. Yamamoto has further designated the putative second subunit as subunit 'X' of approximately 50,000 daltons. Subunit 'X' was found in both 'target' and 'non-target' tissue suggesting that subunit 'X' could be a chromatin associated protein, present in the soluble fraction as a result of vigorous homogenization.

Notides et al. (1975) have shown that 4S $\longrightarrow$ 5S transition requires an activation energy of $\sim$ 21Kcal/mol and this has been suggested as the energy required to bring about the necessary conformational change in the oestrogen receptor providing the increased affinity for the second subunit(s). The receptor was shown to undergo drastic conformational changes in the presence of urea (Notides and Nielsen, 1974, 1975). The physiological significance of such changes is not known but the studies of Katzenellenbogen et al. (1975) and Peck et al. (1973b) suggested that the oestrogen-receptor complex is more stable than the empty receptor. Further studies have shown that activated receptor acquires an increased affinity for oestradiol-17β, (Weichman and Notides, 1977, 1979; DeBoer and Notides, 1981).

It is not clear whether the second subunit(s) involved in receptor transformation is similar or dissimilar to the native 4S molecule. The data of Thampan and Clark (1981) would suggest that the

'activation factor' is different to the native 4S receptor. Notides et al.(1981), however, have presented evidence of positive cooperativity of oestrogen receptor at equilibrium binding, suggesting that the activated oestrogen receptor is a homodimer. Receptor purification and associated studies must allow for dissimilar subunits. The lack of detection of such a transformation process for other non-oestrogenic steroid receptors may perhaps be due to present technical difficulties. Rapid analysis techniques using vertical tube rotors (Jordan and Prestwich, 1977) and high pressure liquid chromatography (Pavlik et al. 1982), may serve to clarify the situation.

It is interesting to compare the transformation process with other activation systems. There are several enzymes which show ligand induced structural changes, accompanied by a rise in their activity (Dunne and Wood, 1975). cAMP, for example, binds to the regulatory subunit of the protein which then leads to the release of the catalytic subunit and subsequent activity. It has been noted that cAMP has a very similar three dimensional structure to oestradiol-17β (Liao, 1975).

1.1.5.3.4 **Cellular Site of Transformation**

Linkie and Siiteri (1978), using cell free extracts, demonstrated that 4S→5S transformation takes place in the nucleus in the precursor/product fashion. Sheridan et al.(1979) suggest that the unbound receptor is partitioned between the cytoplasm and the nucleus according to their respective free water content. This equilibrium hypothesis is based on the water exclusion theory of Horowitz and Moore(1974). Accordingly receptor transformation is not a prerequisite to its translocation as previously proposed (Jensen et al., 1968; Puca et al., 1972) and the whole process may take place in the nucleus. However, under

conditions of minimal redistribution as a result of homogenization, Pavlik et al. (1979) found translocation of oestrogen-receptor complex to occur at a slightly slower rate than the rate at which oestradiol-17β was specifically bound to free cells or receptors. Further, Nenci et al. (1980b) using fluorescent oestradiol-17β have demonstrated that in certain breast tumour biopsies, a heterogeneity in cell types with respect to receptor distribution can be obtained. Certain cells only show the cytoplasmic staining, suggesting a defect in translocation perhaps as a result of a defect in transformation, whereas the majority of cells show an intact translocation process. The cellular site of transformation therefore, remains undefined.

1.1.5.3.5 **Differences between Activation/Transformation of Oestrogen and Progesterone Receptor**

The progesterone receptor has been shown to be composed of two subunits A and B, both of molecular weight ~100,000 (Schrader et al. 1981). These subunits can be separated by ion-exchange chromatography (Schrader and O'Malley, 1972). The native receptor sediments as 6-8S and is composed of an equimolar amount of A and B subunits (Schrader et al., 1975; Schrader et al., 1977). Processes which transform oestrogen receptor lead to the dissociation of the progesterone A-B dimer into individual subunits sedimenting at 4S. Only subunit A shows an affinity for DNA (O'Malley and Schrader, 1972). Subunit B binds specifically to chromatin but only to the target tissue chromatin (Schrader and O'Malley, 1978; Schrader et al., 1981). Theory suggests that the A-B dimer binds to specific acceptor sites after which subunit A dissociates and functions as a DNA unwinding protein. This then leads to increased transcription. It is only the 6S complex which increases the in vitro transcription

(Buller et al., 1976) and the nuclear uptake of 6S complex has been found to be temperature independent (Schrader et al., 1972). This suggests that the 6S complex is physiologically important and that either the active 6S complex is extracted from cells or that the active 6S complex is generated in vitro.

Schrader and O'Malley (1978) have extended the progesterone model to other steroid receptors, a proposal which has lately come under criticism (Gschwendt, 1980). The latter report has shown that the two components of oestrogen receptor are not present in equimolar amounts, the non-DNA binding receptor can be transformed into a DNA binding form and that proteolysis of receptor leads to the appearance of a single peak on the gradients, unlike progesterone receptor where two peaks are observed (Schrader et al., 1981). Based on their studies with oestrogen receptor, Notides et al. (1981) have suggested that the A and B peaks identified on DEAE-cellulose chromatography may represent the non-activated and the activated form of the progesterone receptor. Furthermore, more than 80% of the oestrogen receptor is capable of DNA binding in contrast to the predicted 50% if the situation was analogous to the progesterone receptor (Gschwendt, 1980). This however, does not rule out the possibility that a second non-oestrogen binding component is involved in receptor activation.

Spelsberg and Boyd-Leinen (1980) have shown that the two components of the progesterone receptor undergo seasonal variation. However, the possibility that the two components of progesterone receptor arise as a result of a specific limited proteolytic cleavage of the parent molecule cannot be ruled out (Schrader, 1982).

1.1.5.3.6 **Activation/Transformation in other Systems**

It must be emphasised that the above studies of the mechanism of activation have been conducted with animal models. Species as well as

organ differences may be obtained with respect to transformation Linkie, 1977). Park and Wittliff (1977) have reported that the oestrogen receptor from the lactating mammary gland of the rat exhibits temperature dependent activation but does not show 4S→5S transition. They have ascribed such a difference to the different functions of the receptor in different organs. Mouse mammary gland however, has been reported to show a 4S → 5S transition (Muldoon, 1978). The data from human tissue, with regard to receptor activation is very limited (Notides, 1978; Fazekas and MacFarlane, 1980; Sato et al., 1981a). No 4S → 5S transformation could be shown for the soluble oestrogen receptor although the receptor showed temperature dependent activation. Thorsen and Stoa (1979) observed a 5S nuclear oestrogen receptor from human breast tumour preparations. However, reports of nuclear receptors from human endometrium or myometrium, isolated under similar conditions failed to show the 5S form of nuclear receptor (Fleming and Gurpide, 1980; Katzenellenbogen et al., 1980; Pollow et al., 1981). The detailed analysis by Notides et al. (1976) of human myometrial oestrogen receptor transformation established a difference in the mechanism of action of the rat and human receptor. They suggested that whereas the 4S→5S equilibrium for the immature rat uterine oestrogen receptor transformation is very much in favour of the 5S form, for the human receptor, it is in the opposite direction. The equilibrium in the latter case is very sensitive to temperature, ionic strength and receptor concentration.

In conclusion, receptor activation/transformation is not the simple process previously envisaged (Jensen et al., 1968). Whether such a process is physiologically important and whether second and subsequent subunits are involved, is unknown.

1.1.5.4 **Receptor Translocation**

If it is accepted that, in vivo, unfilled receptor is either

in equilibrium throughout the soluble fraction of the cell or associated specifically with either the cytoplasm or a membrane component, then translocation of the filled hormone-receptor complex to the nucleus must occur. It is known that high molecular weight compounds tend to diffuse very slowly across the nuclear membrane (Paine and Feldher, 1972) whereas translocation occurs very rapidly (Sheridan et al., 1979). Gurdon (1970) has indicated that nuclear transfer of proteins takes place via nuclear pores. The data of Nenci et al. (1980c) would support this observation for steroid receptors. It is therefore quite possible that transformation of receptor may involve a change in the axial ratio as a requirement for passing through the nuclear membrane. It has been shown that the steroid remains bound to the same protein in the cytosol and nuclear compartments (Greene et al., 1977; Greene and Jensen, 1982). The process of translocation has been attributed solely to inherent properties of receptor subsequent to binding of steroid because (a) translocation does not function efficiently at low temperature (Jensen et al., 1968; Nenci, 1981) and (b) it is reported not to be energy or protein synthesis dependent (Shyamala and Gorski, 1969).

1.1.5.4.1 **Physiological Significance of Receptor Transformation - Translocation Process**

The transformation process of oestrogen receptor, accompanied by association of other subunit(s) is thought to be required for directing rapid binding of the complex to the correct acceptor sites on the chromatin (Leake, 1976). Since the transformation process has not been detected for other receptors, this raises the question as to whether the second subunit(s) is a nuclear protein, the acquisition of which leads to tighter binding of the complex to chromatin.

Both Thrower et al. (1976) and Thampan and Clark (1981) have provided evidence of an activation factor in the rat uterine cytosol. This is thought to be directly involved in stimulating RNA polymerase. This suggestion is based on the evidence that low molecular weight proteins from the cytosol can stimulate chromatin bound RNA polymerase (Jacob, 1973; Natori et al., 1973). Furthermore cytoplasmic control of gene expression and involvement of regulatory proteins in eukaryotic gene regulation is well known (Gurdon and Brown, 1965; Davidson and Britten, 1973). In addition, the development of certain tissues is associated with the appearance of cytoplasmic proteins in the embryonic phase, these are capable of regulating nuclear transcription and further development (Brothers, 1976). It is therefore possible that the transformation or activation factor for oestrogen receptor may serve an analogous function (Haselbacher and Eisenfeld, 1976). It has been found, for example, that in the female rat hypothalamus (McEwen et al., 1974), adult concentrations of soluble oestrogen receptor occurs as early as day 20 but adult levels of nuclear receptor are only attained on day 26, that is a 6 fold increase in nuclear binding without a comparable rise in cytoplasmic binding (Plapinger and McEwen, 1973). No change in plasma oestradiol-17β is observed at this time. The studies involving post-natal development of rat uterus, indicate that the full range of responses, associated with oestradiol-17β, do not appear simultaneously reflecting either the presence of multiple species of receptor, activation factors or the sequential appearance of specific gene acceptor sites (O'Malley et al., 1972; Plapinger and McEwen, 1973; Somjen et al., 1973). Thus the physiological role of translocation in carrying gene recognition or activation factors into the nucleus is still in doubt. Powell-Jones et al. (1978) claim that 4S→5S transformation of oestrogen receptor is not essential for binding to DNA, however whether such a

4S form can stimulate RNA polymerase remains to be established.

According to the equilibrium hypothesis of Horowitz and Moore (1974) and Sheridan et al., (1979), unbound receptors would be found principally in the nucleus of an intact cell. Indeed "empty" nuclear receptors have been reported in several systems (Jackson and Chalkley, 1974; Zava et al., 1976, Carlson and Gorski, 1977) including human breast tumours (Garola and McGuire, 1977; Kato et al., 1978; Panko and McLeod, 1978; Thorsen, 1979). These unbound receptors are thought to be inactive, although this cannot be stated with certainty. This poses the question as to whether it is the oestrogen which is required for the transport of the receptor into the nucleus or vice versa?

Studies by Szego (1974) have questioned the biological function of receptor. Szego's hypothesis is that the lysozomes carry the steroid into the nucleus and the steroid allows the lysozomes to carry certain proteins into the nucleus. This poses the question as to whether soluble receptor is necessary physiologically. The evidence for and against a physiological role of soluble receptor has been reviewed (Clark and Peck, 1979; Leake, 1981a).

1.1.5.4.2 **Defects Associated with the Receptor Activation/transformation and/or Translocation Mechanism**

Impressive evidence that the steroid receptors are bifunctional or multifunctional has been obtained in the studies of genetic variants of mouse lymphoma cells in tissue culture (Sibley et al., 1974). Yamamoto et al. (1976) have studied the receptors of nt^- mouse lymphoma cells in detail. Whereas only 8-20% of nt^- receptor enters the nucleus no defect in the activation process is seen. However some differences from the wild type receptor are seen after SDGA. No detectable alteration in the properties of the nucleus was observed. Changes, therefore, in size or

conformation of nt^- receptor must explain why they fail to translocate. Another type of defect has been detected in a receptor containing steroid resistant variant of human lymphoid cells (Schmidt et al., 1980). In this type of defect a stable form of activated complex cannot be formed.

In an oestradiol independent mouse mammary tumour, physiological concentrations of cytoplasmic receptor but no nuclear receptor could be detected (Shyamala, 1972). A defect in the cytoplasmic receptor was indicated. Some of the 'false positive' breast tumour biopsies from humans (Section 1.2.3.2), the so-called +/o type (Laing et al., 1977), could also be explained on a similar basis. Thorsen and Stoa (1979) further substantiated these observations of translocation resistant receptors in some breast tumour biopsies.

A 4S cytoplasmic receptor has been reported in some human breast tumours (Kute et al., 1978). This 4S receptor seems to be present in a form which cannot be activated (Wittliff et al., 1978) and does not bind to DNA (Park and Wittliff, 1980). On the basis of their clinical follow-up, Wittliff and Savlov (1975) have claimed that 4S receptor is found predominantly in the tumour cytosol from patients unresponsive to endocrine therapy. Conversely, the response rate is maximal if the 8S form of the receptor can be demonstrated (results from others have not substantiated this claim - see section 1.2.4). Using dissociation kinetics Kaufman et al. (1982) have detected defective activation of androgen receptor-complexes in androgen insensitive patients.

It is most likely that the unresponsiveness of receptor containing cells to hormones is due to a defect in the activation/transformation and/or the translocation process. However some unresponsiveness could be explained by considering post-chromatin binding defects (Brushovsky et al., 1975).

1.1.5.5 Nuclear Binding of the Steroid-Receptor Complex

1.1.5.5.1 The Acceptor Site Hypothesis

The term acceptor site has been used to designate the nuclear sites that specifically bind the steroid-receptor complexes and result in a biological response (Leake, 1976). The concept was formulated when (a) it was observed that high salt concentration is required for extraction of steroid-receptor complexes from the chromatin, (b) *in vitro* saturable interaction between the nuclei and oestrogen-receptor complexes were demonstrated and, (c) *in vitro* interactions were observed in which RNA polymerase was shown to be stimulated by hormone-receptor complex in only certain target tissues (Spelsberg, 1976).

Milgrom *et al.*(1973b) suggested that the receptor may have a site for binding hormone and an independent site for interaction with chromatin. Recent evidence has substantiated such a view (Khan *et al.*, 1980; Myatt *et al.*, 1982a, b).

Several components of the nucleus have at different times been proposed as the acceptors for the steroid-receptor complexes (Gorski and Gannon, 1976). However, the bulk of evidence is now in favour of the tightly bound non-histone proteins (Spelsberg *et al.*, 1971; King and Gordon 1972; Puca *et al.*, 1974, 1975; Mainwaring *et al.*,1976) with a subsidiary role for DNA (Clemens and Kleinsmith, 1972; Higgins *et al.*, 1973a; Musliner and Chader, 1972; Yamamoto and Alberts, 1975).

The chromosomal non-histone protein fraction AP_3 (a group of acidic proteins) which binds progesterone receptor has received most attention (Spelsberg, *et al.*, 1972; Spelsberg *et al.*, 1979). The acceptor protein is found to be tightly bound to DNA, sensitive to protease but not to nuclease and is confined to and generally distributed within the chromatin.

This acceptor is active in target tissue and masked in non-target tissues (O'Malley et al., 1972; Chytil, 1975; Pikler et al., 1976; Spelsberg and Toft, 1976). Non-histone protein acceptor activity has also been demonstrated in human mammary tissue (Charreau and Baldi, 1977). However, chromatin reconstitution, on which many of these studies are based, is a controversial technique which has been shown to give rise to a number of artifacts (Biessman et al., 1976; Fulmer and Fasman, 1979; Stein, 1979).

The direct involvement of DNA in the acceptor activity was concluded from several studies. DNAase treatment abolished acceptor activity (Shyamala-Harris, 1971; King and Gordon, 1972) and Marver et al.(1972) demonstrated that preheating of nuclei or RNAase activity did not diminish acceptor activity. King and Gordon (1972), Spelsberg (1974) and Leake (1976) concluded that both DNA and chromatin were involved in the acceptor activity in an essential three dimensional conformation which may be altered as a result of cell disruption.

Recently, Thrall and Spelsberg (1980) have confirmed that non-histone protein-DNA complexes show the characteristics of native acceptor sites. Studies to isolate the regulatory sequence of DNA are under way in several laboratories. Recombinant technology has led to the isolation and molecular cloning of certain steroid specific genes (Dugaiczyk et al., 1978; Gannon et al., 1979; Payvar et al., 1981). Purified receptors complexed to steroid stimulate the transcription of these genes. The egg white protein genes are perhaps the best characterized. Compton et al., (1982) have shown that the subunit A of the progesterone receptor binds selectively to the ovalbumin gene fragment. Mulvihill et al. (1982) have identified a consensus sequence for egg white protein which further lends support to the idea of specific DNA sequence involvement in the genomic binding of steroid receptors. It is of interest in this context that when

cloned sequences of genes for glucocorticoid-induced proteins are introduced into non-target cells, their transcription becomes hormone-inducible (Buetti and Diggelman, 1981; Hynes et al., 1981; Kurtz, 1981).

The concept of acceptor sites has been subjected to criticism. Acceptor site hypothesis predicts a saturable and a second order reaction. Chamness et al. (1974) found no evidence of saturation between the oestrogen receptor and nuclear sites. However, given controlled experimental conditions, including the use of physiological ionic strength, saturation can be obtained (Buller et al., 1975; Spelsberg, 1976). The relevance of the saturation process was also questioned by Chamness et al. (1974) but recent evidence serves to indicate its importance (following section). Nevertheless, Higgins et al. (1973b) demonstrated that the presence of steroid receptor in the nucleus, after in vivo receptor translocation had no influence on subsequent translocation in the cell free system.

These conflicting observations have been explained by Yamamoto and Alberts (1975) who found that oestrogen-receptor complexes bind to DNA with relatively low affinity. On the basis of their results Yamamoto and Alberts suggested that there exists a large number of low affinity binding sites and few genetically important sites of high affinity. They further suggested that the number of low affinity DNA binding sites available in the cell is vastly in excess of the number of oestrogen-receptor complexes and would mask the small number of high affinity sites. The loci of these low affinity binding sites is not target tissue specific (Yamamoto and Alberts, 1976). The kinetic studies of Williams and Gorski (1972) also suggested receptor binding to DNA was independent of high affinity sites although, again, their study would not have detected a small number of high affinity sites.

Two or three classes of steroid receptor acceptor sites having different affinities for steroid-receptor complexes are now proposed (DeBoer et al., 1977; Thrall et al., 1978). These sites are detected both in vivo and in vitro (Pikler et al., 1976; Spelsberg et al., 1976). Quantitative estimates have been based on the assumption that receptor binds to acceptor in a 1:1 ratio. The highest affinity sites (dissociation constant (Kd) of 10^{-12}M) must be filled before any physiological response is observed. These approximate to about 100 sites/cell and are most probably fully saturated at plasma steroid concentrations. However, the number of sites to be occupied for a full physiological response has been estimated to be about 2,000 sites per cell (Clark and Peck, 1979). Occupation of the highest affinity sites has been shown to result in an increase of RNA polymerase II activity. Binding of the receptor complex to a second class of acceptor is required for RNA polymerase I activity (Spelsberg, 1976). The third class of chromatin binding sites, the lowest affinity sites, most probably comprises non-specific binding. Tsai et al. (1975), employing isolated chick oviduct chromatin and a rifampicin challenge assay, concluded that oestrogen treatment almost doubles the number of new initiation sites for RNA synthesis and, therefore, it seems that a large number of gene loci are involved with specific binding of the oestrogen receptor complexes to acceptor sites. However, the studies of Tsai et al. (1975) used Escherichia coli (E.coli) RNA polymerase and were subject to criticism. A repeat experiment using hen oviduct RNA polymerase gave similar results (Tsai et al., 1976) and showed that there was a preferential transcription of the ovalbumin gene. Progesterone treatment also showed a similar increase in initiation sites (O'Malley et al., 1976). However, the continual use of E.coli RNA polymerase (Schwartz et al., 1977; Towle et al., 1977) has been severely criticised by Palmiter and Lee (1980) who concluded that E.coli RNA polymerase does not recognise

steroid hormone induced changes in oviduct chromatin. Nevertheless, it is clear that hormonal treatment, both in vivo (Tsai et al., 1976) and in vitro (O'Malley et al., 1977) leads to changes in the chromatin structure as reflected in increased initiation sites. Similar results for initiation sites under androgenic control are reported by Davies et al. (1979). One major problem in these studies is that the initiation site numbers are always far in excess of both available RNA polymerase molecules and the number of acceptor sites required for full physiological response (Clark and Peck, 1979). This suggests that even homologous RNA polymerase molecules may recognise spurious initiation sites or that the assumptions made in the analysis of data are false. Alternatively, these sites could arise during chromatin extraction procedures (Leake, 1976; 1981a).

1.1.5.5.2 **The Induction of Response**

Although the exact relationship between acceptor sites and biological response remains unclear, a number of studies have correlated physiological response with the occupancy, quantity and retention of steroid-receptor complexes in the nucleus (Anderson et al., 1973; Spelsberg, 1976; McKnight and Palmiter, 1979; Mulvihill and Palmiter, 1977, 1980; May and Knowland, 1981).

Historically, Billing et al. (1969) first demonstrated that increased RNA synthesis is an 'early uterine response' to oestrogen. This included the production of mRNA for a specific soluble protein (Baulieu et al., 1972). Further, it was shown that only the oestrogen complexed to receptor was able to stimulate RNA synthesis in a tissue specific manner (Noteboom and Gorski, 1963; Gorski, 1964; Raynaud-Jammet and Baulieau, 1969; Jensen et al., 1972).

Glasser et al. (1972) noted that the first indication of oestrogen stimulation in rat uterus was a rise in RNA polymerase II activity followed by a similar rise in RNA polymerase I activity. The rise in polymerase I was shown to be dependent on the rise in polymerase II and was cycloheximide sensitive (Borthwick and Smellie, 1975).

Modulation of RNA metabolism reflects most but not all biological actions of the steroid. Although post-transcriptional effects (modifications of heterogeneous RNA, processing or translation) may take place (Palmiter and Carey, 1974; Pennequin et al., 1978;), the bulk of the evidence is in favour of changes in the transcriptional activity (Aziz et al., 1979; Higgins et al., 1979; Leake, 1981a).The rate of transcription of specific target cell genes has been shown to be enhanced by a variety of steroid hormones (Baker and Shapiro, 1977; Swaneck et al., 1979 a,b; Taylor and Smith, 1979). In addition, tissue specific DNAase I sensitivity of vitellogenin genes has been shown to take place in Xenopus as a result of oestrogenic stimulation (Gerber-Huber et al., 1981) reflecting changes in the structure of specific genes.

The data from Palmiter's group (Palmiter et al., 1976; Mulvihill and Palmiter, 1980) has shown that the kinetics of induction and appearance of mRNA for different proteins are different. This has led to the suggestion of sequential regulation of gene transcription and also involves co-operative interaction between steroid receptors at individual initiation sites leading to both delayed appearance of certain species of mRNA and exponential dose response curves for some proteins (Thomas and Teller, 1981). The results of Palmiter et al. have recently been criticised by Swaneck et al. (1980), who suggest that, given certain experimental conditions, no lag in induction of ovalbumin mRNA occurs. Nevertheless, the concept of sequential gene induction is still accepted.

'Late response' to oestrogen include sustained high activity of RNA polymerase I, a second peak in glucose metabolism, and a general rise in protein synthesis, DNA synthesis and cell division. Leake et al. (1975) argue that some of the 'late effects' of oestrogen are nevertheless, primary responses, that is, dependent on the retention of the oestrogen-receptor complex by the relevant high affinity sites. This confirmed earlier data (Anderson et al., 1974; Clark and Peck, 1976) that the oestrogen-receptor complex must be retained by the nucleus for 6-18 hours in order to elicit late responses and subsequent uterine tissue growth.

1.1.5.6 Receptor Processing and Recycling

After the oestrogen-receptor complex has completed its function it is released from the chromatin (Clark and Peck, 1976). Oestradiol-17β is released from the cell unmetabolised (Puca and Bresciani, 1968) and passes back into the circulation. The fate of the receptor is less clear. Studies involving RNA and protein synthesis inhibitors have shown that, 24 hours after a single injection of oestradiol into immature rats, approximately 40% of the oestrogen receptor in the cytoplasm has been recycled, that is, the process was independent of mRNA or protein synthesis (Clark and Peck, 1979). The remaining 60% was protein synthesis dependent. The cytoplasmic receptor population, therefore, appears to be composed of two populations, recycled and de novo synthesized receptor (Katzenellenbogen, 1980).

An interesting phenomenon described by Horwitz and McGuire (1978a) is receptor 'processing'. This was first described in the MCF-7 human breast cancer cell line. Receptor processing involves an apparent loss of and subsequent release of nuclear receptors, following oestrogen interaction. This loss and release of receptor correlates with the

appearance of oestrogenic responses such as the induction of progesterone receptor (Horwitz and McGuire, 1980). Processing does not seem to involve a proteolytic step and has been shown to be inhibited by those intercla-ting drugs, such as Actinomycin D, which bind to G-C base pairs, (Horwitz and McGuire 1978b, 1980). Actinomycin D does not prevent receptor trans-location or chromatin binding nor does it bind to the receptor. It is suggested that Actinomycin D causes some form of conformational change in the DNA, which prevents processing (Horwitz and McGuire, 1980). Other inhibitors of RNA and DNA synthesis, including those which interclate in the A-T rich regions do not inhibit receptor processing.

Two different types of response are observed when the oestrogen receptor from MCF-7 cells is complexed with antioestrogens. The nafoxidine-receptor complex, although bound to chromatin, is not processed and as a result, no progesterone receptor induction arises. However, sub-sequent oestradiol-17β treatment results in a much greater progesterone receptor induction than that in controls not pre-treated with nafoxidine (Horwitz et al., 1981). Using tamoxifen, an antioestrogen with oestrogenic properties in MCF-7 cells, the progesterone receptor was superinduced above the levels seen with oestradiol. Tamoxifen does this by markedly reducing the early lag phase for progesterone receptor induction. The mechanism by which this occurs is not known (Horwitz et al., 1978). For nafoxidine action, the two explanations put forward by Horwitz et al. (1981) are 1) that the receptor remains at the nuclear acceptor sites to which it was brought by nafoxidine. When oestradiol replaces nafoxidine, a conformational change occurs in the receptor/acceptor complex which permits rapid processing; 2) when oestradiol displaces nafoxidine from the receptor, the protein moves along the chromatin to another site where it is processed. The exact nature of processing remains unclear. Hansen

and Brooks (1982) have shown that receptor processing correlates with the the formation of oestrone receptor and this equals the loss of oestradiol-17β binding sites. The oestrone receptors so formed, are found in the cytosol and do not translocate, resembling the type II oestrogen binding sites seen in breast tissue (Watson et al., 1980). There are however, other properties which distinguish so formed oestrone receptor from type II sites. Firstly, the cytoplasmic type II oestrogen receptor concentration remains unchanged during oestradiol-17β stimulation (Eriksson et al., 1978) whereas the oestrone receptor increases significantly following the exposure of MCF-7 cells to oestradiol-17β (Hansen and Brooks, 1982). Secondly, oestradiol-17β does not compete for the oestrone receptor in MCF-7 cells, whereas both oestrone and oestradiol-17β are equally effective in the inhibition of tritiated oestradiol binding to type II sites (Clark et al., 1978). Oestrone receptor, may therefore, arise, as a consequence of some form of alteration of oestradiol-17β type I receptors.

Schoenberg and Clark (1981) have found that the nuclear bound receptor resistant to exogenous DNAase treatment corresponds to those sites which are processed. This suggests that some of the nuclear receptors are involved in a function other than binding to chromatin sites and that only this subset of receptors are processed.

1. PART B

1.2. OESTROGEN RECEPTORS AND BREAST CANCER

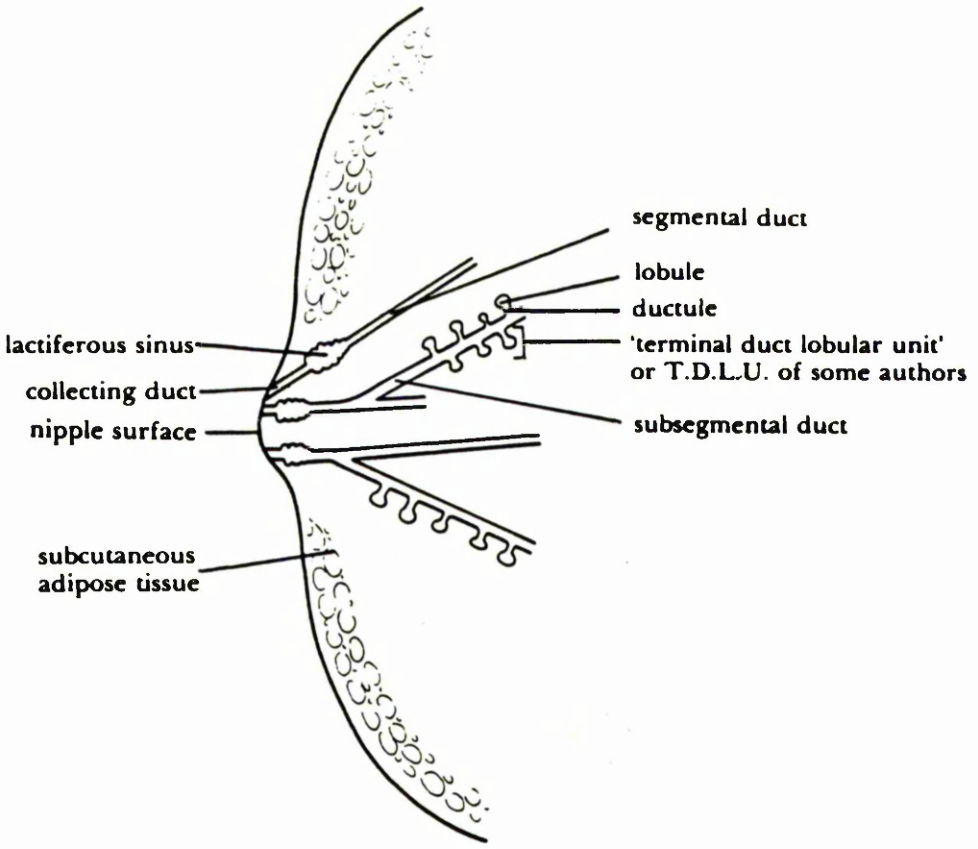
1.2.1 The Mammary Gland

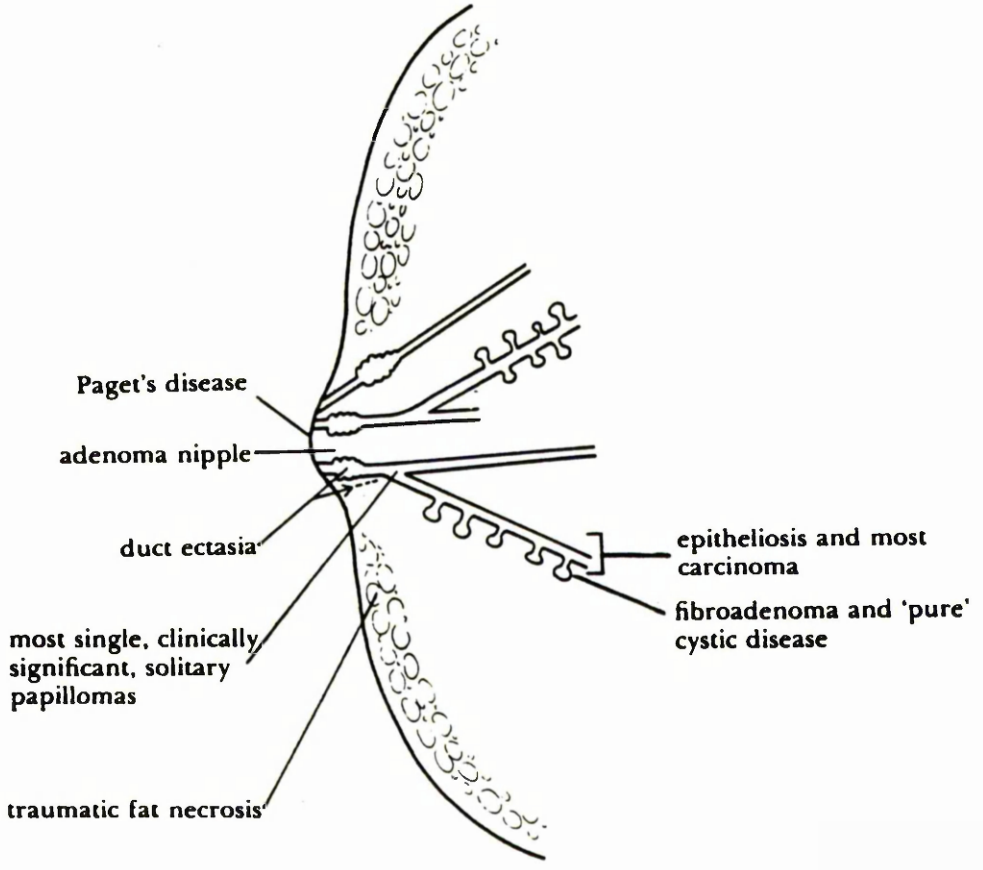
The mammary glands are specialized accessory glands of the skin that have evolved in mammals to provide for the nourishment of their offspring, which are born in a relatively immature and dependent state. In their structure and mode of development, mammary glands resemble sweat glands. Their differentiation during embryonic life is similar in both sexes. In the male, however, little additional development occurs in postnatal life, whereas in the female the glands undergo extensive structural changes correlated with age and the functional condition of the reproductive system. The female breast in fully developed by the twentieth year, with atrophic changes setting in by the age of 40 and becoming more marked after the menopause.

Figures 6A and 6B show the main anatomical parts of the human breast and the major sites which are affected by the different diseases of the breast. The resting mammary gland is a compound tubuloalveolar gland consisting of 15 to 25 irregular lobes radiating from the nipple. The lobes are separated by layers of dense connective tissue and surrounded by abundant adipose tissue. Each lobe is provided with a lactiferous duct which is lined by stratified squamous epithelium. Beneath the areola each of the ducts has a local dilation, the sinus lactiferous. Each lobe is subdivided into lobules of various orders, of which the smallest consist of elongated tubules, the alveolar ducts, covered by small saccular evaginations, the alveoli. The alveolar ducts open into the ductules. The secretory portions of the gland, the alveolar ducts and alveoli, consist of cuboidal or colomnar secretory cells resting on a basal lamina and a discontinuous layer of processes of myoepithelial cells.

Figure 6A. **The nomenclature of the main anatomical sites of the breast**

Figure 6B. **Anatomical sites of the breast affected by different diseases**


segmental duct
lobule
ductule
'terminal duct lobular unit'
or T.D.L.U. of some authors
subsegmental duct
lactiferous sinus
collecting duct
nipple surface
subcutaneous
adipose tissue


Paget's disease
adenoma nipple
duct ectasia
most single, clinically
significant, solitary
papillomas
traumatic fat necrosis
epitheliosis and most
carcinoma
fibroadenoma and 'pure'
cystic disease

Pregnancy and lactation bring about changes in the levels of circulating hormones which result in profound changes in the mammary glands. This is referred to as the active phase. During the first half of gestation, there is a rapid growth and branching from the terminal portion of the duct system. The growth of the epithelial structure takes place at the expense of the interstitial adipose tissue which regresses concurrently with the growth of the glandular tissue. In the later months of pregnancy, the actual hyperplasia of the glandular tissue slows down. The subsequent enlargement of the breast is largely a consequence of enlargement of parenchymal cells and distention of alveoli through eosinophilic secretion rich in lactoproteins but relatively poor in lipid. This is colostrum, the first milk that has special laxative properties and contains antibodies which provide the newborn with passive immunity. During the first few days after delivery the degree of infiltration of the stroma of the gland by lymphoid elements becomes less intense, and the colostrum gives way to a copious secretion of milk, rich in lipid. Milk is produced in secretory cells lining the alveoli. This then drains into the duct system. Ducts in turn open onto the body surface via the teat or nipple.

1.2.2 Hormones, Receptors and Breast Cancer

1.2.2.1 Hormonal Involvement

The mammary gland exhibits absolute dependence on hormonal stimulation. The gland remains refractory prior to the onset of the cyclic secretion of ovarian hormones at puberty. The extent of development of the gland varies between individual species. In rats, for example, the growth is limited to extension and branching of the duct system. In the human female, however, ductal elongation and development of the alveolar system occurs.

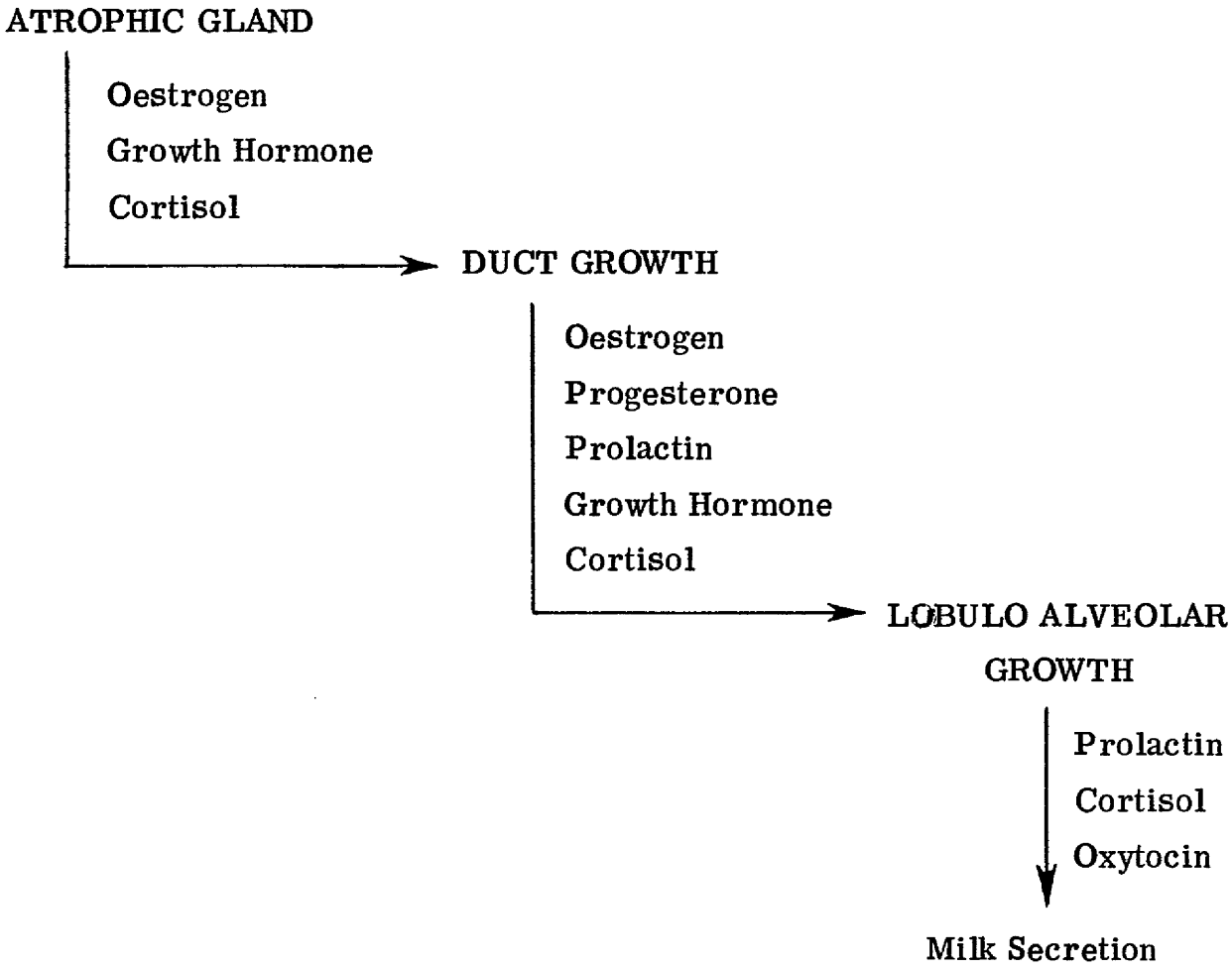
The precise function of the various hormones in promoting mammary gland development during pregnancy is not clear. It is known that these hormones show a very complex relationship, acting individually or synergistically in controlling breast cell functions. Figure 7 shows the relationship between various hormones and the development of the gland to the secretory stage.

Hormones, whether polypeptide or steroid, exert their effect via specific receptors, which are located in the soluble fraction of the cell for steroids and on plasma membrane for polypeptides (Leake, 1976; Kolata, 1978). Insulin function is not completely understood. However, it is known that the insulin-receptor complex is internalised (Kahn et al., 1981). Insulin is thought to control the formation of milk secretory cells. Glucocorticoids have been assigned the function of controlling the secretory proteins and prolactin is thought to initiate RNA synthesis in epithelial cells. Prolactin has been shown to induce oestrogen receptor synthesis and its requirement has been found to be essential for the growth of experimental mammary tumours (Leung and Sasaki, 1975). Lactogenesis is controlled by a combined effect of a number of different hormones (Denamaur, 1971). The precise role of individual hormones in lactogenesis is species specific (Nalbandov, 1976).

1.2.2.2 Oestrogen Receptor in the Mammary Gland

The normal mammary gland has been shown to contain oestrogen receptor, though at low levels (Sander, 1968; Block et al., 1975). Indeed selective retention of hexoestrol in the breast was first observed in 1959 (Glascock and Hoekstra, 1959). It was demonstrated that in addition to ovarian and adrenal oestrogen, the mammary gland itself had the capacity to synthesize oestrogen (Adams and Li, 1975; Miller et al., 1982). Since

Figure 7. **Hormonal involvement in the development of the breast**


ATROPHIC GLAND
Oestrogen
Growth Hormone
Cortisol
DUCT GROWTH
Oestrogen
Progesterone
Prolactin
Growth Hormone
Cortisol
LOBULO ALVEOLAR GROWTH
Prolactin
Cortisol
Oxytocin
Milk Secretion

normal human breast tissue is not readily available, the vast majority of studies have used animal mammary gland models to study the complexity of hormonal involvement with mammary tissue (Leung, 1978). It was found that the level of oestrogen receptor was very low in non-lactating glands and that this value increased considerably during pregnancy and lactation, although the quantitative levels during pregnancy and lactation are hard to establish (Leung et al., 1976; Bohnet et al., 1977; Forsyth and Hayden, 1977). Mammary epithelial and stromal cells both apparently have the capacity to synthesize oestrogen receptor.

1.2.2.2.1 **Regulation and Molecular Form of Oestrogen Receptor in the Mammary Gland**

The oestrogen receptor has been detected in mouse and rat mammary glands (Shyamala and Nandi, 1972; Bohnet et al., 1977), and in human breast tumours (Jensen and DeSombre, 1972; Leake et al., 1981). The similarity between the rat and human mammary gland oestrogen receptor has been reported by McGuire and De La Garza (1973b).It has been assumed so far that oestrogen receptor action, at the molecular level, is the same in rat and mouse mammary gland, rat uterus and human mammary gland. However, temporal differences in oestrogen receptor action in different tissues of the same animal have been reported (Linkie, 1977). Again, differences, most probably at the structural level, between rat and human oestrogen receptor are probable (Notides et al., 1976).Differences between rat uterine and mammary gland oestrogen receptor have also been documented, perhaps at the molecular level (Park and Wittliff, 1977).

On SDGA, in the absence of salt, oestrogen receptor from immature rat uterus sediments solely at 8S, whereas the receptor from mature rat uterus can sediment in two peaks, one at 4S and the other at 8S

(Section 3.1.5). The molecular form of rat mammary gland oestrogen receptor shows a variation in sedimentation properties which may be related to plasma hormonal levels. For example, in early pregnancy only the 4S form is detectable while during lactation the 8S form can be demonstrated (Mohla et al., 1981). A similar variation is observed in the mouse mammary gland and the 8S form was not a result of concentration dependent aggregation since dilution has no effect on the sedimentation profile (Muldoon, 1978). This latter report also provided evidence that the 8S form of receptor has a much longer half life than the 4S form.

The suggested function of the two types of receptor is that the 4S type is involved in mediating acute responses, since these appear to be present at the time when blood oestrogen concentration is high, and that the 8S type is involved in mediating long term or sustained response, when the concentration of hormone may have dropped to low levels. Both forms of receptor were shown to undergo 4S → 5S transition (Muldoon, 1978). In contrast Park and Wittliff (1980) have reported that the low salt 4S form of oestrogen receptor from human breast tumours cannot undergo the 4S → 5S transition and bind to DNA and, therefore, perhaps represents a non-functional form of receptor. It is possible that, whereas in human tissue the 4S receptor represents proteolytic digest of 8S (Schneider and Dao, 1977), in the mouse mammary tissue the 4S represents a dissociated but functional form of the 8S complex, capable of aggregation to the 5S form. Jensen et al. (1968) and Rochefort & Baulieu (1971) have both observed that the aggregation (to 8S) property of salt dissociated 8S complex is important to its ability to form 5S. It is possible that a minor alteration of the physical state of the receptor may cause dissociation of the 8S complex into the 4S form. A similar minor structural change is implied by the change in the dissociation constant associated with oestrous cycle (Buchi and Keller, 1980). Colvard and Wilson (1981) have assigned a cytosolic protein

to the role of converting the 4S form of androgen receptor to the 8S form, implying that 4S androgen receptor similarly possesses this aggregation property.

The results of Gaubert et al. (1982) suggest that the 4S complex observed in mouse mammary gland is a proteolytic product of the 8-9S complex and represents a similar proteolytic species to that observed in human breast tumours (Sherman et al., 1980; Miller et al., 1981). These results are in conflict with the idea proposed by Muldoon (1978) and difficult to reconcile with the observed 4S → 5S transition mentioned in Muldoon's study. Both 4S and 8S forms might bind to DNA but with different affinities. Although the 4S → 5S change is not observed in human mammary gland extracts, temperature dependent activation of oestrogen receptor can be demonstrated (Park and Wittliff, 1977). The proportion of total receptor which can be made to bind to DNA is certainly lower than that in immature rat uterus. The small proportion of receptor which can be activated in human breast tissue reflects the low level of activation found in rat mammary gland (Sato et al., 1981a). The proteolytic activity which deprives the 4S receptor of its ability to bind DNA would, on this basis, be higher in mammary tissue than in uterus. Alternatively, a necessary activation factor(s) might be missing (Thrower et al., 1976). The heating process involved in the in vitro measurement of activation may also lead to aggregation, presumably occluding the DNA binding site.

In rat mammary tumours the concentration of both cytosol and nuclear oestrogen binding changes during the oestrous cycle (Shih and Lee, 1978). Freedman and Hawkins (1980), studying the molecular form of oestrogen receptor during the oestrous cycle in DMBA induced mammary carcinoma of the rat, reported the presence of only the 4S form at pro-oestrous. The 8S form was present at all other stages of the oestrous cycle. Once again, plasma oestrogen levels are implicated in the regulation of the molecular form of

oestrogen receptor. It seems possible, therefore, that when the blood concentration of oestrogen is high, a proteolytic entity (Oestrogen induced?) is present (in the soluble extract) which is able to convert the 8S into the 4S form in a specific manner. Whether such a conversion represent some form of *in vivo* regulation of the receptor (perhaps to a form which does not bind to nuclei) is unclear. It is possible that the conversion of the 8S form into the non-DNA binding 4S form protects the chromatin from excessive oestrogenic stimulation. Milgrom *et al.* (1972) have studied the molecular form of progesterone receptor in the guines pig uterus at different stages of the oestrous cycle. The 4S is dominant at pro-oestrous and 8S at all other stages. Toft and O'Malley (1972) also reported heterogeneity of progesterone receptor in the chick oviduct. No reports on the molecular form of rat uterine oestrogen receptor during the oestrous cycle are known.

Mammary gland oestrogen receptor is stable after ovariectomy (Hunt and Muldoon, 1977). Prolactin was found to have no effect on the oestrogen receptor from the lactating mammary gland (Bohnet *et al.*, 1977). Prolactin, however, has been shown to increase oestrogen receptor concentration in both rat mammary gland and uterus and in mammary tumours of intact ovariectomized and ovariectomized-hypophysectomized rats (Leung and Sasaki, 1973; Sasaki and Leung, 1974; Vignon and Rochefort 1976; Asselin *et al.*, 1977). It also promotes the 4S to 8S conversion of oestrogen receptor in the mammary gland of the adult virgin mouse (Muldoon, 1978).

Finally, very little is documented about inhibition of oestrogen receptor synthesis. Progesterone (Bohnet *et al.*, 1977; McGuire, 1978; Clark and Peck, 1979) and prostaglandin(s) (Jacobson, 1974) are found to inhibit oestrogen receptor synthesis. There is evidence that progesterone inhibits oestrogen receptor in human endometrium at late follicular phase (Soutter *et al.*, 1979).

1.2.3 The Significance of Oestrogen Receptor in Mammary Carcinoma

When a mammary gland epithelial cell undergoes neoplastic transformation, it could escape the normal influence of oestrogen, and either lose the capacity to synthesize oestrogen receptor or become extremely sensitive due to switching on and amplification of oestrogen receptor genes (Wittliff et al., 1972; Jensen, 1975). The increased sensitivity is not confined to female sex tissue and has also been observed in male tumours (prostatic and breast carcinoma)(Leclercq et al., 1975; Miller, 1978; Grilli et al., 1980; Friedman et al., 1981).

Experimental systems which have proved very useful in studying control mechanisms in mammary tumours include (i) rodent mammary tumour (Topper, 1970), (ii) the DMBA induced, hormone dependent and independent rat mammary tumour (Geyer et al., 1953; Young et al., 1963; Hawkins et al., 1978) and several other experimental mammary tumours (Wittliff, 1979), and (iii) the MCF-7 cell line which was originally derived from the pleural effusion of a patient with hormone dependent breast cancer (Soule et al., 1973; Edwards et al., 1979).

There is extensive data that the growth promoting effects of oestrogens in mammary tumours (Wittliff, 1979) are receptor-mediated. The inherent assumption in all such studies is that the mechanism of steroid action is the same in normal tissue and in tumours. The oestrogen receptor itself appears to be similar in physical parameters in the rat and human mammary carcinoma (McGuire and De La Garza, 1973b).

The pituitary gland has been the centre of many studies since it was observed that oestrogen alone cannot promote rat mammary tumour growth. The presence of prolactin is essential for the response to be mediated (Sinha et al., 1973). This finding supports an indirect role of

prolactin in tumour growth. The involvement of prolactin in increasing the concentration of oestrogen receptor has already been presented for animal systems (Section 1.2.2.2.1), data for human tumours is very limited.

A relationship between tumour growth and menstrual cycle was known as far back as 1836 (Cooper, 1836). However, it was Beatson (1896) who first observed tumour regression following oophorectomy, in premenopausal women with advanced breast cancer. Huggins and Bergenstal (1952) followed with a report of the efficacy of adrenalectomy in the treatment of post menopausal breast cancer patients. Reports by Luft et al. (1958) and Ray and Pearson (1958) showed benefits of hypophysectomy in similar patients. In all these studies it was obvious that not all patients responded to ablative endocrine therapy. A discriminant was therefore required to select patients who would benefit from surgical treatment, sparing those who would not (Baker et al., 1960).

With the development of steroid receptor theory, it was proposed that receptor measurements should be used to select the patients who are likely to respond to hormone therapy (Jensen et al., 1971 b). This idea was influenced by earlier findings that organs responsive to oestrogen had the capacity to retain tritiated hexoestrol when the compound was injected into rats (Glascock and Hoekstra, 1959; Jensen and Jacobson, 1960) and from the findings of Folca et al. (1961) that if the patients undergoing adrenalectomy were injected with tritiated hexoestrol prior to surgery, then those who responded had selectively retained a higher proportion of labelled hormone in the tumour than those who did not. The correlation obtained in the studies of Folca et al. (1961), however, was not strong enough to adopt the method for routine clinical analysis.

The potential of oestrogen receptor as an index of hormonal involvement in growth and development of the tumours was studied in

various centres (Feherty et al., 1971; Maass et al., 1972; Englesman et al., 1973). These early findings suggested a high response rate but they were based on very few patients. The combined results of the Breast Cancer Task Force (McGuire, et al., 1975a) suggested that over 50% of patients who had cytosolic receptor in their tumour biopsies showed a response to endocrine therapy, whereas less than 10% of patients without oestrogen receptor, responded. Accumulated data suggests that 40-60% of patients with oestrogen receptor positive tumour biopsies show regression on hormone therapy (Byar et al., 1979; Hawkins et al., 1980; Leake, 1981b). Using an extended technique of measuring both soluble and nuclear receptor the response rate is reported to be just over 70% (Barnes et al., 1979; Leake et al., 1981). It has been reported that the possibility of response is increased if receptor concentration is taken into account (Jensen, 1975; Leclercq and Heuson, 1977). This however, only appears to hold true for premenopausal patients (Hawkins et al., 1979) and may simply be an index of the proportion of tumour cells in the biopsy.

One important assumption in Jensen's proposal was that both local recurrencies and metastases would show the same characteristics with respect to receptor status as the primary lesion. It is however, becoming clear that, whilst receptor negative status is generally maintained in advanced disease, receptor positive primaries do not always give rise to receptor positive secondaries (Leake et al., 1981). There is now much evidence that presence of oestrogen receptor in the tumour biopsy can serve as a prognostic index (Hawkins et al., 1980; Leake, 1981b). Some authors, however, failed to find such a relationship (Bloom and Degenshein, 1980; Hilf et al., 1980), while others note that the prognostic value of oestrogen receptor may be confined to patients with 1-4 axillary nodes involved (Howart & Barnes, 1981). Whether the difference in disease-free interval between receptor positive and receptor negative disease is sustained

beyond 5 years from the primary remains to be seen.

Some receptor negative tumours are found to be fast growing (Meyer et al., 1977) and aggressive in nature (Knight et al., 1977; Maynard et al., 1978). Patients with receptor negative tumours show earlier recurrence rates and shorter survival times than patients with receptor positive tumours (Kiang et al., 1978; Bishop et al., 1979; Cooke et al., 1979; Kinne et al., 1981). Westerberg et al. (1980) have reported a quantitative relationship between the receptor content of the primary tumour and disease-free interval. Certain pathological features of the tumour are also correlated with the hormone dependence or independence (Masters et al., 1979; Millis, 1980).

1.2.3.1 'False Negative' Tumours

Some patients whose tumours contain no detectable oestrogen receptor respond, nevertheless, to hormonal manipulation. These patients are termed 'false negatives'.

The histochemical localization of oestrogen receptor has demonstrated the coexistence of oestrogen receptor positive and negative cells within the same tumour (Nenci et al., 1976; Pertschuk et al., 1980). Some 'false-negatives' could thus be explained on the basis of cellular heterogeneity in that the assay portion of tumour may have contained only receptor negative tumour cells. It is also, obviously, important to check that each biopsy assayed does contain sufficient tumour cells relative to fat and connective tissue.

One reason for 'false negatives' which cannot be ruled out is that some form of structural defect may render the functional receptor undetectable by conventional assay. The small group of tumours which contain only nuclear receptor (Leake et al., 1981) could have explained some

'false negatives' of this type but follow-up data suggests that these (o/+ or ER_c^-/ER_n^+) tumours are normally hormone independent. Histochemical and immunochemical methods may be useful in the detection of 'non-oestrogen binding' functional receptor, in addition to truly functional receptor. The validity of histochemical methods, however, has been questioned (Chamness et al., 1980; Penney and Hawkins, 1982).

Some of the 'false negatives' could arise as a result of the thermolabile nature of receptor. It is possible that receptor could get degraded from the time of excision to the time the tumour was assayed or stored. However, the two most likely explanations for false negatives are (1) a biopsy atypical of the tumour as a whole and (2) responses of tumours to antioestrogens by pathways that are not receptor mediated, such as the proposed inhibition of prostaglandin synthetase (Tisdale, 1977).

1.2.3.2 **'False Positive' Tumours**

'False positive' cases arise when the tumour biopsy shows the presence of oestrogen receptor, yet the patient fails to respond to subsequent hormonal therapies. This situation occurs more frequently than 'false negative' cases. Some of the more obvious reasons leading to such a situation include inadequate methodology (King, 1976) and imprecise clinical assessment (McGuire et al., 1975; Stoll, 1981). Other less obvious reasons perhaps contribute to a larger extent and are discussed below.

The cytoplasmic assay measures only the soluble receptor. A post-oestrogen binding defect in the sequence of events leading to hormone responsiveness, may occur. A defect in receptor structure and/or transforming factor(s) may leave the receptor nonfunctional with respect to chromatin binding and growth (Maass, 1975). Evidence for such a defect is found in experimental systems including the oestrogen induced hamster

kidney tumour (King et al., 1970) and the spontaneous GRA mouse mammary tumour (Shyamala, 1972). Similar defects occur in the unresponsive cells obtained from a glucocorticoid responsive lymphoid tumour (Yamamoto et al., 1974).

In some human breast tumours only the soluble receptor can be detected (Garola and McGuire, 1977; Singh et al., 1978; Thorsen and Stoa, 1979; MacFarlane et al., 1980; Leake et al., 1981). A similar situation has been observed with androgen receptors from human breast cancer (Singh et al., 1979). Tumours in such cases are generally hormone independent but on the basis of only soluble receptor they would be classified as hormone dependent. This class of tumours are referred to as $+/o(ER_c^+/ER_n^-)$. Patients with such tumours have response rates of only 24% (Leake et al., 1981) and this may go down even further with increase in the sample number.

Nenci et al. (1976) using their immunofluorescent technique, have reported independent evidence that such defective receptors can be present in breast tumours. They have further suggested that during early post-natal development, changes in the permeability of the nuclear membrane to oestrogen receptor may occur to give protection to the tissue against circulating oestrogens (see also the possible role of 4S - Section 1.2.2.2.1). This suggestion was based on the fact that certain breast tumour cells displayed a perinuclear concentration of the fluorescent antibody, similar to a pattern observed in the uterus of very immature rats. Perhaps, therefore, similar changes may occur in malignant cells. However, since the fluorescent technique is probably imaging type II sites, this may be an overinterpretation of the data.

There is always the possibility that the receptor itself may be functional but the acceptor sites on the chromatin may be defective.

One report indicates such a finding (Charreau and Baldi, 1977). As with 'false negatives' there are some tumours which show both nuclear and cytoplasmic oestrogen receptor but fail to respond to hormone therapy. It is possible that in such tumours, the nuclear receptors are bound non-specifically or are bound to certain altered specific binding sites leading to tumour insensitivity. Examples of such malfunctions are found in glucocorticoid sensitive hepatoma cells (Thompson and Geleherter, 1971; Croce et al., 1974), certain glucocorticoid sensitive leukaemias (Lippman et al., 1974) and the androgen responsive mouse mammary tumours (Bruchovsky et al., 1975).

It has been demonstrated that target tissues possess the capacity to metabolize active oestrogens to less active compounds (Pack & Brooks, 1974). Thus, though the receptors may be present, these may not be engaged. On the other hand, reports of unfilled receptors (Horwitz and McGuire, 1978a) suggest that these may be able to promote oestrogenic responses independent of the steroid itself (Zava et al., 1976) and therefore ablative or additive hormone therapy may have no effect.

A major factor contributing towards 'false positives' would seem to be the heterogeneous nature of tumours with respect to cellularity and hence receptor concentration (Braunsberg, 1975; Hawkins et al., 1977). In certain cases of intratumoural studies this heterogeneity can actually alter the receptor status of the tumour, although such cases are rare (Leclercq et al., 1975; Tilley et al., 1978; Hawkins et al., 1979). It has also been documented that two tumours taken from the same breast show considerable variation (Braunsberg, 1975; Poulsen et al., 1981). Oestrogen receptor concentration may also vary from one tumour deposit to another (Liskowski and Rose, 1976; Hawkins et al., 1979). Tumours, other than breast tumours also show variation in cell types (Marx, 1982).

Interestingly, some of the large endometrial carcinomas show an 'all or none' phenomena with respect to receptor concentration (Castagnetta et al., 1983). Since breast tumours are composed of such mixed populations of cells, it is possible that the hormone independent cells (faster growing) may take over, converting the tumour to autonomy. In such cases an initial response may be followed by progression.

1.2.3.3 Tumour Markers of Hormone Dependence

Given that detection of both soluble and nuclear oestrogen receptor is not an absolute guarantee of physiologically functional receptor (Boylan and Wittliff, 1973, 1975; Jensen and DeSombre, 1977), other indexes are required which would indicate oestrogen induced responses. Several alternatives have been tested. These include caesin mRNA, but this was found only in 70% of hormone dependent mammary tumours (Rosen and Socher, 1977). Another protein tested has been α-lactalbumin, one of the proteins required for lactose synthesis. However this also was not found in all hormone dependent rat mammary tumours, its absence most probably reflecting some damage, during malignant transformation, to the gene responsible for its synthesis (Ip and Dao, 1978; Hall et al., 1979; Woods et al., 1979). Peroxidase has been suggested as a marker for hormone dependent tumours (DeSombre et al., 1975; Lyttle and DeSombre, 1977a). It has been shown to be produced by oestrogen action on the rat uterus (Lyttle and DeSombre, 1977b) where it may act to cross link uterine proteins (Keepings and Jelling, 1978). However the validity of peroxidase has been put into question with its detection in both benign and oestrogen receptor deficient tumours of mammary origin (Duffy and Duffy, 1977).

Recently, progesterone receptor has gained considerable support as a marker of oestrogen dependence. Oestradiol has been shown to stimulate

the synthesis of this protein within the cell (Asselin et al., 1977). Inclusion of progesterone receptor as a marker for hormone responsiveness was based on the observation that some mammary tumours contain elevated levels of progesterone receptor (England et al., 1975; Horwitz et al., 1975; McGuire et al., 1976). Martin et al. (1979) suggested that progesterone receptor may also serve as an index of hormone responsiveness in endometrial carcinoma. Horwitz et al., (1975) suggested that patients whose tumours contain only oestrogen receptor should not be treated with endocrine therapy. McGuire's group (McGuire et al., 1977) reported that by considering both oestrogen and progesterone receptor, the response rate of patients can be elevated. However, both McGuire (1978) and Allegra et al. (1979) have found that some tumours containing oestrogen receptor but lacking progesterone receptor also responded to hormone therapy. Further, Ip et al. (1979) published a disturbing report concerning a hormone independent tumour system, MTW9B, which contains both oestrogen and progesterone receptors. Similar hormone indepedence is shown by the mammary tumour cell line MXT3590 which nevertheless has detectable progesterone receptor synthesis, but does not undergo cell division in response to oestradiol. This suggests that there must be a step subsequent to progesterone receptor induction which is critical to growth and cell division. As an alternative marker, Westley and Rochefort (1979) have reported three proteins which are a result of oestradiol stimulation and are inhibited by tamoxifen. Better still would be the demonstration of oestrogen-induced growth in organ or cell culture.

The idea of tumour markers for hormone responsiveness has been questioned by McGuire et al. (1972) who found a mammary carcinoma, R3230 AC which is hormone independent with respect to its growth but hormone responsive relative to oestrogen-induced metabolic changes. However, it may be noted that this tumour contains very low levels of oestrogen receptor.

1.2.4 The Clinical Significance of the Molecular Form of Oestrogen Receptor in Human Breast Cancer

Some of the immediate aims of present research are to explain and, thereby, eliminate 'false positive' cases of hormone dependence. Although several markers have been suggested to assess the functionality of the oestrogen receptor (see previous section), these have not met with much success (Barnes et al., 1979; Thorsen and Stoa, 1979). In order to increase the clinical importance of oestrogen receptor in terms of indicating both potential hormonal response and prognosis, Wittliff and his colleagues have suggested that perhaps the molecular form of oestrogen receptor may provide a better index (Wittliff and Savlov, 1975; Wittliff et al., 1976; Wittliff et al., 1977; Wittliff and Savlov, 1978). Based on the molecular form of oestrogen receptor five classifications of the tumours could be obtained. These are (a) no oestrogen receptor, (b) the 8S form only, (c) the 8S and 4S forms together, (d) the 4S form only and (e) oestrogen binding moietics not coincident with either 8S or 4S form on SDGA.

In the study of Wittliff et al. (1977), no correlation could be obtained between the molecular form of oestrogen receptor and the histological grade of the tumour. However, there appeared to be a shift towards the 4S form from the primary to secondary or metastatic lesions. No significant difference in receptor concentration was observed between the primary and secondary lesions (Wittliff et al., 1978). The initial clinical correlation showed that none of the patients whose tumours showed only the 4S form of oestrogen receptor responded to hormonal therapy (Wittliff et al., 1977; Wittliff and Savlov, 1978), whereas 75% of the patients whose primaries showed either 8S or 8S and 4S forms of the receptor showed objective response to hormonal manipulation. In later

reports, the response rate of patients with 8S or 8S and 4S containing primaries have been sustained but some of the 4S containing tumours have also been shown to respond, although with much lower frequency (responders 33/44 in 8S and 8S + 4S class, responders 4/23 in 4S class; Kute et al., 1978; Wittliff et al., 1978; Wittliff, 1980). This has led to the idea that the 8S form represents the functional form of oestrogen receptor. It was further demonstrated that only the 8S form binds to DNA (Park and Wittliff, 1980).

When the experiments were conducted in buffers containing 0.15M KCl, the 8S form in low salt appeared as a 6S peak. No 6S peak in 0.15M KCl was seen in cytosols from tumours in which only the 4S form was found in low salt gradients. On the basis of such findings, Wittliff et al. (1977) suggested that receptors in tumours containing the 8S form can undergo activation to the 6S form and become functional, whereas receptors in tumours containing only the 4S form fail to become functional due to the absence of a component(s) required for activation. Another explanation, however, may be that the 4S receptor is defective in its attachment of the other subunit(s) and/or its conformational change required for activation. It is possible that the 4S receptor may be normal but the other subunit(s) may be missing or defective. It is interesting that the majority of tumours with high total receptor concentration were reported to show the presence of the 8S form, whereas lower receptor content often reflected only the 4S form. It should also be noted that whereas the 6S form was detected in 0.15M KCl containing gradients, when the classical activation conditions were used (30^{o}C, 30 min) only the 4.0-4.6S form appeared and not the 5.5S form (Wittliff et al., 1978).

Although Wittliff's idea is potentially useful, several investigators have failed to confirm his observations, (Westerberg et al., 1978;

Dao and Nemoto, 1980; Gapinski and Donegan, 1980). In addition, both steroid dependent and autonomous rat mammary tumours have an intact translocation mechanism (Hawkins et al., 1978) and show predominantly the presence of 8S receptor (Freedman and Hawkins, 1979, 1980). This questions the validity of the 8S form as an index of hormone responsiveness.

Based on studies to establish the stability of the 8S form, it was concluded that the 4S form of the receptor can arise from the 8S form as a consequence of (1) storage, (2) the various experimental conditions used and (3) in the case of DMBA induced mammary carcinoma, as a result of the hormonal milieu (Schneider and Dao, 1977; Sherman et al., 1980; Freedman and Hawkins, 1980; Tilzer et al., 1981). All breast tumours are thought to possess the 8S receptor initially (Freedman and Hawkins, 1980; Tilzer et al., 1981). In addition various protease inhibitors have been shown to protect the 8S form of oestrogen receptor from being degraded into the 4S form in human myometrium (Notides et al., 1976; Daxenbichler et al., 1980).

In conclusion, it seems strange that such differences in molecular form in relation to response can be obtained by different investigators supposedly analysing similar tissue. The implications are that not only is tissue heterogeneity a problem but so are the variations in procedures used in different laboratories. Several authors have called for a quality control scheme to reduce intra- and inter-laboratory variation (Wittliff, 1980; Leake, 1981b) and this should prove beneficial. It is essential, nevertheless, to find out where in the process of analysis, the variations may have occurred. Some such results are reported in this thesis with respect to interconversion of the molecular forms of oestrogen receptor in human breast cancer.

1.2.5 The Treatment of Breast Cancer

The treatment of human breast cancer falls into four broad areas. These are radiotherapy, hormone additive and ablative therapy, immunotherapy and chemotherapy (Gallagher et al., 1978; Stoll, 1981). These treatments are usually initiated after surgical treatment of the primary tumour. The precise selection of therapy for a particular patient is guided by both clinical and biochemical parameters (size of primary, extent of nodal involvement, age, receptor status etc.). A very general scheme based on receptor status is shown in Figure 8.

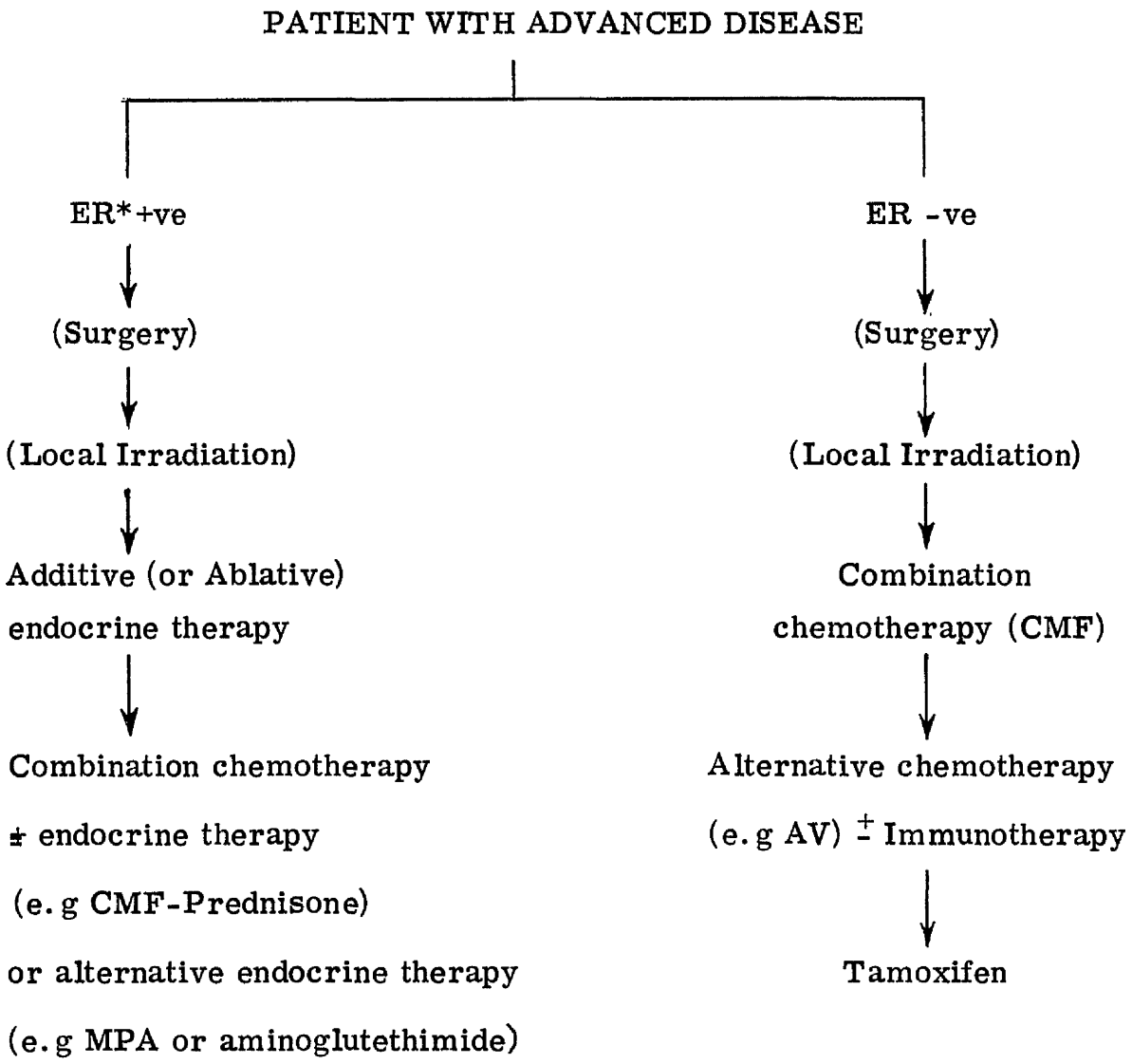
1.2.5.1 The Mechanism of Action of Endocrine Therapy

Endocrine therapy, the treatment of choice for patients with hormone dependent disease, may be either ablative (removal of glands synthesizing the oestrogen nucleus) or additive (competitive blocking of oestrogen action).

1.2.5.1.1 Ablative Therapy

Removal of the ovaries is, of course, the first procedure in premenopausal women, whereas adrenalectomy and hypophysectomy are applicable to post-menopausal women. However, such surgery is traumatic for the patient. A synthetic compound, aminogluthetimide, is now available and causes chemical adrenalectomy by blocking synthesis of steroids at the conversion of cholesterol to pregnenolone, with an additional effect on aromatization (Santen et al., 1980). Such treatment has to be supplemented with glucocorticoids and inevitably leads to side effects (Siiteri, 1982).

Figure 8. **Possible schemes for the treatment of advanced breast cancer incorporating stratification according to oestrogen receptor status**



( ) = procedures adapted where appropriate

* = oestrogen receptor

1.2.5.1.2 **Additive Therapy**

Additive therapy may act by (i) depleting cytoplasmic oestrogen receptor, thus reducing the sensitivity of the tumours to endogenous hormone, (ii) by blocking the steroid receptor acceptor sites, this perhaps happens when large doses of oestrogen or androgen are given and (iii) by an indirect pathway, such as depression of pituitary function or suppression of the immediate environmental factors which may be required for promotion of tumour growth. One such possibility is the depression of prolactin release (Djiane and Durrand, 1977).

In additive therapy, pharmacological levels of hormones may be administered. These include glucocorticoids, androgens, progestins and oestrogens. A recent addition, and now the most accepted among hormone therapies, is the administration of antioestrogens such as tamoxifen (Henningsen, 1980 a, b).

The mechanism of action of additive therapy is far from understood. Glucocorticoids are thought to act both directly by inhibiting mammary cell proliferation and indirectly by inhibiting the stimulatory effect of insulin (Osborne et al., 1979). Progesterone is known to be a regulator of oestrogen receptor synthesis (Hseuh et al., 1975). During the menstrual cycle, for example, the appearance of progesterone in the blood plasma is accompanied by inhibition of the synthesis of oestrogen receptor in late follicular phase (Soutter et al., 1979). This inhibition, at least in the rat, is a direct effect on the target tissue (Clark and Peck, 1979). However in some experimental model systems, progesterone may promote tumour growth. When given in conjunction with oestrogen, the progesterone-oestrogen combination seems to cause tumour regression (McCormie and Mason, 1973). One function of progesterone is to increase prolactin release and this may be the mechanism by which progesterone

on its own can stimulate growth. Androgens, when administered in high doses, deplete the oestrogen receptor from the cytoplasm into the nucleus (Garcia and Rochefort, 1979). Lippman and Huff (1976) however, by demonstrating the presence of both androgen and oestrogen receptors in MCF-7 cells, have confused the picture since it cannot be said which receptor may be involved in promoting regression.

1.2.5.1.2.1 Antioestrogens

Antioestrogens have been studied in considerable detail but their precise mode of action is not certain. It is known that they interact with the oestrogen receptor in a manner analogous to oestrogens, promoting translocation and chromatin binding (Clark, et al., 1973; Sutherland and Jordan, 1981). Antioestrogens, although having certain minimal side effects (Kiang and Kennedy, 1977), do not show the usual, initial upsurge of tumour growth associated with other additive therapies (Moseson, et al., 1978).

Evidence that antioestrogenic effects are mediated after the oestrogen receptor translocation step comes from (a) suppression of oestrogen stimulated uterine growth (Clark et al., 1973; Ferguson and Katzenellenbogen, 1977), (b) inhibition of growth and development of oestrogen dependent mammary tumours in rats (Jordan, 1975; Tsai and Katzenellenbogen, 1977), (c) inhibition of growth of some oestrogen stimulated human breast cancer cell lines (Lippman and Bolan, 1975; Zava et al., 1977), (d) stimulation of pituitary gonadotrophin output and subsequent ovulation in women by antagonism of oestrogen feedback at the level of hypothalamus and pituitary (Vaitukaitis et al., 1971) and (e) suppression of oestrogen receptor processing or recycling (Section 1.1.5.6). It should be noted that in some tissues such as the uterus, antioestrogens are not pure antagonists and possess some oestrogenicity themselves (Terenius, 1971).

The antioestrogenic property could be exerted at any point along the sequence of events which are proposed for steroid hormone action (Figure 5). The most likely sites of action involve the transformation step, DNA binding step, receptor replenishment and receptor processing (Katzenellenbogen et al. 1981). Different antioestrogens have different modes of action in kinetic terms, although they all appear to act subsequent to the translocation step. Clark et al. (1973) observed that administration of antioestrogens to rats blocked the replenishment of the cytosolic oestrogen receptor. This observation, together with the apparent competition for the oestrogen receptor were thought to be the basic mechanisms involved in antioestrogenic action. Nicholson et al. (1976) and Koseki et al. (1977) on the other hand, found that in rat mammary tumours, tamoxifen, in amounts equivalent to those known to cause regression in human cancer, did not block this replenishment. More recent reports suggest that oestrogen receptor synthesis in human mammary tumours is, indeed, suppressed by tamoxifen (Sutherland and Jordan, 1981). The complexing of oestrogen receptor with an antioestrogen is also thought to interfere with 'processing' (Mester et al., 1977).

Another mechanism of antioestrogenic effect, proposed by Jordan and Dowse (1976), is related to the hypothalamic oestrogen receptor. They suggest that antioestrogens mediate their effect by interfering with hypothalamic function, intefering with prolactin release and so rate of tumour progression (Leung and Sasaki, 1975). This hypothesis is opposed by the studies of Moseson et al. (1978) which showed that patients who had undergone previous hypophysectomy, did respond to tamoxifen.

Bouton and Raynaud (1978), have suggested that antioestrogen effects are due to their high dissociation kinetics which probably inhibit receptor from promoting its effect at the transcriptional level.

Katzenellenbogen et al. (1981) have shown that compared to 60% binding of the oestrogen-receptor complex to DNA, only 25% of the antioestrogen-receptor complex binds to DNA, indicating a difference in the capacity of oestrogen and antioestrogen to activate the oestrogen receptor (DeBoer et al., 1981; Rochefort and Borgna, 1981). Additionally, differences in sedimentation behaviour of activated receptor, when complexed to oestrogen or antioestrogen, have been observed (Katzenellenbogen et al., 1981). These studies together with those of Horwitz and McGuire (1978a) and Ruh and Baudendistal (1977) have suggested the effect of antioestrogen in antitumoural action is both on receptor replenishment and on receptor-chromatin interaction.

1.2.5.2 Chemotherapy of Human Breast Cancer

Chemotherapy of human breast cancer is associated with considerable side effects. However, this form of treatment has to be used for patients whose tumours show oestrogen receptor negative status and/or are rapidly growing. Patients who develop secondaries in lung, liver or viscera are also selected for this form of therapy. The relationship between oestrogen receptor and response to chemotherapy is uncertain. Whereas some groups have found that oestrogen receptor negative tumours respond much better to chemotherapy (Lippman et al., 1978; Jonat et al., 1980) others claim that oestrogen receptor positive tumours are better responders (Kiang et al., 1978). Further studies indicate that oestrogen receptor status is not a determinant of response to chemotherapy (Hilf et al., 1980; Samal et al., 1980; Young et al., 1980).

It is now known that oestrogen receptor negative tumours show a high thymidine labelling index (Meyer et al., 1977), suggesting the presence of a higher proportion of dividing cells. It is now established

that oestrogen receptor negative cells are often more aggressive (Byar et al., 1977). Normally premenopausal disease is especially aggressive (Bonadonna et al., 1977; Knight et al., 1977). The drugs used on aggressive disease include: 1) antimetabolites, such as methotrexate and 5-fluorouracil, which affect the synthesis of nucleic acids, 2) alkylating agents, such as cyclophosphamide, which cause cross linkage in the DNA molecule interfering with DNA replication, 3) antitumour antibiotics, such as adriamycin or bleomycin, which bind to DNA and cause breaks in the strands and interfere with the synthesis of RNA, 4) vinca alkaloids, such as vincristine or vinblastin, which are metaphase inhibitors and interfere with the mitotic spindle. Usually a combination of drugs is used such as CMF, AV or FAC (see abbreviations section, page iv) (Carbone et al., 1977; Baum, 1981) since single agent chemotherapy is not very effective (20-25% response rate). A major problem is drug resistance by cells. Therefore, some groups use alternating cycles of two combinations in an attempt to obtain maximal cell kill and delay drug resistance, while avoiding excessive damage to the marrow or bowel (Pannuti and Creaven, 1979).

There is evidence (Nenci et al., 1976) that some tumours are heterogeneous, that is contain both oestrogen receptor positive and negative cells (Nenci, 1978, 1981). This may explain why complete remission is not obtained when either endocrine or chemotherapeutic treatment is used alone. Randomized trials, combining endocrine therapy and chemotherapy have been reported. These studies have suggested an additive effect of this form of therapy. Higher regression rates, more prolonged remission and survival time are reported for premenopausal women where cytotoxic therapy is combined with oophorectomy (Ahmann et al., 1977). In postmenopausal women, polychemotherapy in conjunction with tamoxifen

administration is more effective than either modality alone (Heuson, 1976).

1.3 OBJECTIVES

Since Jensen first proposed that cytosolic oestrogen receptor could be the necessary clinical discriminant in human breast cancer in relation to hormone treatment (Jensen et al., 1971b), several groups have developed assays for measuring oestrogen receptors (McGuire et al., 1975; Laing et al., 1977). Various clinical correlations have been made with the intent of finding parameters which could be related to receptor status and possibly lead to enhanced understanding of the biology of the individual tumour. However, only about 50% of patients whose primary tumour biopsies show the presence of soluble oestrogen receptor respond objectively to hormone therapy (Hawkins et al., 1980). In addition, it is becoming increasingly clear that oestrogen receptors from human tissue may show characteristics different from those present in other animal systems (Notides et al., 1976; Fazekas and MacFarlane, 1980). The differences could arise at any of the proposed steps in steroid hormone action (Figure 5).

Outstanding areas for the study of oestrogen receptor function in human breast cancer cells include,

1) Explanation and identification of 'false positive' cases, where the presence of soluble oestrogen receptor is detected yet the patient fails to respond to subsequent hormonal treatment. In this respect, one of the molecular forms of oestrogen receptor in human breast cancer (the 8S form) has been proposed as an index of functional receptor (Wittliff and Savlov, 1975) and this has been put forward as an index of prognosis (Section 1.2.4). Other investigators have failed to define patients response on the basis of the molecular size of oestrogen receptor

(Dao and Nemato, 1980). Therefore, further investigations in this area are required. Tumour sampling and subsequent handling may affect the molecular parameters. A satisfactory tumour storage system is also required which can be used as an alternative to the liquid nitrogen method.

2) The activation/transformation process of oestrogen receptor in rat or human tissue is far from being completely understood. There is conflicting data on the mechanism of this process (Notides, 1978; Rochefort et al., 1980). Other steroid receptors show the process of activation but this is not accompanied by the 4S⟶5S transformation observed (on SDGA) in the oestrogen receptor system from immature rat uterus. The process remains ill understood. Detection of 'false positive' patients could be due to detection of non-functional receptor either as a result of a structural defect in the receptor protein or absence of total compliments and/or defective compliments, required for activation (Spelsberg and Boyd-Leinen, 1980). Other reasons are plausible. Non-functional receptors may be expressed in tumours. There is certainly preliminary evidence for defective receptors in human breast cancers (Thorsen and Stoa, 1979; Leake et al., 1981) and in a mouse mammary tumour (Shyamala, 1972). It is therefore important to describe the components involved in receptor activation and to study their kinetic properties. The presence, detection and quantitation of such components are important for studying chromatin-receptor interaction. The activation/transformation process may also have other implications in drug-receptor interactions (Katzenellenbogen et al., 1981, Rochefort and Borgna, 1981). Some of the preliminary studies are therefore directed towards relating the activation/transformation process of human breast tumour oestrogen receptor to the relatively better characterized immature rat uterine tissue oestrogen receptor.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Fine chemicals were obtained as follows:-

Dithiothreitol (DTT)	The Boehringer Corporation (London) Ltd., and Koch-Light Laboratories, Colnbrook, England.
Norit A activated charcoal (untreated powder)	Sigma, London.
Bovine Serum Albumin (BSA) (fraction V)	Sigma, London.
Human-γ-Globulins (fraction II)	Sigma, London.
Ovalbumin	Sigma, London.
Deoxyribonucleic acid (DNA) (calf thymus type V sodium salt, highly polymerized).	Sigma, London.
Deoxyribonucleic acid - cellulose (DNA-cellulose)	Sigma, London
Dextran T70	Pharmacia, Sweden.
Sucrose (AnalaR)	Fisons, England.
Diisopropyl fluorophosphate (DFP)	Sigma, London.
Phenylmethylsulphonylfluoride (PMSF)	Sigma, London.
Trasylol (Aprotinin in isotonic solution containing 0.9% benzyl alcohol)	Bayer, Germany.

Acetyl-L-leucyl-L-leucyl-L-arginal (leupeptin)	Peptide Institute, Japan and Sigma, London.
Sodium molybdate (AnalaR)	BDH chemicals Ltd., England.

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

2.1.2 Buffers

N-2-Hydroxypiperazine-N'-2-ethane Sulphonic acid (Hepes) was obtained from The Boehringer Corporation (London) Ltd.,

N-Tris [hydroxymethyl] methyl-2-aminoethane Sulphonic acid (TES) was obtained from Sigma, London.

Tris (hydroxymethyl) aminoethane was obtained from Sigma, London.

2.1.3 Hormones

2.1.3.1 Radioactive Steroids

[2,4,6,7-^{3}H]Oestradiol-17β ($^3HE_2$), specific activity range 91-112 Ci/mmol was obtained from the Radiochemical Centre, Amersham, now Amersham International. This was used in all sedimentation analysis studies (Section 2.2.2).

[6,7-^{3}H]Oestradiol-17β ($^3HE_2$), specific activity 54 Ci/mmole, was also obtained from the Radiochemical Centre, Amersham. This was used for all Scatchard analysis studies (Section 2.2.2.3).

2.1.3.2 Competitor for Receptor Analysis

Diethylstilboestrol (DES) was obtained from Sigma, London.

2.1.4 **Radioactive Sedimentation Markers**

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

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

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

2.1.5 **Scintillation Materials**

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

Toluene (AnalaR grade)

2,5-diphenyloxazole (PPO)

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

Triton X-100 was obtained from Rohm and Haas, Croydon, England.

2.1.6 **Livestock**

Mature (250-280g) and immature (16-21 days old) rats were female Albino Wistar rats (Glasgow University Colony).

2.1.7 **Human Tissue**

2.1.7.1 **Human Breast Tumour Tissue**

Human breast tumour tissue was kindly supplied by the following Health Board hospitals:-

Victoria Infirmary, Glasgow

Western Infirmary, Glasgow

Gartnavel General Hospital, Glasgow

Royal Infirmary, Glasgow

Stobhill General Hospital, Glasgow

Royal Beatson Memorial Hospital, Glasgow

Southern General Hospital, Glasgow

Hairmyres Hospital, East Kilbride.

Monklands General Hospital, Airdrie

Belvidere Hospital, Glasgow

Ballochmyle Hospital, Mauchline, Ayrshire.

2.1.7.2 **Human Endometrial Tissue**

Normal human endometrial tissue was obtained from the uteri of patients undergoing hysterectomy in the Gynaecology Department of the Western Infirmary, Glasgow.

2.1.8 **Miscellaneous**

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

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

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

Glass/glass tissue grinders were obtained from either Kontes, New Jersey, U.S.A. or Cowie Scientific, Middlesbrough, England.

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

All solutions were made in glass distilled water.

2.2 METHODS

2.2.1 Buffers and Solutions

2.2.1.1 Tissue Storage Medium

Human breast tumour tissue was collected fresh from the operating theatre. A parallel section was removed for pathological examination. The remaining tissue was put into an empty sterile container which was then transported on ice to the laboratory for routine clinical analysis of steroid receptor content (Leake et al., 1981) by the method of Scatchard analysis (Scatchard, 1949). However, from the distant hospitals it was not always possible to dispatch and process the sample on the same day. In such cases tumour tissue was stored in a medium of 0.25M Sucrose, 1.5mM $MgCl_2$, 10mM Hepes pH7.4/50% v/v glycerol (Sucrose/glycerol buffer) at -20°C until its arrival in the laboratory whereupon it was registered and stored at -20°C. The glycerol in the medium prevents the freezing of the tissue. Freezing and thawing of tissue is detrimental to receptor stability (King, 1979). This storage procedure preserves receptor for several weeks (Hyder and Leake, 1982; see also Section 3.1.1.7).

Fresh tissue was processed immediately if the biopsy was large

enough for serial sections. Otherwise, sucrose density gradient analysis was performed after the receptor status had been determined by routine clinical analysis (Leake et al., 1981). Tissues stored in sucrose/glycerol buffer for more than 60 days were discarded (except in the case of storage study - see Section 3.1.1.7). Before assay, stored tissue was rehydrated for 15 min. at $4^{o}C$ in HED or $HDK._{15}$ (see below for abbreviations), as appropriate.

2.2.1.2 **Buffers used in the Sedimentation Analysis of Oestrogen Receptor**

2.2.1.2.1 Low Salt Buffers

To study the sedimentation profile of oestrogen receptor in low salt conditions, the following homogenization buffers were used:-

10mM Hepes, 1.5mM EDTA, 0.5mM DTT pH 7.4 (HED)

10mM Tris, 1.5mM EDTA, 0.5mM DTT pH 7.4 (TED)

2.2.1.2.2 **Physiological Ionic Strength and High Salt Buffers**

Physiological ionic strength (0.15M KCl) and high salt buffers, used mainly for activation/transformation studies, were as follows:-

10mM Hepes, 0.5mM DTT, 0.15M KCl pH 7.4 ($HDK._{15}$)

10mM Hepes, 0.5mM DTT, 0.4M KCl pH 7.4 ($HDK._{4}$)

A few experiments were conducted in either 10mM or 40mM Tris (Notides, 1978) in place of Hepes - this buffer is referred to as TDK. EDTA was omitted from the activation/transformation buffers (because EDTA inhibits this process - see Sato et al., 1978a). High salt buffers were used in the gradients for the analysis of 4S⟶5S transition, but not in the initial homogenization buffer ($HDK._{15}$). The 4S⟶5S

transition was also studied in gradients containing 0.15M KCl.

A few experiments involved the use of low salt buffers without EDTA (HD or TD) for the initial homogenization followed by activation and then centrifugation in high salt gradients. Buffers are indicated in the legends to the figures.

2.2.1.2.3 **Dextran coated Charcoal Solution (DCC) for the Separation of Unbound from Bound Steroid**

0.5% w/v DCC solution was prepared by resuspending Norit A charcoal in HED containing 0.25M sucrose and 0.005% w/v dextran T-70. When required, an aliquot was centrifuged at 2000 x g for 5 min. and the supernatant removed. The pelleted DCC was then resuspended to the desired final concentration in radiolabelled cytosol from various tissues.

2.2.1.2.4 **Preparation of [^{3}H]Oestradiol-17β ($^3HE_2$) Solutions**

Stock $^3HE_2$ was prepared to a final concentration of 10^{-7}M in absolute alcohol and stored at -20°C. Appropriate amounts of this were aliquoted to give the desired final concentration when resuspended in the cytosol preparation (see below). Another aliquot was added to an equal volume of DES (10^{-4}M stock solution) in absolute alcohol. The alcohol was evaporated in a stream of compressed air or nitrogen and the $^3HE_2$ ± DES redissolved in the cytosol. Tests to ensure full solution showed that never more than 2% of radioactivity remained attached to the walls of the tubes. The DES tube thus contained an 1000-fold excess of synthetic oestrogen for the determination of non-specific binding.

2.2.2 Analysis of the Sedimentation Pattern in Low Salt Buffers

2.2.2.1 The Human Breast Tumour Cytosol Oestrogen Receptor

Sucrose density gradient analysis (SDGA) was originally described by Martin and Ames (1961) as a method for determining the molecular nature of proteins. It was first applied to the analysis of the oestrogen receptor by Toft and Gorski (1966). Lately, clinical implications of the molecular forms of the receptor have been proposed (Wittliff and Savlov, 1975; Kute et al., 1978). The results in this field are conflicting and the method described below was developed with the intention of studying some possible reasons for the contradictory results.

Either fresh or sucrose/glycerol buffer stored tumour tissue (rehydrated in HED, 15 min. at $4^{o}C$) was dissected free of fat and visible necrotic tissue. It was then homogenized at 200-500 mg/ml in HED using 2 x 10 sec. bursts from an Ultra-turrax (TP 18/2) at a setting of 150, with intermittent cooling periods. This yielded the rough homogenate which was then further refined using a glass/glass homogenizer (Leake et al., 1981). Great care was taken to maintain the cytosol below $8^{o}C$ at all times, as warming of the homogenate causes receptor loss (King, 1979). The homogenate was then centrifuged at 50,000 rev/min ($226,395 \times g_{max}$) in a Beckman 50 Ti rotor for 30 min at $4^{o}C$. The clear supernatant was then carefully removed avoiding the thin layer of fat which was usually present. This yielded a cytosol with a final protein concentration in the range 3-10mg/ml. Within this protein concentration range, no significant alteration of sedimentation profile was observed, see Figure 25. Aliquots of the cytosol were incubated with $5 \times 10^{-9}M$ $^{3}HE_{2} \pm 5 \times 10^{-6}M$ DES for 1h at $4^{o}C$. These were then transferred onto a pellet of dextran coated charcoal (DCC) and the

DCC resuspended to a final concentration of 0.5% w/v. Incubation was continued for a further 15 min. at $4^{o}C$, with mixing every 5 min. Tubes were then centrifuged at 2000 x g at $4^{o}C$ for 5 min. to pellet the DCC, and aliquots of cytosol taken for the determination of specific binding. Further 200ul aliquots were loaded onto 5ml linear 5-20% (w/w) sucrose density gradients prepared in HED buffer. Sucrose density gradients were made in cellulose nitrate tubes, using a simple gradient forming device (made in the Department workshop by Mr. N. Harvey) and chilled at $4^{o}C$ for at least two hours prior to use. Specific binding was determined prior to loading the cytosol onto the gradients to monitor percentage specific counts recovered. After layering the cytosol on top of the gradient the tubes were centrifuged at 45,000-50,000 rev/min. (243,000 x g_{max} - 300,000 x g_{max}) for 13-16 h. in a Beckman SW 50.1 rotor at $4^{o}C$ in a Beckman L2-65B ultracentrifuge. Each sample contained ^{14}C-labelled BSA and/or ^{14}C-labelled human-γ-globulins as internal markers. Sedimentation values were determined according to Martin and Ames (1961), and, in addition, the internal markers indicated the quality of each gradient.

After centrifugation the bottom of the tube was punctured with an 18 gauge syringe needle and two drop fractions were collected directly into scintillation vials (37 vials per gradient), with a controlled air flow from the top (1ml/min). This was obtained by using the Gilford gradient scanner unit (accesssory unit of the Gilford 240 spectrophotometer) linked to an air tight centrifuge tube holder. This method provided excellent resolution (see Figure 10) when compared to the Gilford gradient scanner attached to a flow cell which pumps dense sucrose from the tube bottom with collection from the top. The technical problems associated with using the Gilford flow cell system

and the mixing of the sample in the outlet tube were found to reduce the sensitivity. However, using this system the protein profiles could be obtained by ultra-violet light absorption analysis prior to the sample entering the outlet tube, confirming the validity of the gradients obtained by the two drop procedure (data not shown). Resolution becomes increasingly important in the transformation studies (Section 3.2).

The graphs are plotted showing the total and non-specific or the specific counts ($^3HE_2$-DES counts, for every fraction) as a function of the fraction number and the arrows on the graph indicate the position of the sedimentation markers. To avoid confusion, the minor differences obtained in the sedimentation values of receptor from individual tumours have, in general, been ignored. Values are generally quoted as either 4S (3.5S - 4.8S) or 8S (7.2S - 8.0S), in accordance with results presented in the literature.

The recovery of specific counts was in the range 70-114%. The relative area under the sedimentation peaks was determined. Tumours were classified as of predominant 4S type, only if >70% of total counts in the sedimentation profile was found in this area and predominantly 8S if >70% of total counts were under the 8S peak.

2.2.2.1.1 Development Leading to the Final Procedure of SDGA

Some developments carried out to reach the final procedure described above (Section 2.2.2.1) include -

(i) a reduction in time of incubation of the cytosol with $^3HE_2 \pm$ DES from 3h to 1h at 4^oC. Although a 1-3h incubation may not result in any modification of the receptor, long term incubations

certainly have an adverse effect on tumour receptor sedimentation profile (Section 3.1.1.2).

(ii) an increase in the final concentration of DCC from 0.25% to 0.5% (w/v) to strip unbound steroid. Although most unbound ^{3}H-steroid (>98%) was removed by 0.25% DCC (data not shown) some tumours contained non-specific components which masked the 4S area. The higher DCC content removed these and did not affect receptor concentration (Figure 16).

(iii) although total recovery of specific counts under the sedimentation peak was usually in the range 70-114%, for some tumours as little as 50% of the specific counts applied were recovered under the peaks. This prompted an investigation of the tube bottom to determine if any heavy aggregates had been precipitated. The tube bottom was cut and placed in 4ml absolute alcohol at 37^{o}C for 1h in a tightly capped scintillation vial, after which 1ml aliquots were counted for radioactivity. Using this procedure recovery of specific counts was $\geqslant$90% (Table 1). The presence of heavy aggregates could, therefore, result in a false estimation of the 4S : 8S ratio if only one of these forms were able to aggregate.

2.2.2.2 Intratumoural Variation of Oestrogen Receptor Concentration and Molecular Form in Human Breast Cancer

To study intratumoural variation of the oestrogen receptor two procedures were followed. Where possible, the peripheral section and adjacent sections moving towards the central area of the tumour were analyzed (selected sectioning approach) (Figure 9S). A second approach was to randomize the sections prior to analysis (random sectioning approach) (Figure 9R). The homogenization procedure, cytosol preparation and sedimentation analysis were as described in Section 2.2.2.1.

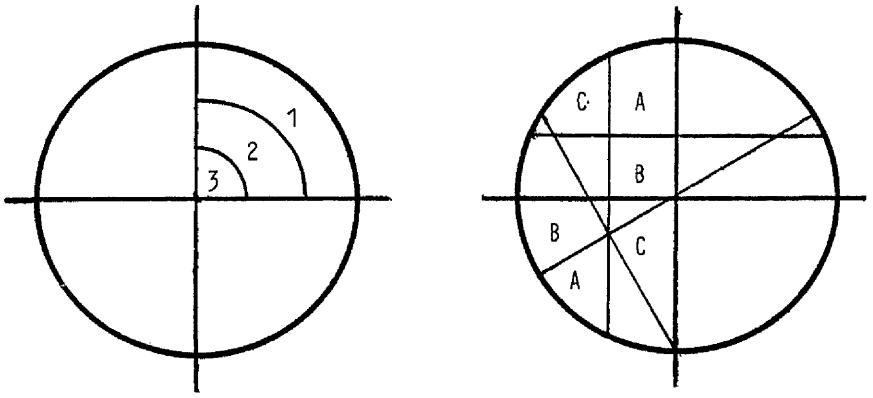


Figure 9 : Sectioning procedure for analysis of tumour intersite variation. In the "selected sectioning procedure" (S), section position was known with respect to the peripheral section. In the "random sectioning procedure" (R), position within the tumour was unknown.

2.2.2.3 **Scatchard Analysis**

Routine scatchard analysis to determine receptor status of the tumour prior to SDGA was described by Leake et al. (1981). This was carried out by Marion McMenamin, to whom I am most grateful. Briefly, a 7 point competition assay over the range 10^{-10}M to 10^{-9}M $^{3}HE_2$ using a 100 fold excess DES as competitor, was conducted. Incubation of steroid with tumour cytosol or nuclear fraction was for 18h at 4°C. For receptor positivity at least 5 of the 7 points were used in the construction of the Scatchard plot and the Kd (dissociation constant) was in the range 5×10^{-11}M - 7×10^{-10}M.

2.2.2.4 **Sedimentation Pattern of the Rat Uterine Cytosol Oestrogen Receptor**

2.2.2.4.1 **Preparation of Immature Rat Uterine Cytosol**

16-21 day old rats were anaesthetized with chloroform and killed by cervical dislocation. Uteri were dissected free of adhering

fat and mesentry, quickly excised and placed in HED buffer on ice. Four or five uteri/ml were homogenized using a glass/glass homogenizer. The centrifugation procedure for cytosol preparation and subsequent sedimentation analysis were as described in Section 2.2.2.1. The protein concentration obtained was 2-4mg/ml.

2.2.2.4.2 **Preparation of Mature Rat Uterine Cytosol**

Prior to killing, the oestrous cycle stage of the rats was determined by a microscopical examination of a vaginal smear. The uteri were excised and placed on ice. These were then finely chopped and homogenized in 2ml HED buffer/uterus. The centrifugation of the homogenate and sedimentation analysis was as described in Section 2.2.2.1. The protein concentration obtained was 4-6mg/ml.

2.2.2.5 **Preparation of Human Endometrial Cytosol Fraction**

Human endometrial tissue was homogenized in HED buffer at 200-400mg/ml in a glass/glass homogenizer. This was followed by the high speed spin (226,395 x g_{max}) cytosol preparation and sedimentation analysis as described in Section 2.2.2.1. Protein concentration was 3-6 mg/ml.

2.2.3 **Transformation Studies on Sucrose Density Gradients**

2.2.3.1 **Immature Rat Uterus**

The method used was basically derived from the studies of Notides and Nielsen (1974).

Immature rat uteri were homogenized in HD or HDK.$_{15}$ buffer at 4 or 5 uteri/ml and cytosol prepared as described in Section 2.2.2.1.

EDTA was omitted from buffers for reasons previously mentioned (Section 2.2.1.2.2). After labelling the cytosol for 1h at $4^{o}C$ with $5 \times 10^{-9}M$ $^{3}HE_{2} \pm 5 \times 10^{-6}M$ DES and DCC-stripping free steroid, one set of aliquots was kept at $4^{o}C$ and a second aliquot warmed to 30^{o} for 30' (unless otherwise stated) to permit activation. After warming, the cytosol was recooled to $4^{o}C$ for 5 min. An aliquot was taken for determination of specific radioactivity and a further aliquot loaded onto 5-20% w/w (unless otherwise stated) linear sucrose density gradients (each of 5ml) prepared in buffers containing either 0.15M or 0.4M KCl. ^{14}C-labelled markers were added. The salt concentration did not affect the precision of the sedimentation markers in their sedimentation properties (Figure 10). The centrifugation was at $4^{o}C$ for times and speeds indicated in the Figure legends. In some instances the centrifugation temperature was $20^{o}C$, without previous warming of cytosol. These will be indicated. The sample recovery procedure was as described in Section 2.2.2.1.

2.2.3.2 Human Breast and Endometrial Tissue

Human breast tumour cytosol and human endometrial cytosol (Section 2.2.2.5) were prepared as described in Section 2.2.2.1. The buffers used for homogenization were $HDK._{15}$ or HD. Inclusion of the protease inhibitor Leupeptin during the homogenization procedure resulted in the formation of froth (also observed by Sherman et al., 1980) possibly leading to a loss of receptor activity. For this reason, the protease inhibitors Leupeptin and/or DFP were added immediately following homogenization. PMSF and Trasylol, used in the studies reported in Section 3.1 were present during the homogenization procedure. However, as already noted (Lukola and Punnonen, 1982), the latter do not seem to be very effective inhibitors of the particular protease(s) activity

encountered here.

The tumours used were generally those for which a high concentration of receptor activity had been detected using Scatchard analysis (Section 2.2.2.3). No difference was observed in specific counts obtained after 30 or 60 min. incubation with radioactive steroid at $4^{o}C$. This probably indicates that all the unoccupied sites are rapidly filled (in some experiments involving the effect of DCC, the concentration of radioactive steroid was reduced to $2 \times 10^{-9}M$ $^{3}HE_{2}$ $\pm$ $2 \times 10^{-6}M$ DES to prevent the excess smear of counts at the top of the gradient). Cytosol was then DCC-stripped (0.25% w/v final concentration) and aliquoted onto 5-20% (w/w) gradients. A few experiments involved initial heating of cytosol ($30^{o}C$, 30 min) prior to centrifugation for activation purposes. The gradients were centrifuged at 42,000-50,000 rev/min ($211,000 \times g_{max}$ - $300,000 \times g_{max}$) for 7-11h at $20^{o}C$ or 11-16h at $4^{o}C$ in a Beckman SW 50.1 rotor. The procedure of collection and scintillation counting was the same as described in Section 2.2.2.1.

2.2.4 The Assessment of Activation of Soluble Oestrogen Receptor from Immature Rat Uteri and Human Breast Carcinoma using DNA-Cellulose Binding

A modified method from Park and Wittliff, (1977) and Sato et al., (1981a) was developed and is described below.

2.2.4.1 DNA-Cellulose Binding of Rat Uterine ER_{c}

Immature rat uterine cytosol was prepared in $HDK._{15}$ buffer

as described in Section 2.2.3.1. Labelling of the cytosol receptor was carried out at 4°C for 60 min. with 5 x 10^{-9}M $^{3}HE_2$ ± 5 x 10^{-6}M DES. Labelled cytosol was then warmed at 30°C for 30 min. to activate the receptor. A second aliquot was kept at 4°C as a control. After warming, the cytosol was cooled at 4°C for 5 min. Then 200ul aliquots were mixed with pelleted DNA-cellulose containing ~100ug DNA (see 2.2.4.1.1). Pretreatment of immature rat uterine cytosol with DCC (0.25% w/v final concentration), either before or after activation, did not result in a significant increase of the final specific counts obtained and, therefore, stripping of unbound counts, prior to mixing of the cytosol with DNA-cellulose was not normally carried out. Specific counts in the cytosol prior to DNA-cellulose binding, however, were determined for every sample preparation from a DCC-stripped aliquot. Initial experiments confirmed that no significant retention of non-specific binding to the DNA-cellulose slurry occurred.

2.2.4.1.1 Preparation of DNA-Cellulose and the Binding Assay

The DNA-cellulose powder, 4.1mg DNA/gm DNA-cellulose was suspended in $HDK._{15}$ and the slurry centrifuged at 2000 x g for 5 min. The supernatant was removed and 200ul aliquots of the labelled cytosol, either activated (30°, 30') or non-activated (4°, 30'), were added. DNA-cellulose, now containing ~100ug DNA, was resuspended in the cytosol and incubated for 60 min. at 4°C with vortexing every 15 min. At the end of the incubation, 0.8ml $HDK._{15}$ was added, the tubes vortexed and centrifuged at 2000 x g for 5 min. at 4°C. The supernatant was removed (wash 1) and the DNA-cellulose was washed a further four times (washes 2-5) each with 1ml $HDK._{15}$. At the end of wash 5, 1ml of $HDK._6$ was added (Park and Wittliff, 1977) to salt extract the DNA bound receptor. This incubation was carried out for 60 min. at 4°C

(Sato et al., 1981a) with mixing every 15 min. When characterization of the DNA-bound receptor by SDGA was required, the volume of $HDK._6$ extraction buffer was reduced to 0.5ml in order to concentrate receptor for analysis. In addition, it was essential to introduce a carrier protein, described by Yamamoto and Alberts (1974) in both the $HDK._6$ extraction buffer and in the SDGA buffer. For SDGA, therefore, salt extraction buffer contained protein at 4mg/ml. After the salt extraction, tubes containing DNA-cellulose were again centrifuged and aliquots from the supernatant removed for scintillation counting or SDGA. The remaining supernatant was discarded and the DNA-cellulose resuspended in 1ml absolute alcohol and left overnight at room temperature ($\sim$14h). Next morning, an aliquot was removed for scintillation counting after pelleting of DNA-cellulose at 2000 x g for 5 min.

The results are expressed as percentage (%) of total specific receptor binding, that is

$$\text{DNA bound receptor} = \frac{\text{amount of } ER_c \text{ bound to DNA-cellulose}}{\text{amount of } ER_c \text{ incubated with DNA-cellulose}} \times 100$$

The amount of receptor bound to DNA-cellulose was taken as the sum of salt extracted + alcohol extracted specific counts.

From the total DNA-cellulose bound receptor, % salt extractable and hence % alcohol extractable counts were determined.

2.2.4.2 **DNA-cellulose Binding of Human Breast Carcinoma ER_c**

The cytosol preparation is described in Section 2.2.3.2. The DNA-cellulose binding procedure, as described for immature rat uterine ER_c, was conducted with two modifications. After labelling the cytosol

for 60 min with $^{3}HE_2$ ± DES, stripping of free $^{3}HE_2$ with 0.25% DCC (final concentration w/v) was included so that the exchange of endogenously bound steroid is minimized during the elevated temperature used for receptor activation. It is not clear whether Sato et al. (1981a) took such a precaution. An error can be introduced into the results if specific receptor concentration is determined at 4°C prior to activation and % DNA bound is expressed relative to this value. The concentration of DCC in the experiments reported in this thesis in connection with activation studies was kept at 0.25% (w/v) since it is known that, at elevated ionic strength, DCC tends to both adsorb receptor itself and increase the stripping of bound $^{3}HE_2$ (Peck and Clark, 1977).

2.2.5 DNA - Oestrogen Receptor Interaction Analysis on Sucrose Density Gradients (Low Salt Conditions)

A similar approach to that proposed by Park and Wittliff (1980) was employed, with a few modifications. Tumour tissue was homogenized and the cytosol prepared as described in Section 2.2.2.1. Cytosol was then incubated with 5×10^{-9}M $^{3}HE_2$ ± 5×10^{-6}M DES. After a 1h incubation at 4°C, calf thymus DNA in HED buffer was added to one set of cytosols, to a final concentration of 1mg/ml, while keeping the other set DNA free. The cytosol was further incubated at 4°C for 20 min. after which the unbound counts were stripped with 0.5% DCC w/v (final concentration) as described in Section 2.2.1.2.3. Aliquots (200ul) were loaded onto 5-20% linear sucrose density gradients prepared in HED. ^{14}C-labelled marker proteins were used as internal standards and the samples centrifuged at the speed and time indicated in the legends, in a Beckman SW 50.1 rotor at 4°C.

2.2.6 Expression of Results

2.2.6.1 Radioactive Counting

All vials from cytosol assays were counted in a Searle Mk.II liquid scintillation analyzer. Dual labelling was employed in sucrose density gradients and consequently the samples were counted in a dual label programme. The counting efficiency of ~26% for ^{3}H was determined by 'spiking' with tritiated toluene. The quenching values were monitored from the external standard pulse and there was no significant variation. The spill over from ^{14}C channel into ^{3}H channel was estimated to be 20% and this value was therefore subtracted from the ^{3}H channel values to give the corrected values which was then plotted as a function of fraction number.

2.2.6.1.1 Calculation of Receptor Concentration

After the determination of specific counts from the DCC aliquots prior to SDGA, the following relationship was used to convert the c.p.m. value into specific receptor concentration in fmoles/ml.

$$\frac{\text{Specific c.p.m.}}{\text{ml}} \div \text{counter efficiency} = \frac{\text{Specific d.p.m.}}{\text{ml}}$$

$$\text{then, } \frac{\text{Specific d.p.m.}}{\text{ml}} \times \frac{1}{\text{Specific Activity of } {}^{3}HE_2} \times 2.2 \times 10^{6} = \frac{\text{Specific nmoles}}{\text{ml}}$$

$$\text{and } \frac{\text{Specific nmoles}}{\text{ml}} \times 10^{6} = \frac{\text{Specific fmoles}}{\text{ml}}$$

The value thus obtained was used to monitor the percentage recovery on SDGA. The 4S and 8S profiles were quantitated by estimating the area under their respective peaks. In some cases the percentage recovery on SDGA was corrected by considering the specific counts at the bottom of the tube. Thus when the 4S + 8S profile was used to compute the 4S to 8S ratio, the total percentage recovery

of specific counts may exceed that under the 4S + 8S profile.

2.2.7 Protein Determination

The protein content of each cytosol was determined by the method of Lowry et al. (1951) using BSA as the standard.

2.2.8 DNA Determination

The DNA determination was made by the modification of the method of Burton (1956) described by Katzenellenbogen and Leake (1974).

2.2.9 Final Form of Results

Cytosol receptor concentration were determined in fmoles/ml (Section 2.2.6.1.1). These values were then divided by the cytosol protein concentration to yield the value in fmoles/mg protein or divided by the value of DNA to yield receptor concentration in fmoles/mg DNA.

2.2.10 Statistical Parameters

Statistical parameters of mean ($\bar{x}$) and standard deviation (S.D.) were computed using a CASIO fx-19 scientific calculator. Where duplicate or triplicate samples were used, the specific receptor concentration is derived from subtraction of the mean of non-specific counts from the mean of total bound counts. Where the mean and standard deviations were calculated from separate experiments, the range is included.

3. RESULTS

3.1 SUCROSE DENSITY GRADIENT ANALYSIS OF HUMAN BREAST TUMOUR OESTROGEN RECEPTOR

3.1.1 Conditions for Sucrose Density Gradient Analysis (SDGA)

3.1.1.1 Separation of ^{14}C-labelled Marker Proteins

Prior to the use of the sucrose density gradients, it was essential to establish the quality of both the gradients and the separation of proteins. Markers used for this purpose were ^{14}C-labelled such that they could subsequently be used as internal markers. Figure 10 shows that there is a very clear separation of 4.6S and 7.1S markers and these sediment in approximately a linear fashion. The top of the gradient may experience some disturbance during sample loading and this explains why perhaps the line joining the sedimentation points occasionally does not pass through the origin. The relationship of marker proteins, shown in Figure 10, is obtained in both Hepes and Tris buffer and linearity of the markers is not affected by the presence of salt.

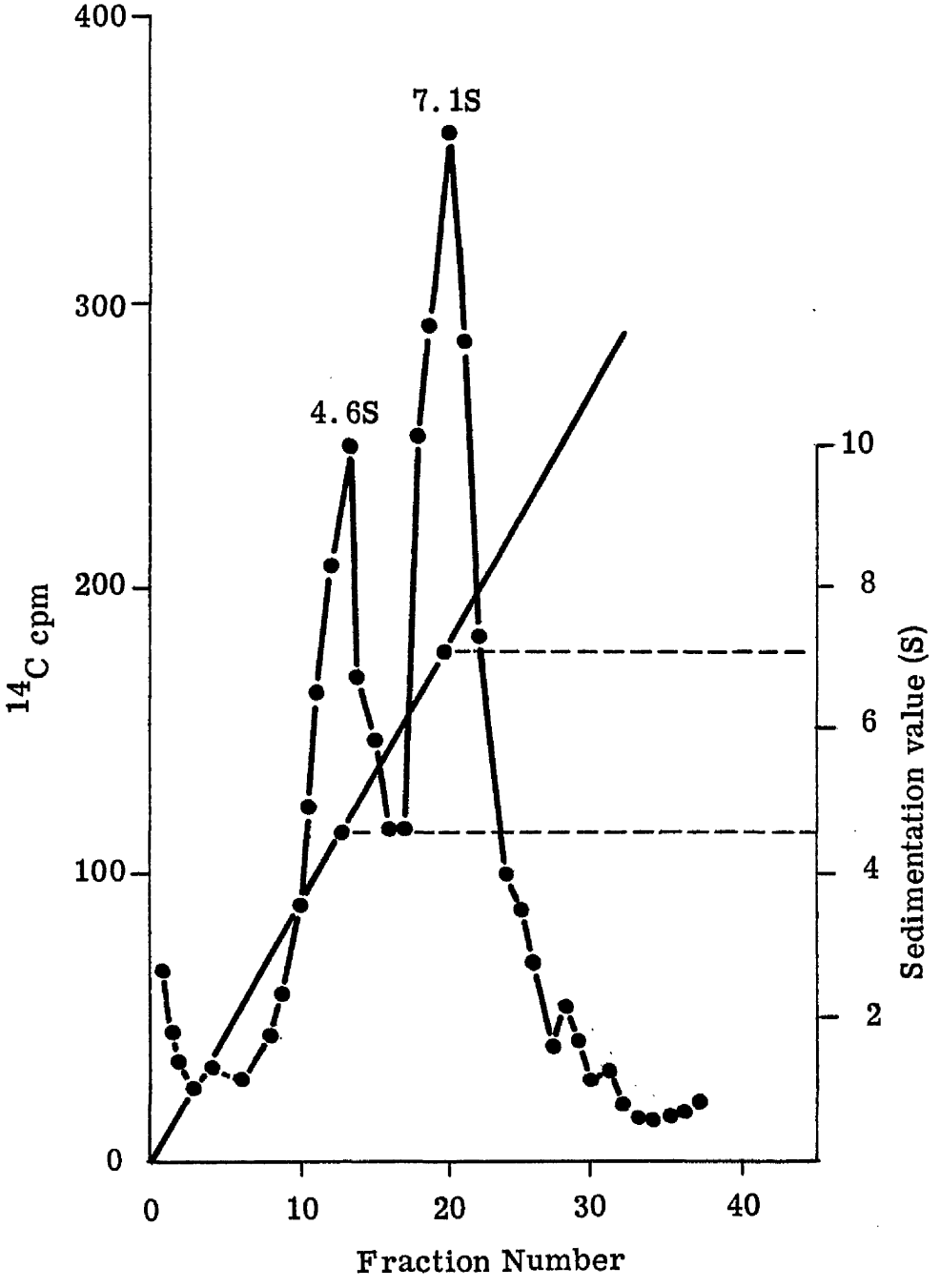
3.1.1.2 Effect of Time of Incubation with ^{3}H-oestradiol on Sedimentation Profile

Two different types of effects of pre-incubation on oestrogen receptor profile, as a function of time are reported in the literature. Erdos (1968) and Stancel et al. (1973a) reported a time dependent aggregation of receptor while Freedman and Hawkins (1980) have reported a time dependent interconversion of 8S into 4S species. Two different sources of cytosol preparation were involved in the above studies.

The effect of time on the stability of soluble oestrogen receptor was studied with human breast tumour cytosol both in the presence and

Figure 10. **Separation of ^{14}C-labelled marker proteins on sucrose density gradient**

^{14}C-labelled BSA (4.6S) and human-γ-globulin (7.1S) (Section 2.1.4) were mixed with 200ul aliquot of cytosol prepared from human breast tumour (patient SN, protein concentration 9.8mg/ml). Cytosol was then layered on to of a 5-20% (w/w) sucrose density gradient prepared in HE and centrifuged at 45,000 rev/min for 14h at $4^{o}C$. The rest of the procedure is described in Section 2.2.2.1.


400
300
200
100
0
7.1S
4.6S
10
8
6
4
2
^{14}C cpm
Sedimentation value (S)
0
10
20
30
40
Fraction Number

absence of oestradiol. Figure 11 demonstrates a predominant 8S profile from a tumour cytosol. Incubation for between 45-150 min. in the presence or absence of steroid resulted in no change in the profile. There was a slight but not significant increase in the receptor binding capacity after 150 min. (412 fmoles/mg protein) compared to the 45 min. incubation (373 fmoles/mg protein) in the presence of steroid. Pre-incubation of receptor in the absence of steroid for 105 min. followed by labelling with ^{3}H-oestradiol for 45 min. resulted in a comparable receptor content (388 fmoles/mg protein). It may however be quite fortuitous that the receptor profile did not change since tumour protease content is very variable and this tumour may represent the type in which such a protease(s) is at minimal level.

The receptor status of the tumour shown in Figure 12.1 was 4S + 8S type when tumour cytosol was incubated with ^{3}H-oestradiol for 1h (A). Incubation of the same cytosol with ^{3}H-oestradiol for 24h resulted in a loss of the 8S component with a concomitant increase in the 4S area (B). The receptor concentration in both these cases was comparable being 70 and 68 fmoles/mg protein respectively, as determined with a DCC one point assay (Section 2.2.2.1). However, when incubation of the same cytosol was carried out for 23h in the absence of steroid followed by incubation with ^{3}H-steroid for 1h, very little receptor activity was detectable (12 fmoles/mg protein). This confirms the view that receptor is less stable in the absence of added steroid.

Cytosol from a more receptor rich tumour than that shown in Figure 12.1 was taken through the same procedure of 1h or 24h incubation with ^{3}H-steroid, or 23h incubation in the absence of steroid followed by 1h incubation with ^{3}H-steroid, prior to stripping with DCC and analysis

Figure 11. **Influence of short-term incubation (up to 150 min), in the presence or absence of steroid, on the sedimentation profile of oestrogen receptor from human breast cancer**

Human breast tumour cytosol was prepared as described in Section 2.2.2.1 (Tumour SC; protein concentration 2.3mg/ml). One set of cytosol was then labelled for 45 min. with 5 x 10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5 x 10^{-6}M DES (A) at 4^{o}C. Similarly, a second set of the same cytosol was labelled for 150 min (B). A third set of cytosol was kept unlabelled for 105 min. and then labelled for 45 min. in the absence or presence of DES (C). Unbound steroid was removed by DCC treatment and an aliquot taken for determining specific receptor concentration prior to SDGA. Centrifugation was at 45,000 rev/min for 14h in a Beckman SW 50.1 rotor at 4^{o}C. Specific binding in SDG was 86% in A, 89% in B and 94% in C. Arrow indicates the sedimentation position of ^{14}C labelled 7.1S marker protein.

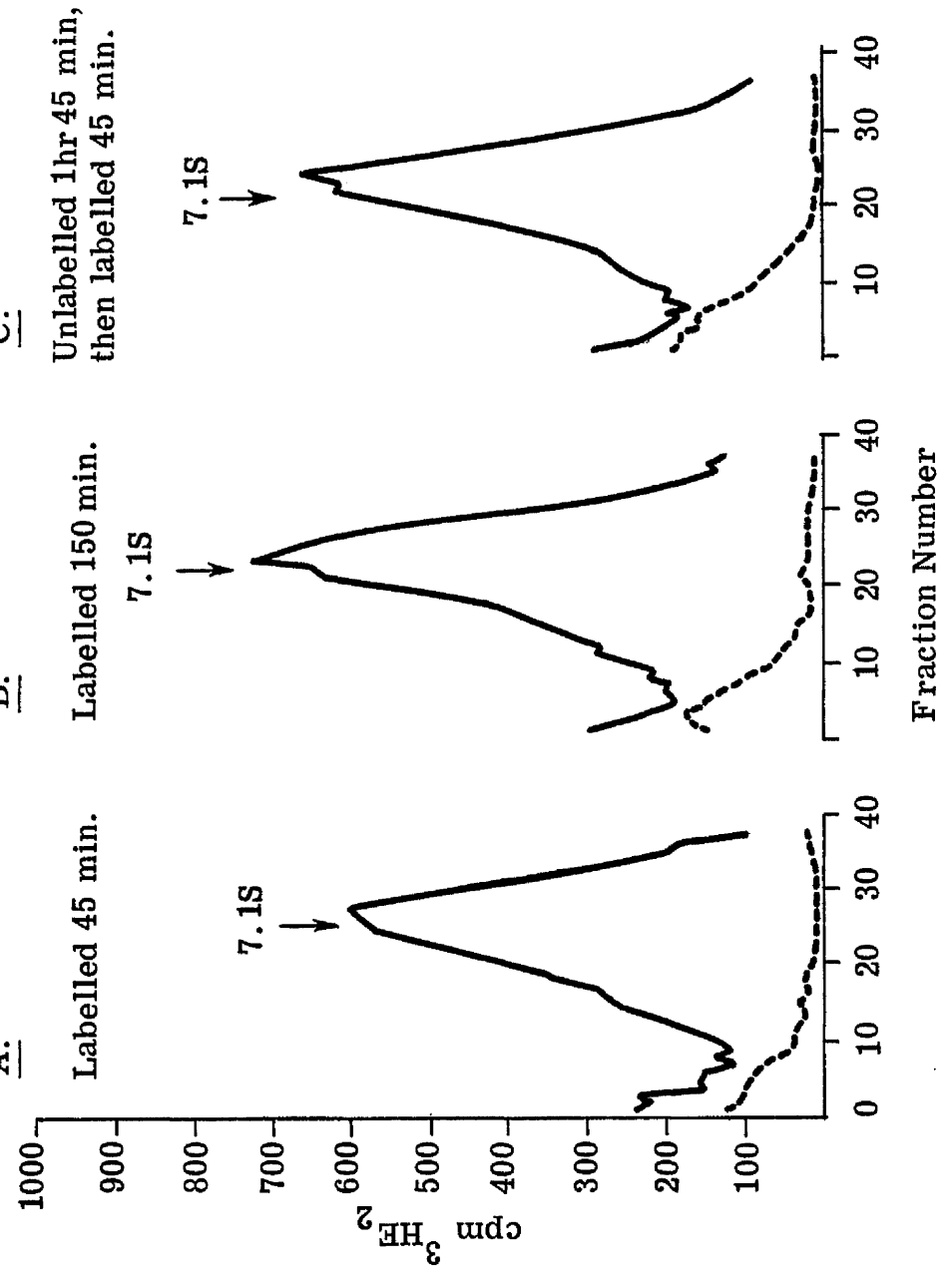

A.
Labelled 45 min.
7.1S
B.
Labelled 150 min.
7.1S
C.
Unlabelled 1hr 45 min,
then labelled 45 min.
7.1S
cpm $^3HE_2$
1000
900
800
700
600
500
400
300
200
100
0
10
20
30
40
Fraction Number

Figure 12.1. **Influence of long-term incubation (24h), in the presence or absence of steroid, on the sedimentation profile of oestrogen receptor from human breast cancer**

Cytosol was prepared as described in Section 2.2.2.1 (Tumour CM; Protein concentration 6.2mg/ml). One set of cytosol was then labelled for 1h at $4^{o}C$ with 5 x $10^{-9}M$ $^{3}HE_{2}$ in the absence (——) or presence (....) of 5 x $10^{-6}M$ DES (A). Similarly a second set of the same cytosol was labelled for 24h at $4^{o}C$ (B). A further set of the same cytosol was kept unlabelled for 23h at $4^{o}C$ and then labelled for 1h at $4^{o}C$ (C). Unbound steroid was removed prior to SDGA and an aliquot taken for determination of specific bound counts. Centrifugation was at 45,000 rev/min for 14h at $4^{o}C$ in Beckman SW 50.1 rotor. Specific binding (fmoles/mg protein) was 70 in A, 68 in B and 12 in C. Recovery of specific counts on the gradient was near 80% in A and B. Arrows indicate the sedimentation position of ^{14}C-labelled marker proteins.

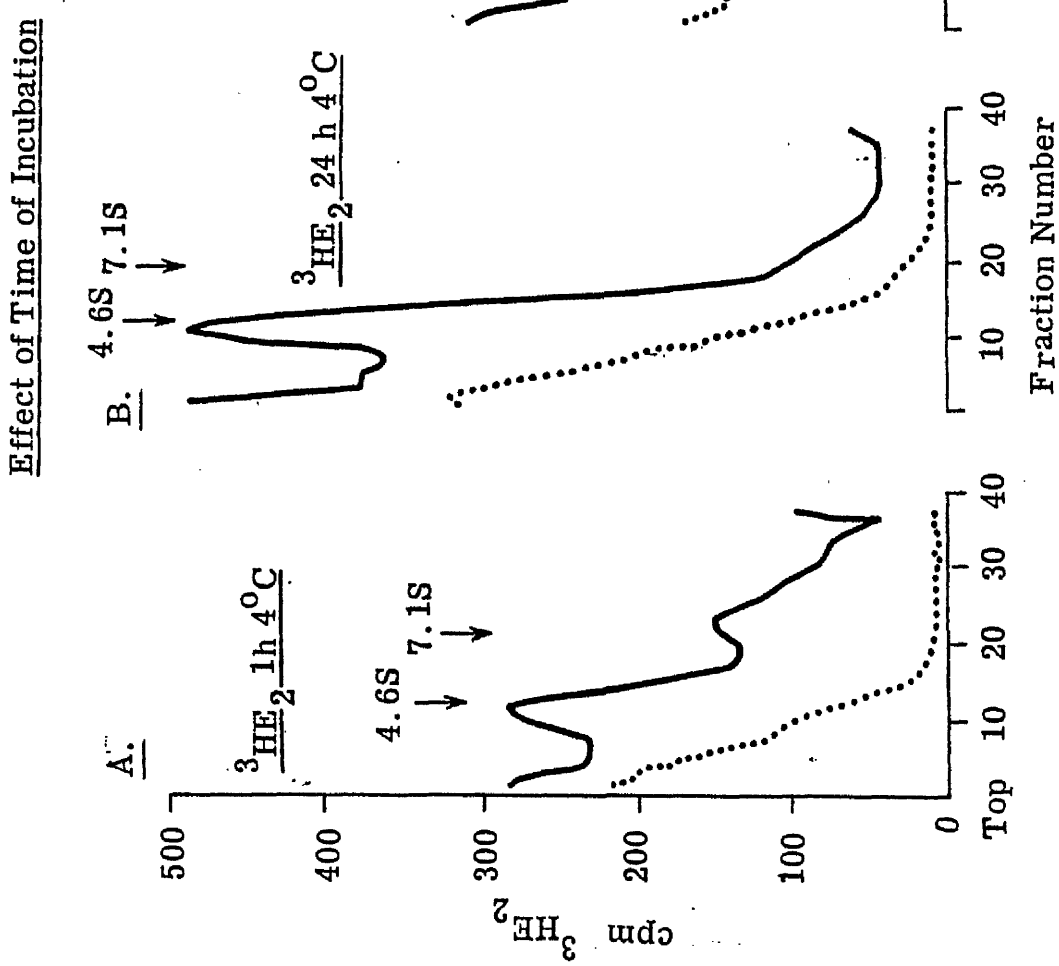
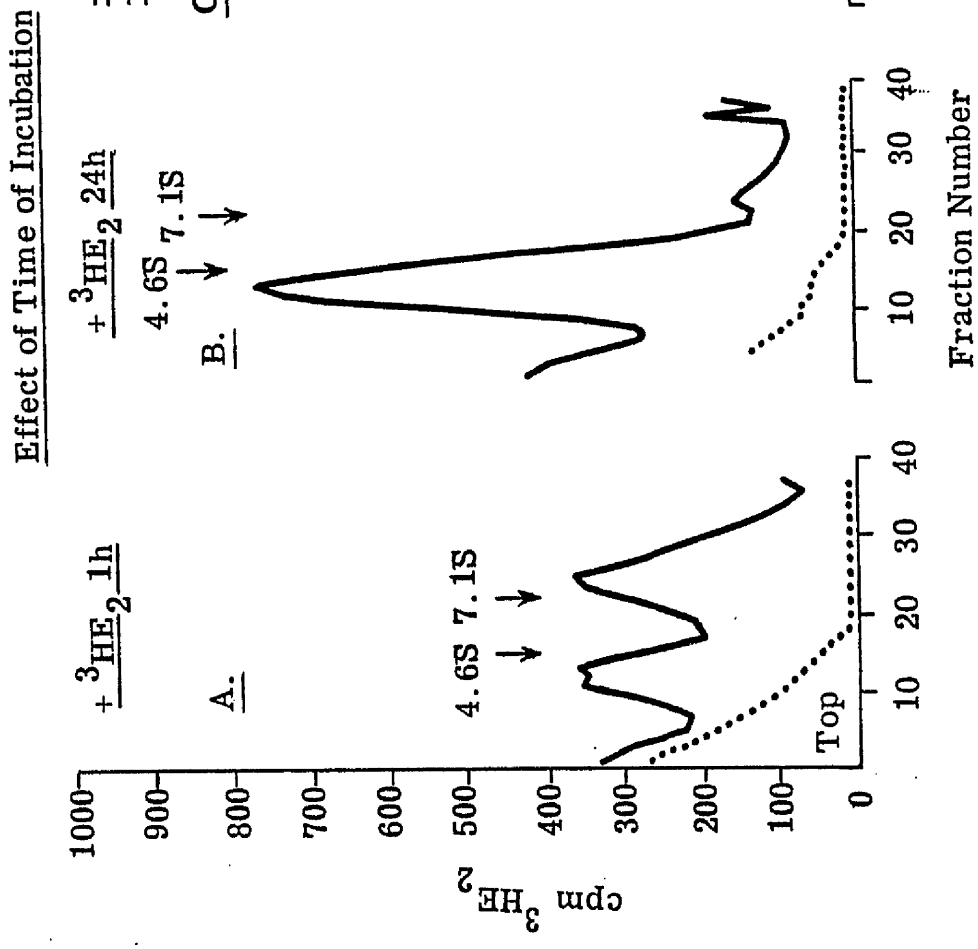

Effect of Time of Incubation
A.
$^3HE_2$ 1h 4°C
4.6S
7.1S
B.
4.6S
7.1S
$^3HE_2$ 24 h 4°C
C.
No steroid 23 h 4°C
then $^3HE_2$ 1 h 4°C
4.6S
7.1S
cpm $^3HE_2$
500
400
300
200
100
0
Top
10
20
30
40
Fraction Number

Figure 12.2. **Evidence for inter-tumoural variation in the effect of long term incubation (24h) on the sedimentation profile of ER_c**

Experiment was conducted as described in the legend to Figure 12.1. Breast tumour cytosol was prepared from tumour of patient F.R. (Protein concentration 6.2mg/ml). Specific binding in fmoles/mg protein was 116 in A, 134 in B and 89 in C. Percentage recovery on SDG was 84% in A and B and 78% in C.


Effect of Time of Incubation
+ $^3HE_2$ 1h
A.
4.6S 7.1S
+ $^3HE_2$ 24h
B.
4.6S 7.1S
- $^3HE_2$ 23h then,
+ $^3HE_2$ 1h
C.
4.6S 7.1S
cpm $^3HE_2$
1000
900
800
700
600
500
400
300
200
100
0
Top
10 20 30 40
10 20 30 40
10 20 30 40
Fraction Number

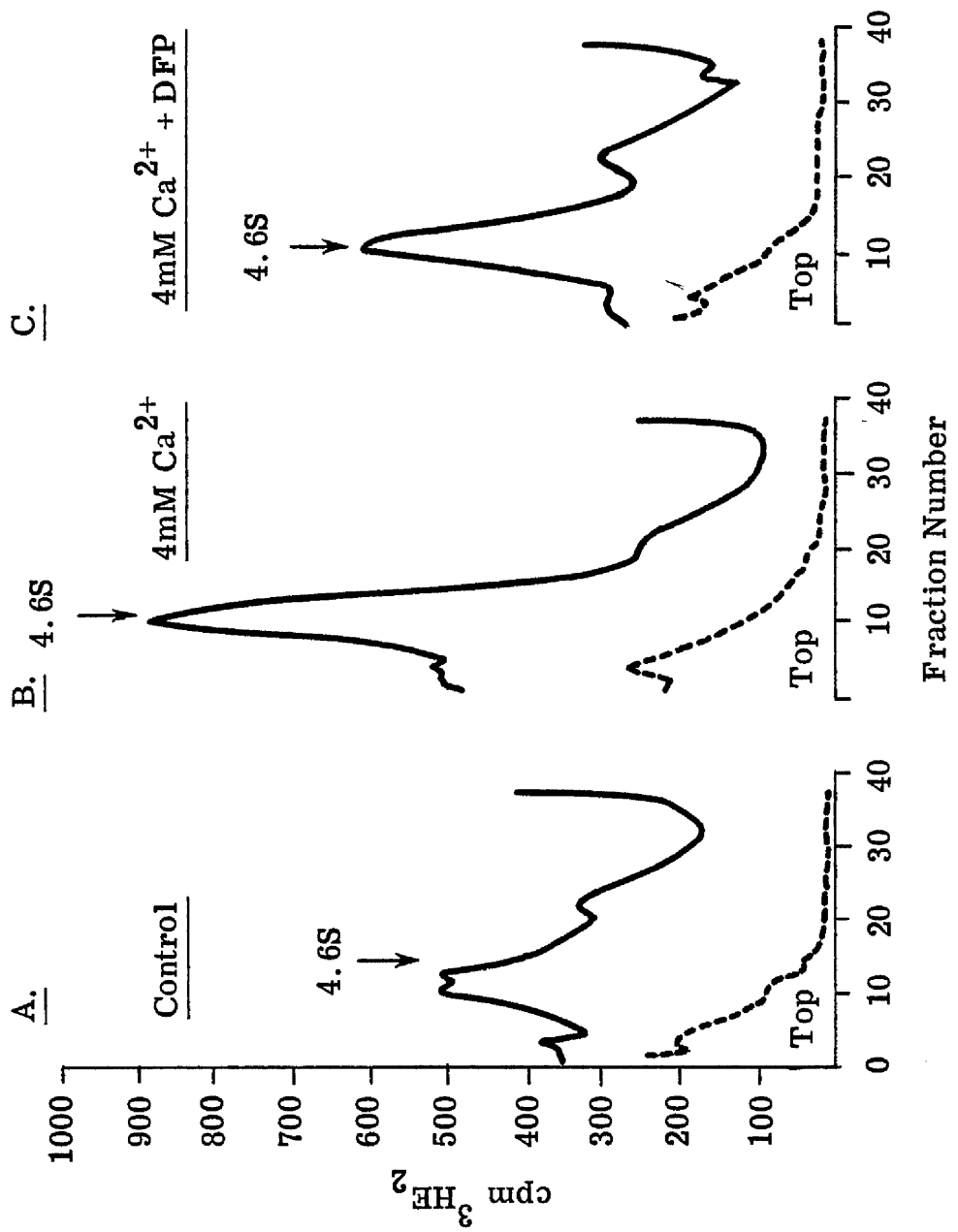
on sucrose density gradient. A similar 4S + 8S profile is again seen (Figure 12.2A) after a 1h incubation (receptor concentration, 116 fmoles/mg protein). Again a 24h incubation resulted in a decrease in the 8S peak with a concomitant increase in the 4S peak area (Figure 12.2B). There was also an increase in the receptor concentration to 134 fmoles/mg protein, this most probably represents exchange of endogenously occupied receptor and may or may not represent the reported increase in binding which can occur after mild trypsinization (Petterson et al., 1982). The 23h incubation without steroid followed by labelling for 1h (Figure 12.2C) resulted in a similar relative increase in the 4S area but the peak height and receptor concentration was now low (89 fmoles/mg protein). The two peaks observed in the 4S region in Figure 12.2A are also present in Figure 12.2C with now a higher proportion of the smaller peak ($\sim$ 3.6S). This splitting of the 4S peak is probably masked in Figure 12.2B due to the larger concentration of total receptor present. Such a splitting has been previously observed (Kute et al., 1978). It is probably not a simple aggregation effect since receptor sedimenting in the 4S area (in low salt, as opposed to salt disaggregated 4S) is also inefficient in binding to DNA (see Section 3.2.2), an indication of lack of aggregation potential.

In Figure 12.2C, as compared to Figure 12.1C, the receptor is not totally absent and this most probably represents a lower proteolytic activity in the tumour cytosol reported in Figure 12.2. Unfilled receptor is, thus, thought to be more labile than filled receptor.

The results shown in Figure 13 support the view that 8S$\longrightarrow$4S conversion is a proteolytic step, since such a conversion can be accelerated by adding calcium ions (Figure 13B compared to Figure 13A control). This confirms the studies of Schneider and Dao (1977). The tumour cytosol

Figure 13. **Influence of calcium ions ± DFP on the sedimentation profile of oestrogen receptor from human breast cancer**

Human breast tumour cytosol was prepared as described in Section 2.2.2.1 (Patient MA; Protein concentration 7.2mg/ml). Three sets of cytosol were incubated for 50 min. with 5×10^{-9}M $^{3}HE_{2}$ at 4°C in the absence (——) or presence (....) of 5×10^{-6}M DES. One set was then made 10mM with respect to DFP (C). Incubation was continued for 10 min. at 4°C. One set of cytosol was then designated control (A) and calcium was added to a final concentration of 4mM in DFP free (B) or DFP containing cytosol (C). Incubation was continued for a further 30 min. at 4°C. Unbound steroid was removed by DCC treatment prior to SDGA. Centrifugation was at 45,000 rev/min for 14h at 4°C in Beckman SW 50.1 rotor. Specific binding (fmoles/mg protein) was 176 in A, 167 in B and 153 in C. Percentage recovery was ~70% in all cases. In addition the analysis of the bottom of the tube accounted for 24% specific counts in (A), 11% in (B) and 23% in (C).


A.
Control
4.6S
B.
4.6S
4mM Ca^{2+}
C.
4.6S
4mM Ca^{2+} + DFP
Top
Top
Top
0
10
20
30
40
100
200
300
400
500
600
700
800
900
1000
cpm $^3HE_2$
Fraction Number

represented in Figure 13 showed a high amount of heavy aggregates sedimenting to the bottom of the tube, a situation also observed by Kon et al. (1980). When DFP was added, prior to the addition of calcium ions, the 8S peak was stabilized (Figure 13C) and the split present in the 4S area in Figure 13A was also absent. The total receptor concentration in the presence of DFP was actually lower than in its absence, an effect well recognized with the inclusion of this protease inhibitor (Lukola et al., 1980). This most probably is due to the isopropanol present in the DFP solution. However, a direct effect of DFP on receptor steroid binding site cannot be ignored (Lukola and Punnonen, 1982). The same experiment, when repeated with immature rat uterine soluble oestrogen receptor (ER_c), resulted in the slow cleavage of 8S $\longrightarrow$ 4S which could not be inhibited with DFP (data not shown). Rochefort and Baulieu (1971) have reported observing the same effect and species dependent sensitivity of the protease is implied.

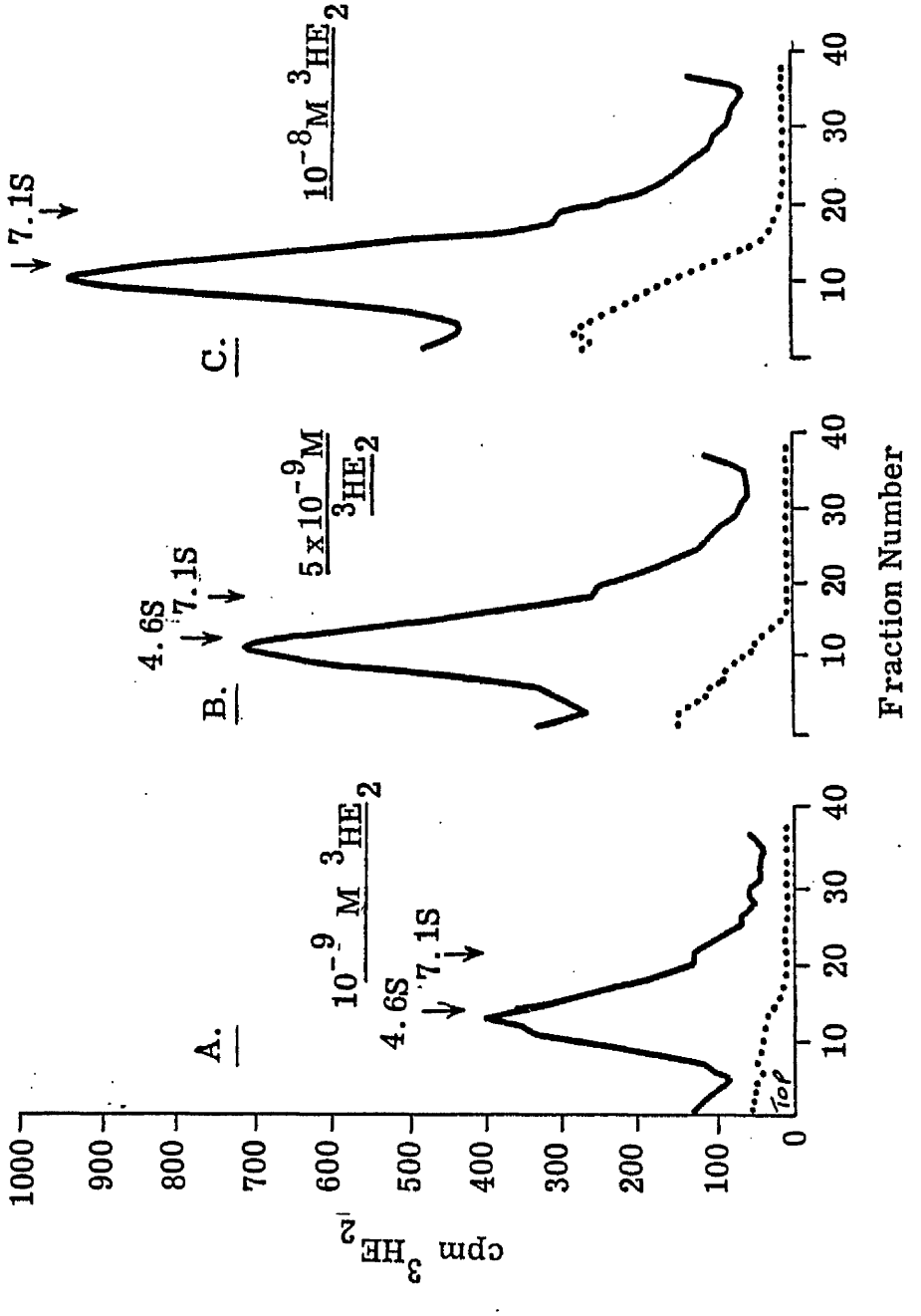
3.1.1.3 Effect of Hormone Concentration

The tumour cytosol study generally involved an incubation time of 1h at $4^{o}C$ with 5 x 10^{-9}M $^{3}HE_2$ + 5 x 10^{-6}M DES. It was therefore important to establish that most unoccupied sites were labelled. Such conditions are found by several authors to be satisfactory (Freedman and Hawkins, 1980) since only a maximum of 30% of the soluble oestrogen receptor sites may be occupied with endogenous oestradiol in breast tumour cytosols (Sakai and Saez, 1976). However with respect to the present studies it becomes important to establish if the different molecular forms had different affinities for the radioactive steroids. Figure 14 represents the effect of incubating the tumour cytosol at three concentrations of steroid in the range 10^{-8} - 10^{-9} M. The receptor concentrations measured

Figure 14. **Effect of increasing concentration of radio-active ligand on the sedimentation profile of oestrogen receptor from human breast cancer**

Human breast tumour cytosol was prepared as described in Section 2.2.2.1 (patient MG; protein concentration 9.4mg/ml). Cytosol was then labelled with $^{3}HE_{2}$ at $4^{o}C$ in the absence (——) or presence (....) of 1000-fold excess DES. The $^{3}HE_{2}$ concentration in the labelling mixture was $10^{-9}M$ in A, $5 \times 10^{-9}M$ in B and $10^{-8}M$ in C. Unbound steroid was removed with DCC prior to SDGA. Centrifugation was for 14h at 45,000 rev/min. in Beckman SW 50.1 rotor. Receptor concentration (fmoles/mg protein) was 44 in (A), 86 in (B) and 94 in (C). Percentage recovery from the gradient was between 80-100%. Arrows indicate the position of the ^{14}C-labelled marker protein.

Effect of Increasing Concentration of Ligand



by the single saturation dose analysis (counts prior to loading cytosol for SDGA) were 44, 86 and 94 fmoles/mg protein at the concentration 10^{-9}M, 5 x 10^{-9}M and 10^{-8}M $^3HE_2$ respectively. This indicates that almost twice the number of sites are detected with 5 x 10^{-9}M $^3HE_2$ when compared to 10^{-9}M $^3HE_2$. The rise in receptor concentration when incubated with 10^{-8}M $^3HE_2$ was only $\sim$ 10% higher when compared to the value obtained with 5 x 10^{-9}M $^3HE_2$ (Figure 14B). This may indicate the presence of some additional unoccupied sites or the exchange of endogenously bound steroid.

The tumour cytosol profile shown in Figure 14 was of the predominant 4S type (>70% specific counts in 4S area). The presence of a large excess of steroid (10^{-8}M) did not result in any binding in the 8S area when compared to the 10^{-9}M $^3HE_2$ incubation. It should be pointed out that in all cases of tumour cytosol containing the 4S receptor form, there was always an indication of an 8S shoulder. Wittliff et al. (1976), have reported that no difference in the binding affinity of 4S and 8S receptor forms can be demonstrated. Muldoon (1978), studying the receptor forms in the mouse mammary gland has indicated a difference in terms of the actual stability of the 8S and 4S forms, 8S being the more stable form. Some recent reports however, have indicated that the in vitro 8S form seems to be degraded to the 4S form which demonstrates greater stability to storage conditions (Namkung et al., 1979). In vitro artifacts could play a major role in the latter observation.

In addition to the above, the result presented in Figure 14 shows the reproducibility obtained with the same homogenate with respect to the receptor molecular form.

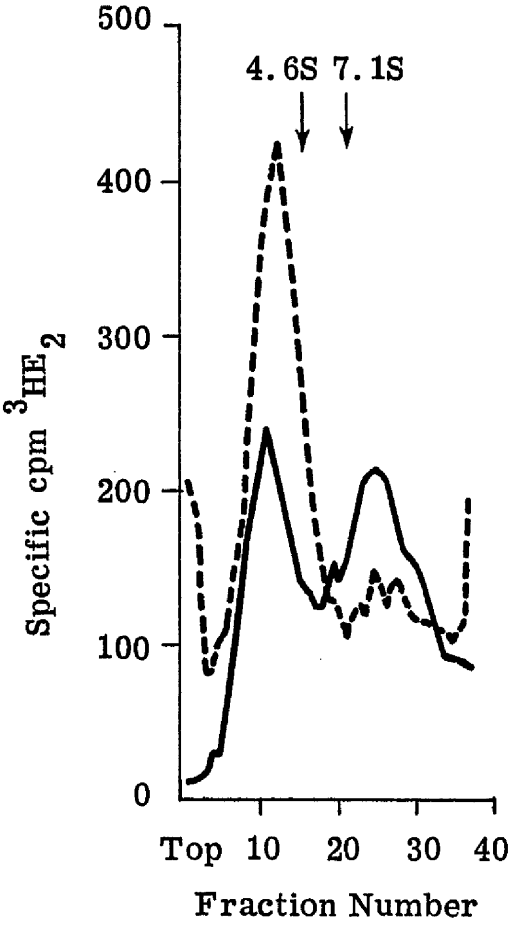
3.1.1.4 **Effect of Temperature**

A rise in temperature promotes receptor aggregation in the immature rat uterine cytosol (data not shown). Aggregation has been correlated with loss of receptor function in aged animals (Nishizawa et al., 1981). However, it should be noted that at least limited aggregation may be physiologically important as suggested from the fluorescence studies of Nenci et al. (1980c).

In human breast tumour cytosol, two different types of effects were observed on warming the cytosol to 20°C in the presence of steroid: (1) aggregation of the 8S form and (2) interconversion of the 8S into the 4S form. Both effects are seen in Figure 15. The receptor concentration increased from 90 to 134 fmoles/mg protein on heating the cytosol, as determined by the DCC analysis prior to SDGA, but after the heating process only 61% of total specific counts were recovered in the gradient. The 4°C incubation showed a 95% recovery on the gradient signifying aggregation in the heated cytosol. Heating the tumour cytosol to 20°C also resulted in a diffuse 8S area and an increase in the 4S peak height, when compared to the 4°C control, once again the 4S peak remained sharp. The possibility exists that the 4S receptor detected after the heating process resulted from endogenous exchange during the warming of the cytosol. Further tumour cytosols were therefore analyzed and it was found that even with very low changes in receptor concentration after warming and equal recovery of counts on the gradients, the 8S profile is again lost, with a rise in the 4S peak (data not shown). Sharpness in the 4S peak was obtained once again confirming the view that 4S receptor does not randomly aggregate (see also Section 3.2.3.2.6).

Figure 15. **Effect of temperature on sedimentation profile of oestrogen receptor from human breast cancer**

Human breast tumour cytosol was prepared as described in Section 2.2.2.1 (patient CB; protein concentration 5.6mg/ml). Cytosol was labelled for 1h with 5 x 10^{-9}M $^{3}HE_{2}$ in the absence or presence of 5 x 10^{-6}M DES either at 4°C (___) or at 20°C (....). Unbound steroid was stripped with DCC prior to SDGA. Specific binding (fmoles/mg protein) was 90 in 4°C control with 75% recovery on gradient and 134 at 20°C incubation with 61% recovery in gradient. In addition, specific counts determined at the bottom of the tube accounted for a further 10% receptor in 4°C control and 20% for 20°C incubation run. Centrifugation was at 45,000 rev/min for 16h at 4°C in Beckman SW 50.1 rotor. Arrows indicate the position of ^{14}C-labelled marker proteins.


500
400
300
200
100
0
4.6S 7.1S
Specific cpm $^3HE_2$
Top 10 20 30 40
Fraction Number

3.1.1.5 Effect of Dextran Coated Charcoal (DCC) in Separating Unbound from Bound ^{3}H-oestradiol-17β

Since the initial use of DCC by Korenman and Dukes (1970), it has become the most popular method for the separation of unbound from bound radioactive steroids. However certain limitations of the process are noted (Peck and Clark, 1977) and sensitivity to both ionic strength and the protein concentration are recognized. In the present study 0.5% w/v (final concentration) of DCC was used and it is demonstrated in Figure 16 that such DCC concentration does not lead to an underestimation of receptor concentration. In some initial experiments 0.25% w/v DCC (final concentration) was used but 0.5% w/v DCC was found to reduce the non-specific counts to a minimum. However, even when using 0.5% DCC, a variable non-specific proportion was observed (Figure 17A) in 10 receptor positive tumour cytosols. The variability of non-specific counts perhaps indicates that different quantities of serum components are present in different tumour cytosols. With immature rat uterine cytosol comparatively lower values of non-specific counts were seen. Human tumours always contain substantial amounts of plasma contamination (Maass et al., 1975). The components contributing towards the non-specific counts include albumin, α_1-acidic glycoprotein, corticosteroid binding globulin (CBG) and sex hormone binding globulin (SHBG) (King and Mainwaring, 1974). It is further shown in Figure 17B that such non-specific binding appears independent of protein concentration.

3.1.1.6 Inclusion of Centrifuge Tube Bottom Alcohol Extractable Counts in the Analysis of the Final Percentage Recovery

The results obtained from sections taken from 13 tumours, showing both 4S + 8S and 8S forms of soluble oestrogen receptor (ER_c), indicate

Figure 16. **Effect of Increasing DCC Concentration on the Removal of Unbound Radioactive Steroid from Tumour Cytosol**

Human breast tumour cytosol was prepared as described in Section 2.2.2.1 (patient MW; protein concentration 3.9mg/ml). Cytosol was labelled with 5 x 10^{-9}M $^{3}HE_2$ in the absence (———) or presence of 5 x 10^{-6}M DES (....) for 1h at 4°C. The incubated cytosol was then transferred onto pelleted charcoal which was resuspended to yield a final concentration (w/v) of 0.125% in A, 0.25% in B and 0.5% in C. The suspension was incubated for 15 min. at 4°C with mixing every 5 min. After the incubation, the DCC was pelleted at 2000 x g for 5 min. at 4°C. 200ul aliquots were then loaded onto 5-20% gradients which were centrifuged for 14h at 45,000 rev/min in a SW 50.1 rotor at 4°C. Aliquots were also taken for determining specific receptor concentrations in fmoles/mg protein and there were 168 in A; 166 in B and 172 in C. Percentage recovery in gradients was near 70% in all cases.

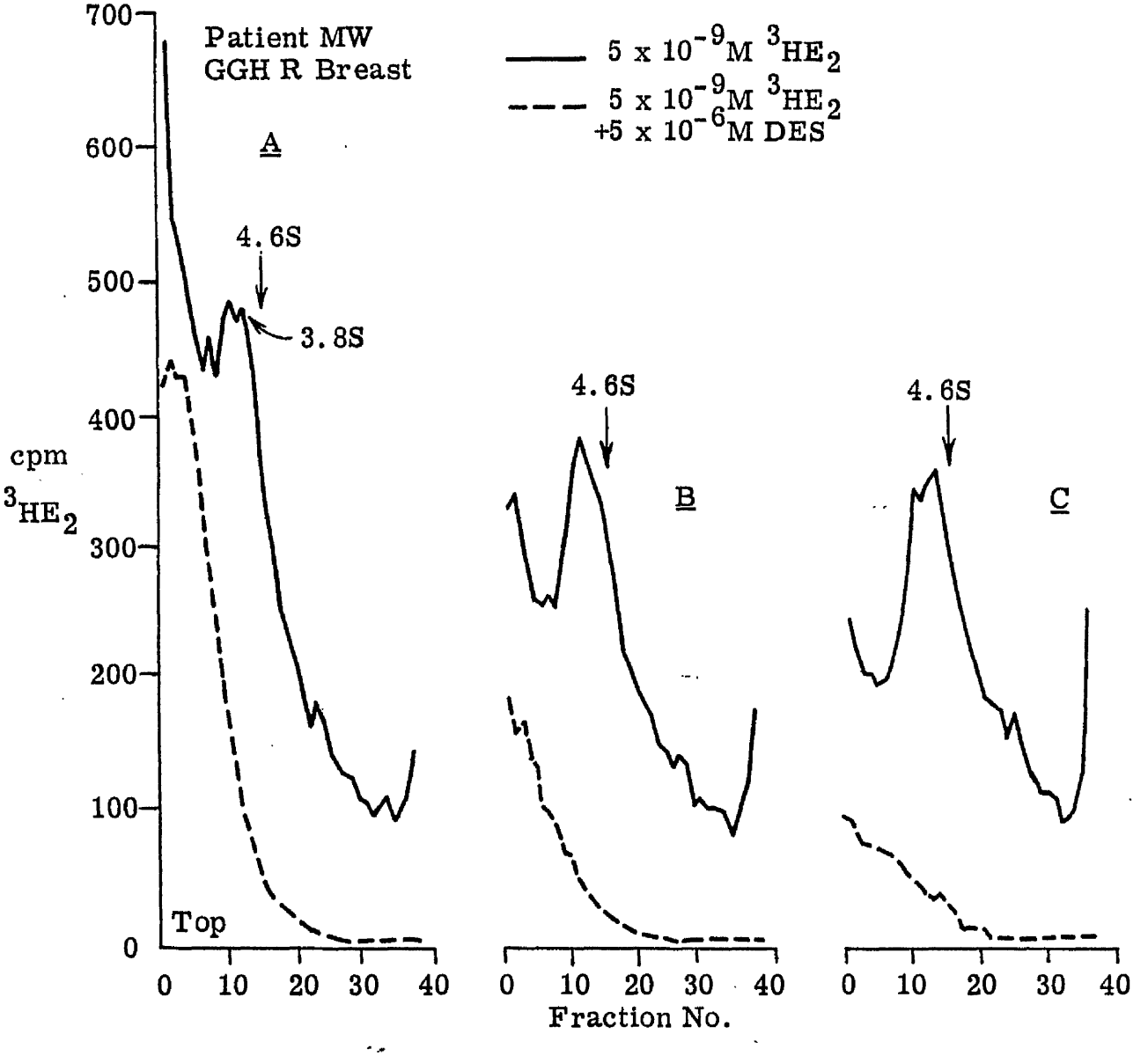
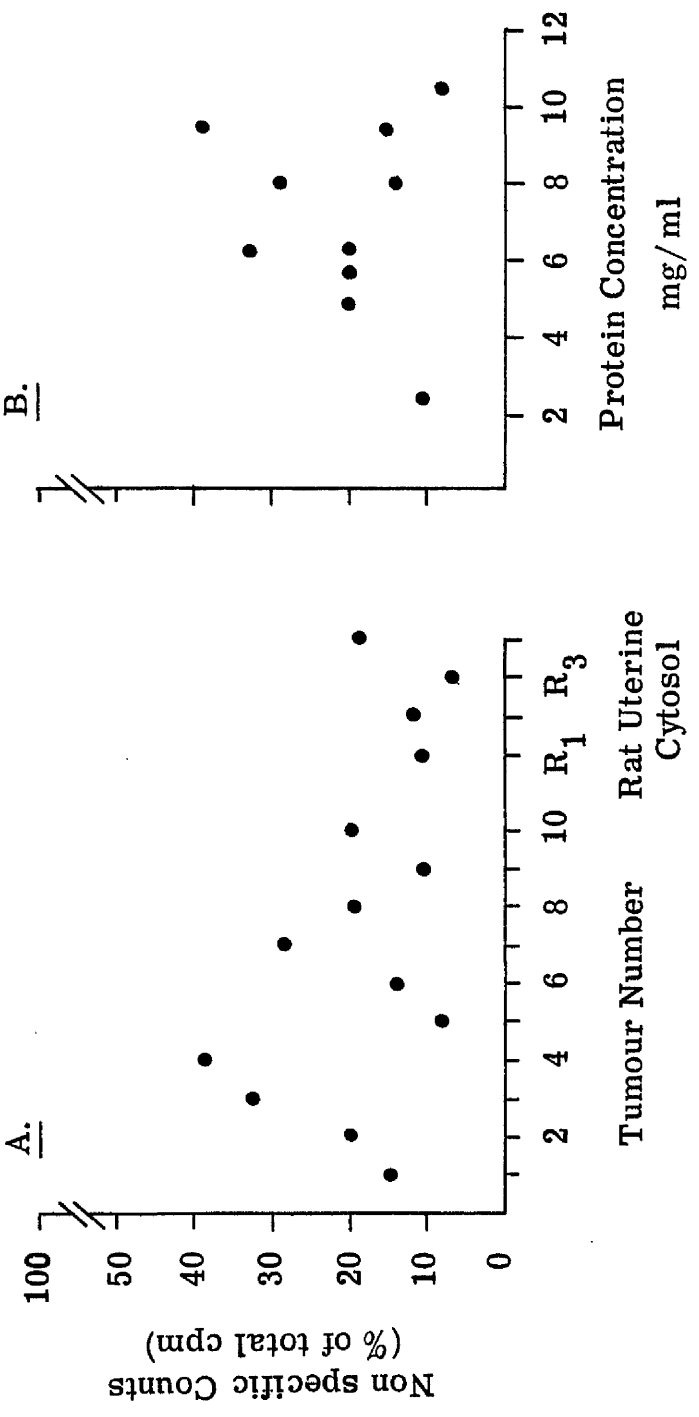

Patient MW
GGH R Breast
5 x 10⁻⁹M ³HE₂
5 x 10⁻⁹M ³HE₂
+5 x 10⁻⁶M DES
A
B
C
4.6S
3.8S
4.6S
4.6S
cpm
³HE₂
700
600
500
400
300
200
100
0
Top
0 10 20 30 40
0 10 20 30 40
0 10 20 30 40
Fraction No.

Figure 17. **Variability in non-specific Counts Obtained after Removal of Unbound Steroid with DCC**

Human breast tumour and immature rat uterine cytosol were prepared, labelled and treated with DCC as described in Section 2.2.2.1 and 2.2.2.4.1 respectively. The non-specific counts (those not competed out by DES) were plotted (as a % of total counts) against either sample number (A) or protein concentration (B). Protein concentrations shown in B correspond to the tumours listed in (A).


A.
B.
Non specific Counts
(% of total cpm)
100
50
40
30
20
10
0
2
4
6
8
10
R_1
R_3
Tumour Number
Rat Uterine
Cytosol
2
4
6
8
10
12
Protein Concentration
mg/ml

that there is a good correlation between counts loaded onto the gradient and counts recovered after the centrifugation, (mean ± S.D = 82 ± 11 - Table 1A). However some other tumour cytosols showed very low receptor recovery on the gradient (Table 1B). This prompted the investigation of analysis of the bottom of the tube for the presence of heavy aggregates. This led to a better yield as shown in Table 1A and 1B. Some tumour cytosols showed greater than 100% recovery of counts, presumably resulting from the combination of errors introduced in taking aliquots for DCC analysis, prior to SDGA, and taking a second aliquot for loading onto the gradients. Occasionally some ^{3}H-steroid dissociated towards the top of the gradient during centrifugation due perhaps to proteolysis of receptor and/or altered affinity of the receptor for ^{3}H-steroid.

Since no loss of total receptor is observed in calcium promoted proteolysis (Figure 13) and the process results in a very sharp 4S peak, limited proteolysis may release the receptor from the heavy aggregates and increase percentage recovery on the gradient (data not shown). This, however, results in the loss of the 8S form also. The idea of limited tryptic digestion has also led to increased resolution of the receptor on isoelectric focussing gels (Wrange et al., 1978).

3.1.1.7 **Storage Studies in Sucrose/Glycerol Buffer**

In contrast to the usually accepted tumour storage procedure which involves freezing in liquid nitrogen, followed by tumour pulverization (Brown et al., 1977) a different storage procedure was used during the course of the reported studies. Leake et al. (1979) reported that sucrose/glycerol medium (Materials and Methods, Section 2.2.1.1) is suitable for preserving the oestrogen receptor activity for at least 1 week and possibly longer, as measured by Scatchard analysis. However

Table 1. **Recovery of Aggregated Receptor in Relation to Total Percentage Recovery of Specifically Bound Oestradiol**

Details of breast tumour analysis and extraction of counts sedimenting to the bottom of tube are given in Section 2.2.2.1 and 2.2.2.1.1.

	Patient	SDG Value fmoles/mg Protein (% of DCC value)	Value corrected after tube Bottom Analysis	Protein Concentration mg/ml
A.				
	1. FR	84	88	6.2
	2. CMH	79	95	6.2
	3. JW	91	94	3.6
	4. FM	81	92	6.4
	FM	81	89	5.4
	FM	95	109	4.2
	5. MM	80	102	4.2
	6. CB	70	77	5.0
	CB	75	85	5.6
	7. SCR	86	91	2.8
	8. MA	78	90	7.2
	9. IH	80	95	8.0
	10. GS	91	95	8.0
	11. HB	99	104	10.4
	HB	87	95	9.2
	12. JA	82	103	6.0
	13. SR	86	97	9.2
	Mean ± S.D.	82 ± 11	94 ± 7	6.3 ± 2.1
B.	14. SCH	48	89	4.9
	SCH	68	84	5.4
	SCH	75	95	6.2
	SCH	56	71	4.2
	SCH	56	85	3.0
	Mean + S.D.	61 ± 10	85 ± 10	4.7 ± 1.1

it was essential to establish whether the molecular form of oestrogen receptor was also preserved.

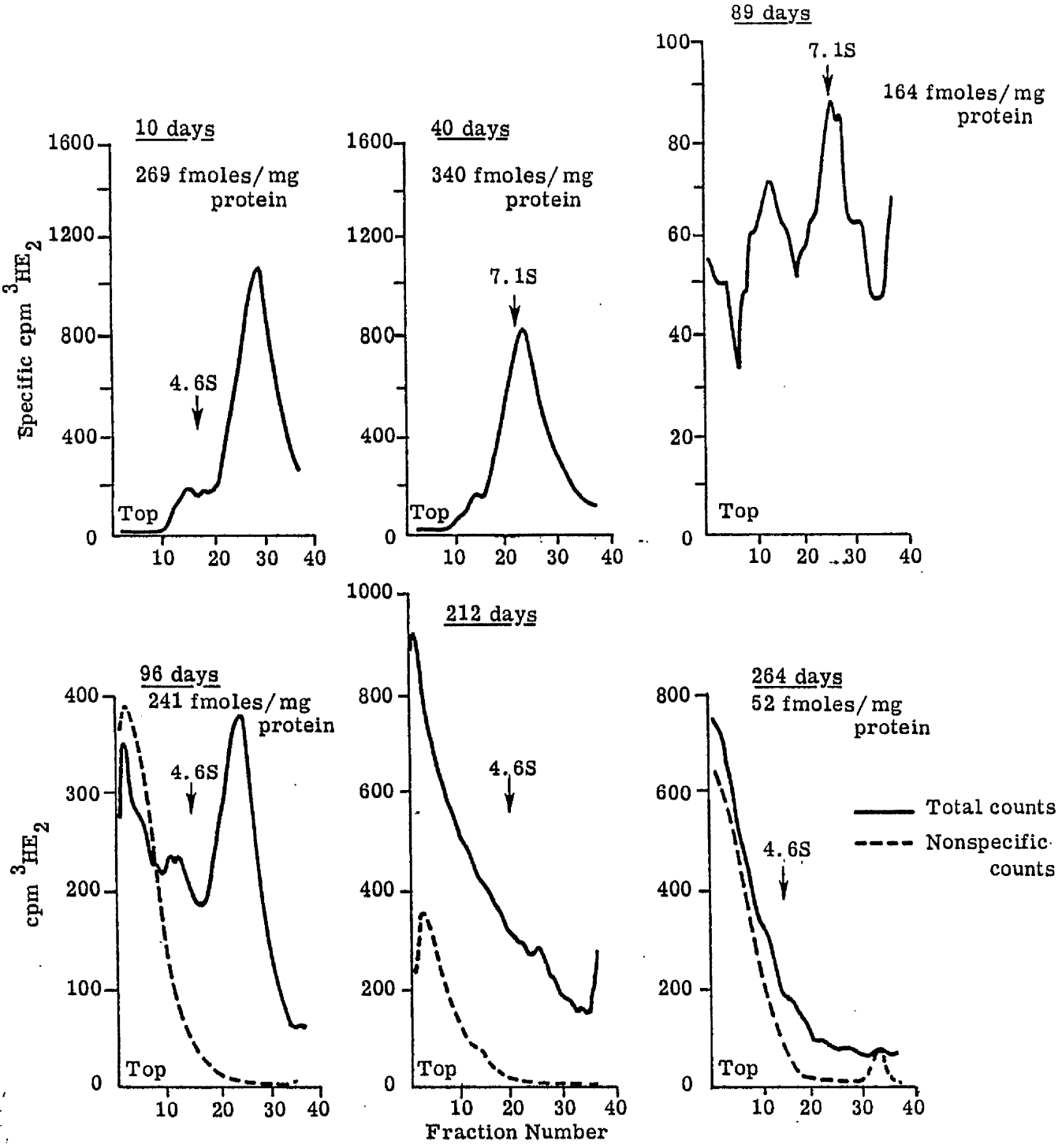
At the time the reported studies were commenced, there was already evidence in the literature that storage can have a profound effect on tumour receptor activity (Wittliff and Savlov, 1975), and recent work has further shown that the 8S form of receptor is more prone than 4S to such degradation during 3 weeks of storage in liquid nitrogen (Namkung et al., 1979). Based on such results the conclusion was drawn that the time period in storage can affect receptor molecular form (Freedman and Hawkins, 1980). It therefore became essential to investigate the sucrose/glycerol medium for storage effects on receptor molecular form and concentration as a function of time.

A large left axillary node from a breast cancer patient was divided into several pieces and stored at -20^{o}C in sucrose/glycerol medium. Sections were removed at intervals, rehydrated at 4^{o}C in the homogenization buffer and analysed by SDGA. Up to 96 days the receptor molecular form and concentration is preserved (Figure 18). After 96 days, although the binding capacity could be detected by the initial DCC assay, no molecular form was detected on the gradient (e.g. Day 264 in Figure 18). Total recovery from the later gradients was below 100% suggesting that either the receptor has lost its high affinity binding property and steroid was therefore dissociating during the centrifugation run or that the receptor is present in the aggregated form after long periods of storage. To test the extent of aggregation the remaining sample was analyzed on the 517th day of storage in both low salt and high salt gradients. Figure 19 shows that in contrast to the low salt gradient, the 0.4M KCl containing gradient showed a 4S binding peak. Thus age-related aggregation was a contributory factor. There was,

Figure 18. **Effect of Storage in Sucrose/Glycerol Buffer at -20°C on the Molecular Form of Oestrogen Receptor from Human Breast Cancer**

On arrival in the laboratory the axillary tumour mass (patient BM) was sectioned and placed in sucrose/glycerol buffer at -20°C. Individual sections were then analyzed on the indicated day of storage. Preparation and SDGA of tumour cytosol was as described in Section 2.2.2.1. Receptor concentrations as determined by DCC prior to SDGA are also included. Recovery of specific counts was near 100% up to 96 days and 90% for days 212 and 264. Receptor concentration in fmoles/mg protein for the analysis of 212th day could not be determined due to loss of cytosol.

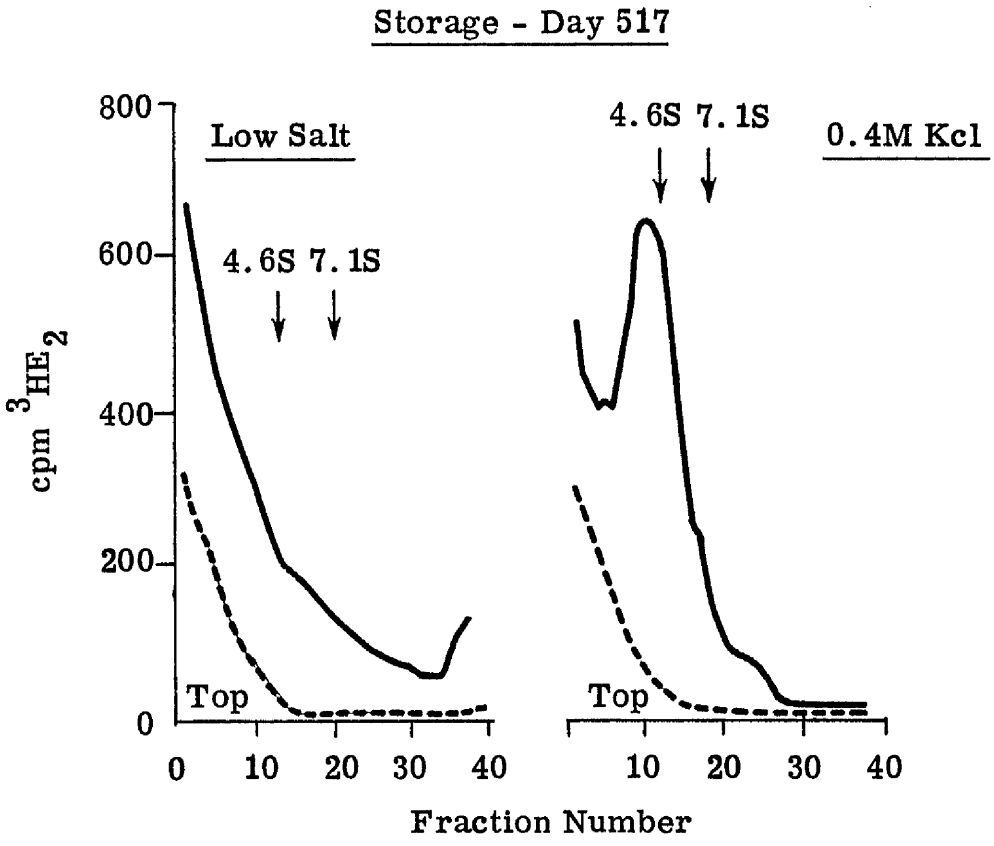
Effect of storage in sucrose/glycerol buffer at -20°C on the molecular form of oestrogen receptor (L Axilla)



The 8S form is preserved for 96 days

Figure 19. **Analysis of Cytosol Oestrogen Receptor from Human Breast Tumour Tissue after long-term Storage in Sucrose/glycerol Buffer at -20°C**

The same tumour shown in Figure 18 was used in this analysis. After 517 days of storage, cytosol was prepared as described in Section 2.2.2.1 (protein concentration 8.6mg/ml). Cytosol was labelled with 5 x 10^{-9}M $^{3}HE_2$ in the absence (———) or presence (....) of 5 x 10^{-6}M DES for 1h at 4°C. Free steroid was removed with DCC and 200ul aliquots loaded onto either a low salt gradient (A) or a high salt gradient (B). The total specific count recovery (no peak was seen) in low salt gradient was 73%, whereas the recovery of specific counts under the sedimentation peak in the high salt gradient was 91%. The receptor concentration as determined by DCC analysis prior to SDGA was 82fmoles/mg protein. Centrifugation was for 14h at 45,000rev/min at 4°C in Beckman SW 50.1 rotor.


Storage - Day 517
Low Salt
4.6S 7.1S
0.4M Kcl
4.6S 7.1S
Top
Top
800
600
400
200
0
cpm $^3HE_2$
0
10
20
30
40
10
20
30
40
Fraction Number

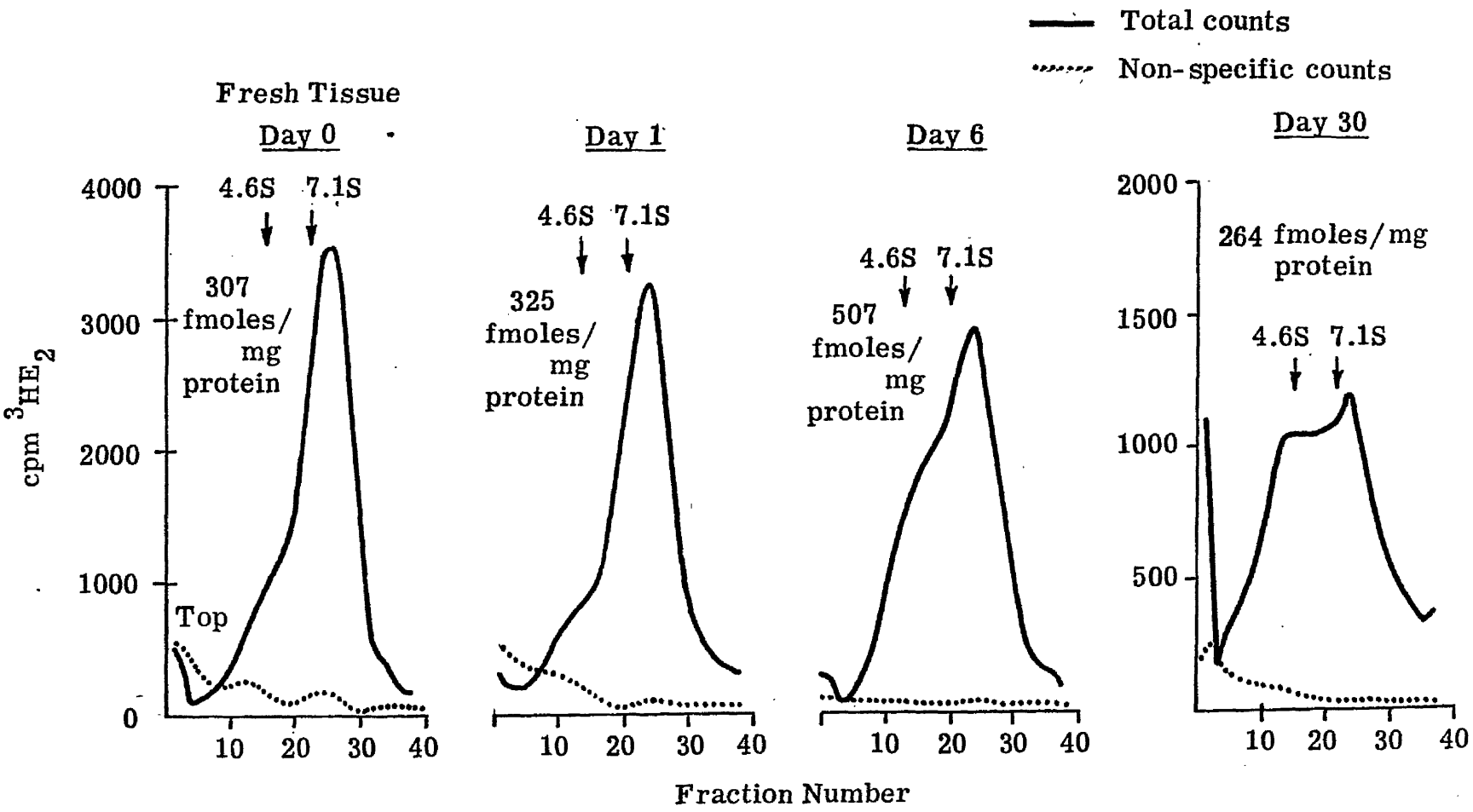
however, considerable loss of ^{3}H-oestrøadiol-17β during centrifugation suggesting that alteration in receptor structure has indeed also taken place. The total receptor concentration was approximately the same on 517th day as on 264th day but lower than in earlier samples. Although tumour heterogenity cannot be ignored it is suggested that receptor degradation must have taken place during storage for very long periods. Experiments reported in this thesis, outwith this storage study, were conducted with tumours which were either fresh or stored for less than 60 days in sucrose/glycerol buffer.

In the initial large tumour used for the storage study inter-conversion between the molecular forms was minimal. However, a second analysis conducted on a different tumour (Figure 20) gave an indication that such an effect could occur in this storage system, and presumably also in other storage systems. Figure 20 clearly demonstrates the 8S$\rightarrow$4S conversion during 30 days storage. Receptor binding capacity did not seem to be destroyed during storage, in contrast to the reported results after storage in liquid nitrogen (Namkung et al., 1979). The possibility of tissue heterogeneity with respect to the molecular forms cannot be excluded but this generally does not seem to be the case in other tumours studied (Section 3.1.3.2). However, if analyzed fresh this tumour would have been classified into the 8S predominant type, and on day 30 of storage, into the 4S + 8S group. Due to sample limitation it was not possible to study if a total conversion of 8S into 4S would have occurred on longer storage but long term in vitro incubation of tumour cytosol demonstrates (Figure 12.1 and 12.2) that this may be possible. There were other tumours which preserved the 8S form for considerable periods (see below). This change from 8S$\rightarrow$4S, therefore, seems to depend on intrinsic protease activity of individual tumours.

Figure 20. **Promotion of the 8S → 4S Conversion During Storage of Breast Tumour in Sucrose/Glycerol Buffer at -20°C**

A primary breast cancer (patient SN) was processed as described in the legend to Figure 18. Percentage recovery of specific counts was near 100% when tumour was analyzed fresh and on days 1 and 6. Recovery was 82% when analyzed on day 30 of storage. The protein concentration obtained (mg/ml) was 11.2 at Day 0, 9.8 at Day 1, 8 at Day 6 and 9.8 at Day 30.

Effect of storage in sucrose/glycerol buffer at -20° on the molecular form of oestrogen receptor (R. Breast)



There appears to be a 8S ⟶ 4S conversion in certain tumors

The observed 4S : 8S ratio changes indicates that such a ratio should only be related to patient prognosis in relation to the length of storage. However, the level of protease activity in itself may represent an index of some form (e.g. of invasion potential), and merits further study. Table 2 represents data from 23 primary breast tumours (27 sections) which were stored in sucrose/glycerol for the number of days indicated. Table 2 includes analysis of two additional axillary nodes (numbers 24 and 25). Some points to note include:-

a) Tumour number 3 shows no change in receptor profile (8S) when analyzed on days 6 and 55 after storage. Similarly, tumour number 14 analyzed after 37 and 51 days shows no change in the predominant 8S profile (cf Figure 20 where the change could be observed by 30 days storage).

b) Tumour number 13 shows a predominant 4S profile after 33 days of storage. The initial status with respect to sedimentation profile was not known. Tumour number 25 (axilla) shows a much higher proportion of 8S after 66 days of storage compared to tumour number 24 (also an axilla) which had been analyzed after 48 days.

c) Some tumour cytosol analyzed showed the presence of heavy aggregates, e.g. tumour number 4, and if only the 8S form is capable of aggregation then this would lead to an error in the 4S : 8S ratio. Alternatively these heavy aggregates may represent either receptor precursor or aged receptor perhaps bound to membrane fractions or other heavy structures which fail to sediment during the high speed centrifugation step in the cytosol preparation. Similar aggregates have been found by others (Sakai and Saez, 1976; Kon et al., 1980).

This storage system was also found suitable for the retention

Table 2. **Effect of storage in Sucrose/Glycerol Buffer at -20°C on the Molecular Form of Oestrogen Receptor**

Fresh tumour biopsies were sectioned and stored at -20°C in sucrose/glycerol buffer. One section was then used for routine determination of receptor content by Scatchard analysis (Section 2.2.2.3). The other section(s) were stored at -20°C in sucrose/glycerol for the time noted. It was then rehydrated at 4°C and the assay carried out as described in Section 2.2.2.1. The time period indicated does not apply to Scatchard analysis which were always performed prior to tissue being used for storage data.

* performed on a different Section 2.2.2.3.

** performed as described in Section 2.2.2.1.

*** represents the aggregated receptor at the bottom of centrifuge tube.

**** receptor could not be demonstrated on SDG.

Effect of Storage in Sucrose/Glycerol Buffer at -20°C on the Molecular Form of Oestrogen Receptor

Patient	Tissue	Period of Storage in Sucrose/Glycerol (Days)	Soluble Oestrogen Receptor fmoles/mg Protein					
			* Scatchard ER_c/ER_n	** DCC one point Assay	Sucrose Density Gradient	Molecular Form 4S	8S	*** Agg.
1	Breast	1	246/1147	189	170	13	157	ND
2	Breast	6	213/3500	112	99	36	58	5
3.1	Breast	6	293/8743	388	334	0	334	ND
3.2	Breast	55		337	305	0	290	15
4.1	Breast	7	120/4615	90	80	19	24	37
4.2	Breast	25		625	538	248	101	188
5	Breast	20	236/7318	409	389	0	389	ND
6.1	Breast	20	8/2400	90	87	28	50	9
6.2	Breast	57		38	27	20	7	ND
7	Breast	21	82/975	42	47	23	13	11
8	Breast	24	58/1334	65	74	36	7	21
9	Breast	28	125/1096	52	59	18	30	11
10	Breast	30	23/876	-	-	-	-	-
11	Breast	30	356/11873	330	261	133	128	ND
12	Breast	32	64/3037	83	75	32	43	ND
13	Breast	33	131/5345	164	148	104	44	ND
14.1	Breast	37	186/10878	275	231	0	231	ND
14.2	Breast	51		250	230	0	230	ND
15	Breast	44	150/3077	155	151	66	65	10
16	Breast	45	194/3808	153	144	59	85	ND
17	Breast	45	118/987	70	66	29	26	11
18	Breast	47	97/4286	41 ****	-	-	-	-
19	Breast	48	121/1648	93	94	40	40	14
20	Breast	51	112/3820	176	158	58	58	42
21	Breast	54	44/ND	53 ****	-	-	-	-
22	Breast	67	161/4884	44	42	17	23	2
23	Breast	71	120/2886	156	134	79	39	16
24	Axilla	48	100/1691	330	261	133	128	ND
25	Axilla	66	51/3168	201	189	74	115	ND

of 8S form from human endometrial tissue for at least 30 days (data not shown).

An interesting contrast was noted between human breast tumours and immature rat uterine tissue with respect to storage in sucrose/glycerol buffer. Rat uterine oestrogen receptor was found to be unstable in sucrose/glycerol and there was a considerable loss of both receptor activity and quality after only 1 week storage (Figure 21). The receptor does not leach into the storage medium, as judged by Scatchard analysis of the medium. It is shown in Figure 21 that the 8S receptor decayed and proteolytic fragments could be observed. The formation of the 4S form could be seen after day 1 in storage. It is further shown in Figure 57 (Section 3.2.3.3.1) that one day storage of rat tissue renders ~50% of the receptor non-transformable when compared to fresh tissue (Figure 46 and Section 3.2.3.1).

3.1.1.8 Effect of Ionic Strength, Sodium Molybdate and Protease Inhibitor

3.1.1.8.1 Ionic Strength

When breast tumour ER_c was analyzed in low salt gradients, following homogenization in low salt buffers, a 4S + 8S or 8S alone profile was generally observed (Section 3.1.2). However if the salt concentration of the centrifugation buffer was changed to either 0.15M or 0.4M KCl ($HEDK._{15}$ or $HEDK._4$), there was a quantitative change from 8S⟶4S and a single sharp peak was observed (Figure 22 A and B - obtained from same cytosol). The same effect is observed with immature rat uterine ER_c (data not shown) in agreement with the observations of Yamamoto (1974).

It is claimed by Wittliff et al. (1976) that when the human

Figure 21. **Effect of Storage of Immature Rat Uteri in Sucrose/Glycerol Buffer at -20°C on the Sedimentation Profile of Soluble Oestrogen Receptor**

One group of uteri was analyzed fresh and the remaining 3 groups on the days indicated. The preparation and analysis was as described in Section 2.2.2.4.1. The recovery of total specific counts was between 80-100%. Receptor concentration in fmoles/mg protein was 921 for fresh uteri analysis, 846 for day 1, 258 for day 7 and 135 for 5 weeks storage period.

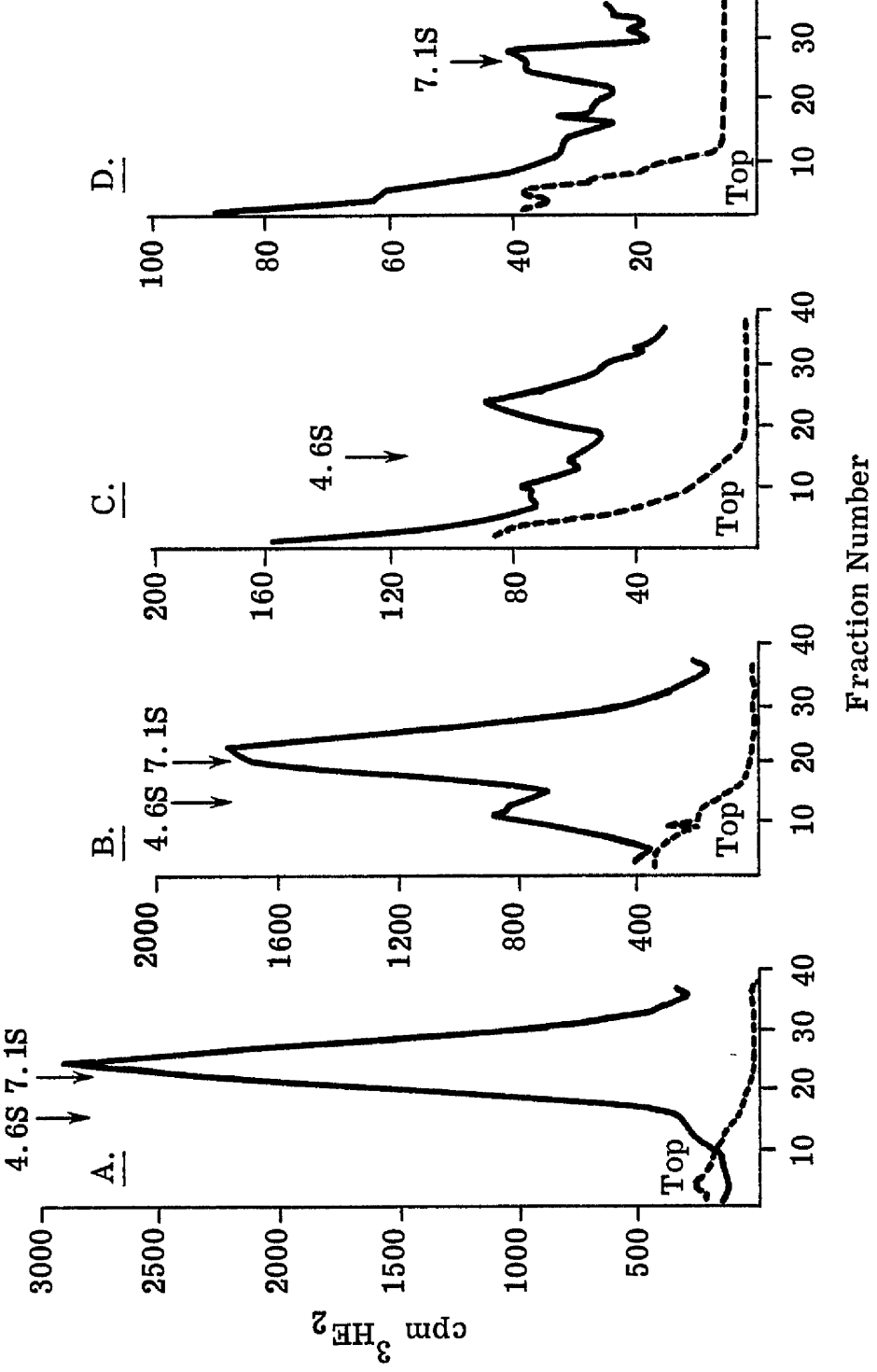

Fresh
Day 1
Day 7
5 Weeks
A.
B.
C.
D.
4.6S 7.1S
4.6S 7.1S
4.6S
7.1S
Top
Top
Top
Top
cpm $^3HE_2$
Fraction Number
3000
2500
2000
1500
1000
500
2000
1600
1200
800
400
200
160
120
80
40
100
80
60
40
20
10 20 30 40

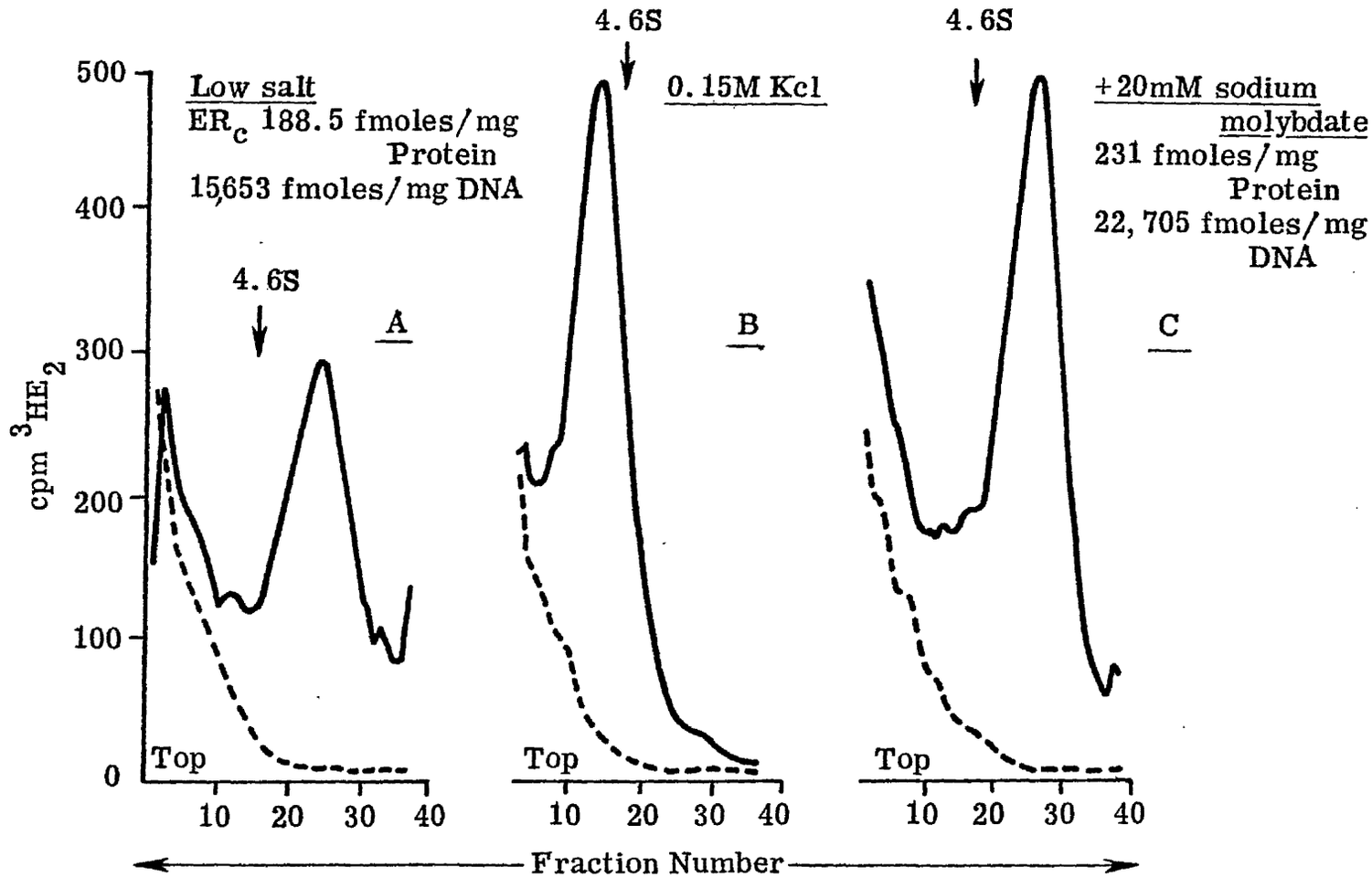
Figure 22. **Influence of Ionic Strength and Sodium Molybdate on Sedimentation Profile of Oestrogen Receptor from Human Breast Cancer Cytosol**

A and B: Cytosol was prepared in HED as described in Section 2.2.2.1 (patient AW; protein concentration 4.8mg/ml). After labelling with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES, unbound steroid was removed with DCC. One set of aliquots was loaded onto 5-20% gradients prepared in HED (A) or HEDK.$_{15}$ (B). Centrifugation was at 47,000 rev/min for 13h. Recovery of specific counts was 90% in A and 103% in B.

C: A second portion of the same tumour was homogenized in HED +20mM sodium molybdate (protein concentration 5.8mg/ml) and labelled cytosol after DCC stripping was centrifuged in HED as described for A and B. Recovery of specific counts was 93%.

Receptor concentrations are indicated on the figure.

Effect of ionic strength and sodium molybdate on sedimentation profile of ER_c from human breast tumor cytosol ♂



breast tumour ER_c is homogenized in TEDK.$_{15}$ and centrifugation performed in the same buffer, a 6S form is obtained. They suggested that this represented the active form of receptor. Although homogenization in 0.15M KCl - containing buffer and centrifugation in the same buffer results in a 6S peak, centrifugation in 0.4M KCl-containing gradients yields a single 4S peak. Thus, the ionic strength of both the homogenization and the centrifugation buffers is critical to the molecular form of the receptor.

3.1.1.8.2 **Sodium Molybdate**

The result presented in Figure 22C (obtained from the same tumour as used for Figure 22A and B) further shows that if the homogenization is performed in the presence of sodium molybdate, the 8S profile persists. There is no change in the sedimentation profile on inclusion of sodium molybdate in the homogenization buffer, as also noted by Mauck et al. (1982). The result is however, in disagreement with Nishigori and Toft (1980) who were able to find that sodium molybdate promoted increased sedimentation values.

It has been observed by Nishigori and Toft (1980) and Anderson et al. (1980) that sodium molybdate increases the amount of progesterone receptor detected in avian oviduct and human breast tumour cytosol, respectively. The latter report also indicates that sodium molybdate increases apparent ER_c concentration only in certain breast tumours. In Figure 22C, the increased receptor value on inclusion of sodium molybdate may be attributed to a combination of molybdate and/or intra-tumour variation (Section 3.1.3.3). The routine use of sodium molybdate to stabilize the 8S form was avoided since, if the 4S and 8S complex have any clinical significance, then they should be studied

without masking the effect. In vivo conversion to 4S may explain why 4S containing tumours do not respond to hormone therapy.

A further point of interest is that Figure 22 represents a tumour receptor profile from a male patient with breast carcinoma. Very few studies have demonstrated the molecular forms of receptor in male breast carcinoma. Wittliff (1974) was the first to demonstrate that male breast tumours possess receptor similar to that of female tumours and he suggested that further consideration should be given to endocrine therapy for male breast cancer.

3.1.1.8.3 **Protease Inhibitors**

The effect of two protease inhibitors, PMSF and Trasylol are shown in Figure 23.1 and 23.2. In the presence or absence of PMSF during homogenization, no significant difference could be observed in the sedimentation profile (Figure 23.1). The tumour ER_c was of the predominant 8S type with a small 4S sedimentation peak unaffected by the presence of PMSF. The absence of an effect may be fortuitous due to a low content of protease activity in this tumour. Indeed this same tumour was studied for storage purposes and showed no significant interconversion to 4S (Figure 18). Using a different tumour, Trasylol (Figure 23.2) failed to inhibit the appearance of 4S and, in addition, disturbed the 8S profile. The latter probably results from the medium in which Trasylol is supplied (see Materials and Methods, Section 2.1.1). The salt constituent of the Trasylol medium may result in some 8S → 4S conversion.

Thus proteases sensitive to PMSF and Trasylol were not apparent in these human breast tumours. However the protease inhibitors, Leupeptin

Figure 23.1 **Influence of a Protease Inhibitor, PMSF, on Sedimentation Profile of Oestrogen Receptor from Human Breast Tumour Cytosol**

Human breast tumour cytosol was prepared from two different sections of the same tumour (patient BM) as described in Section 2.2.2.1. PMSF (1mM final concentration) was introduced into one set prior to homogenization. Labelling of cytosol, removal of free steroid and SDGA was as described in Section 2.2.2.1. Protein concentration was 7.0mg/ml for PMSF-free cytosol and 11.0mg/ml for PMSF-containing sample. Receptor concentration was 324fmoles/mg protein in PMSF free and 304 fmoles/mg protein in PMSF containing cytosol. Percentage recovery was 90% in both samples. Centrifugation was for 14h at 50,000rev/min in Beckman SW 50.1 rotor at $4^{o}C$. Arrow indicates the position of ^{14}C-labelled marker protein.

Effect of PMSF on ER_c sedimentation profile

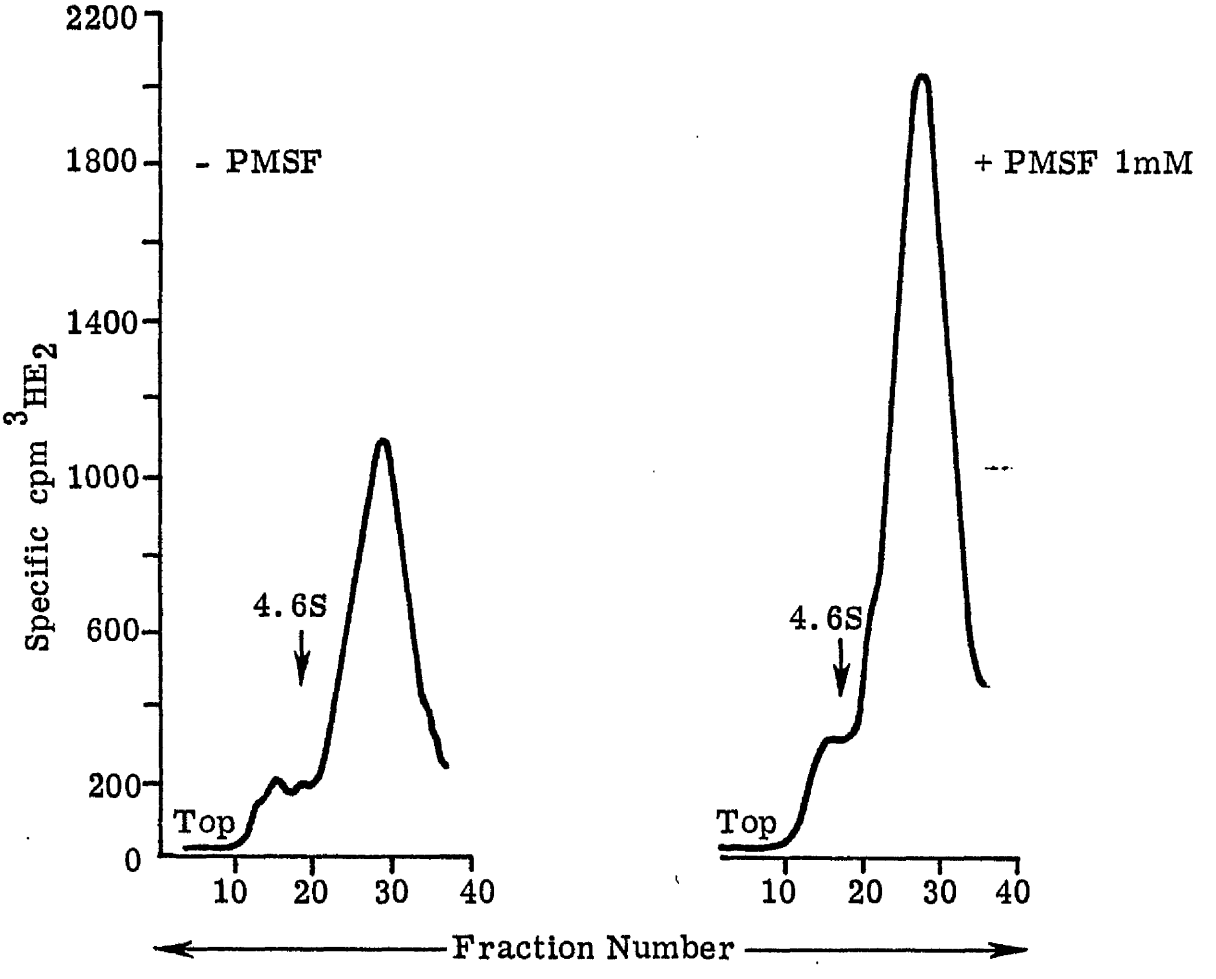
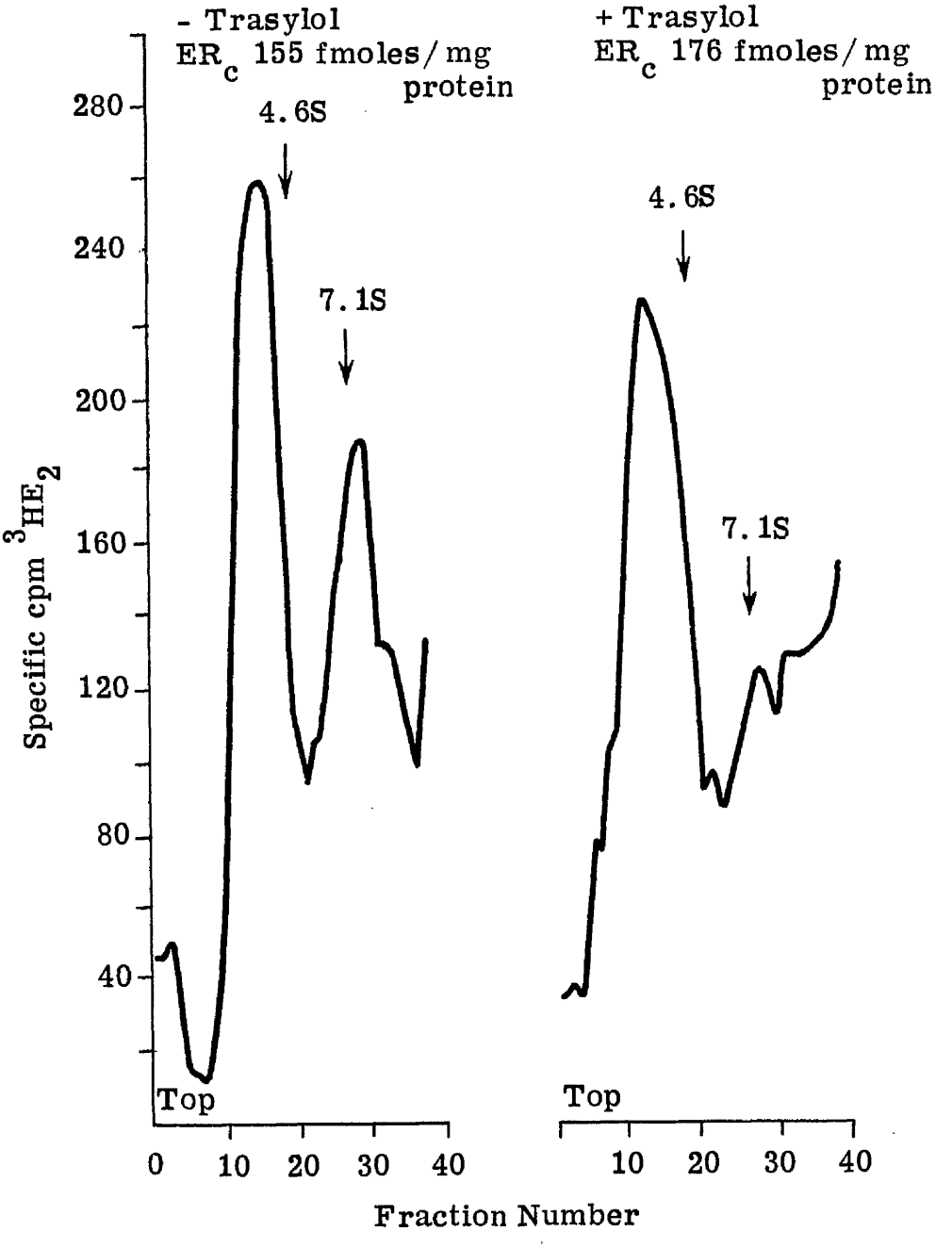


Figure 23.2. **Effect of an Alternative Protease Inhibitor, Trasylol, on the Sedimentation Profile of Oestrogen Receptor from Human Breast Cancer Cytosol**

Human breast tumour cytosol was prepared from two different sections of the same tumour (patient AK) as described in Section 2.2.2.1. Trasylol (2000 units) was introduced into one set prior to homogenization. Labelling of cytosol, removal of unbound steroid and SDGA was as described in Section 2.2.2.1. Protein concentration was 6.0mg/ml for Trasylol-free cytosol and 4.6mg/ml for Trasylol-containing cytosol. Receptor concentration was 155fmoles/mg protein in Trasylol-free cytosol and 176fmoles/mg protein in Trasylol-containing cytosol. Percentage recovery of total specific counts in gradient was 91%. Centrifugation was for 14h at 50,000 rev/min in Beckman SW 50.1 rotor at $4^{o}C$. Arrows indicate the position of ^{14}C-labelled marker proteins.


- Trasylol
ER$_c$ 155 fmoles/mg protein
4.6S
7.1S
+ Trasylol
ER$_c$ 176 fmoles/mg protein
4.6S
7.1S
Specific cpm $^3HE_2$
280
240
200
160
120
80
40
Top
Top
0
10
20
30
40
10
20
30
40
Fraction Number

and DFP, mainly used in the latter part of the study (Section 3.2), are effective against the formation of the non-aggregatable type of 4S receptor and so imply the presence of a relatively specific protease(s).

3.1.1.9 Effect of Buffers on Sedimentation Analysis

Tissue was normally stored in medium containing Hepes buffer. Since some experiments were subsequently conducted in Tris buffer it was necessary to determine the effects of both buffers on the sedimentation profile. Figure 24 (A and C) shows that tumours stored in sucrose/glycerol buffer (Hepes) then both rehydrated in and, subsequently, analyzed on gradients containing Tris or Hepes alone, resulted in a clear 8S peak. Figure 24 (B and D) further shows the effect of homogenization in one buffer and sedimentation in the other. This procedure showed loss of 8S when the homogenization buffer was Tris and centrifugation buffer Hepes. The loss of 8S, presumably due to dissociation of the 8S into the 4S form, may be due to a local effect at the point of contact between Tris and Hepes. The experiment was only performed once since such a use of different homogenization and centrifugation buffers is, hopefully, uncommon.

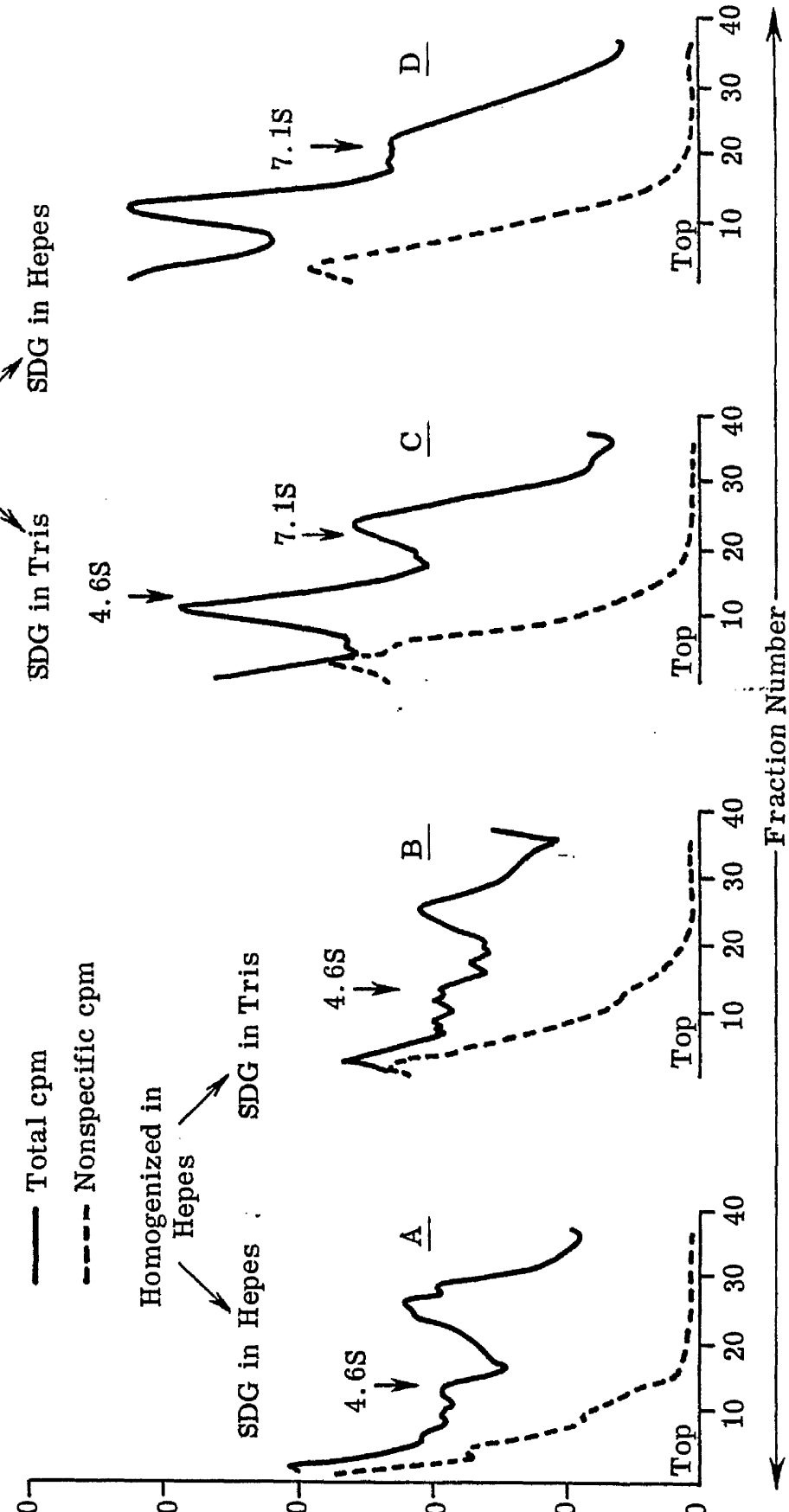
3.1.1.9.1 Quality Control of Every Batch of Buffer

In the initial phase of these studies a different source of Hepes (Ubichem Ltd), rather than that obtained routinely from the Boehringer Corporation, was found to inhibit the formation of the 8S complex. Twenty-one tumours were studied and formation of 8S complex was never observed. Some of the components of the Ubichem buffer were thought to influence the final molecular form. Both immature rat uterine and human endometrial cytosol ER_c were tested and both showed loss of 8S (data

Figure 24. **Effect of Alternative Buffers on Sedimentation Profile of Oestrogen Receptor from Human Breast Tumour**

Human breast tumour cytosol was prepared from two different sections of the same tumour as described in Section 2.2.2.1 (patient MK). One section was homogenized in HED (protein concentration 4.9mg/ml) and the other in TED (protein concentration 5.6mg/ml). Aliquots were labelled at $4^{o}C$ with $5 \times 10^{-9}M$ $^{3}HE_{2}$ in the absence (——) or presence (....) of $5 \times 10^{-6}M$ DES for 1hr. After removal of free steroid with DCC, cytosol, prepared in HED, was loaded onto 5-20% gradients prepared in either HED (A) or TED (B). Similarly cytosol prepared in TED was loaded onto gradients prepared in either TED (C) or HED (D). Centrifugation was for 14h at 45,000 rev/min at $4^{o}C$ in a Beckman SW 50.1 rotor.

Effect of buffer on sedimentation profile of ER_c from human breast tumor cytosol



not shown). Several alternative explanations were considered before the source of Hepes was once again changed to Boehringer. The receptor was then shown, once again, to sediment at 8S. This finding led to the quality control of every batch of buffer using immature rat uterine ER_c.

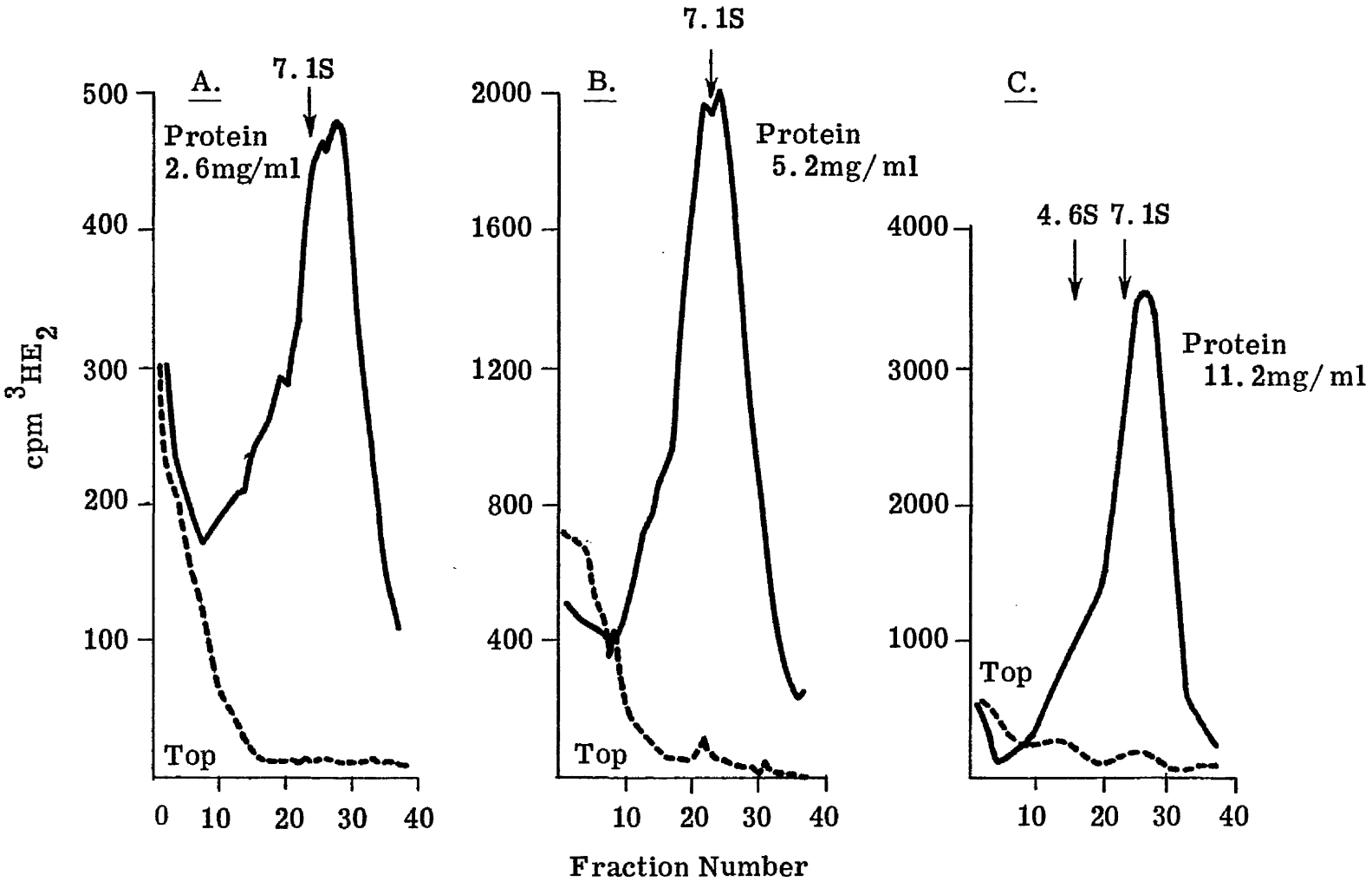
3.1.1.10 **Effect of Protein Concentration**

Stancel et al. (1973a) have shown that protein concentration of the soluble fractions can affect the final sedimentation patterns. In the present study, low salt conditions similar to those of Stancel et al. (1973a) were used. The protein concentration ranged generally from 3-10mg/ml. Figure 25 shows that in this protein concentration range (and lower) no marked change in sedimentation properties occurred. Slight shifts in the position of the 8S peak can be seen but the ^{14}C-labelled marker confirms that the sedimentation value is always greater than 7.1S. Similarly, 4S receptor ran below the 4.6S marker at all protein concentrations tested (data not shown). The receptor, in salt containing gradients, sedimented in a very sharp 4S peak. However the actual peak value varied slightly with protein concentration, possibly as a result of non-specific components.

A plot of protein concentration verses the 'S' value for the 49 sections exhibiting both the 4S and the 8S forms of receptor (Table 3.1) showed that there was no significant relationship between the 8S form and protein concentration ($n = 49$, $r = 0.078$). However, sedimentation of the 4S form showed a correlation (significant at 1% level) with protein concentration ($n = 49$, $r = 0.389$). The latter is, however, subject to error since only 6 values were obtained at protein concentration >7mg/ml where a slight relationship was seen between the two, probably as a result of serum proteins sedimenting in the same area.

Figure 25. **Comparative Profiles of Oestrogen Receptor from Different Biopsies in Relation to Protein Content**

Three separate human breast tumours were homogenized in HED and analyzed as described in Section 2.2.2.1 (patients MC, AP and SN). The protein concentrations obtained were as indicated on the graph. The receptor concentration in fmoles/mg protein and receptor percentage recovery was 275 and 84% in A, 409 and 95% in B and 307 and 97% in C. The centrifugation was at 45,000 rev/min for 15h in A and B and 14h in C at $4^{o}C$ in a Beckman SW 50.1 rotor. Arrows indicate the position of ^{14}C-labelled marker proteins.


A.
7.1S
Protein
2.6mg/ml
Top
B.
7.1S
Protein
5.2mg/ml
Top
C.
4.6S 7.1S
Protein
11.2mg/ml
Top
cpm $^3HE_2$
500
400
300
200
100
2000
1600
1200
800
400
4000
3000
2000
1000
0 10 20 30 40
10 20 30 40
10 20 30 40
Fraction Number

3.1.2. Sedimentation Profile of Human Breast Tumour Oestrogen Receptor (Low Salt Buffer)

A total of 118 sections from 74 tumours were analyzed by SDGA. These comprised tumours obtained from pre- and post-menopausal patients. Figure 26 demonstrates the major forms of sedimentation profiles obtained. The following were the profiles seen:-

(i) receptor negative cytosol (including one case from a women undergoing mammoplastic reduction) showed a profile as in Figure 26A.

(ii) tumour cytosol presenting a predominant 8S class of receptor (Figure 26B).

(iii) tumour cytosol presenting both 4S and 8S forms (Figure 26C)

(iv) Figure 26D represents tumour cytosol which displayed a higher proportion of 4S form of receptor but 8S was still clearly observable.

(v) tumour cytosol showing predominantly (>70%) 4S form has been presented in Figure 14.

(vi) Figure 26E is an example of a profile from an axillary node showing the predominant 8S profile.

3.1.2.1 Distribution of the Molecular Forms

The relative distribution of the molecular forms observed is shown in Table 3.1. It must be pointed out that wherever possible only tumours with high receptor content were studied. However certain tumours showing low receptor concentrations were also included.

3.1.2.1.1 The 4S only ER_C Distribution

In contrast to several published results (Kute et al., 1978;

Figure 26. **Molecular Forms of Oestrogen Receptor in Human Breast Carcinoma**

Breast tumour cytosols were prepared, labelled with $^{3}HE_{2} \pm$ DES, DCC stripped and centrifuged as described in Section 2.2.2.1. The profiles shown represent (A) a receptor negative tumour, (B) a predominant 8S profile, (C) a mixture of 8S and 4S forms, (D) a mixture of 8S and 4S form but with a much greater concentration of 4S form. A predominant 8S profile shown for an axillary node (E) is similar to that found in primary breast carcinoma (B). A predominant 4S profile is also possible - see Figure 28.1A.

Molecular forms of ER_c obtained in female breast carcinoma

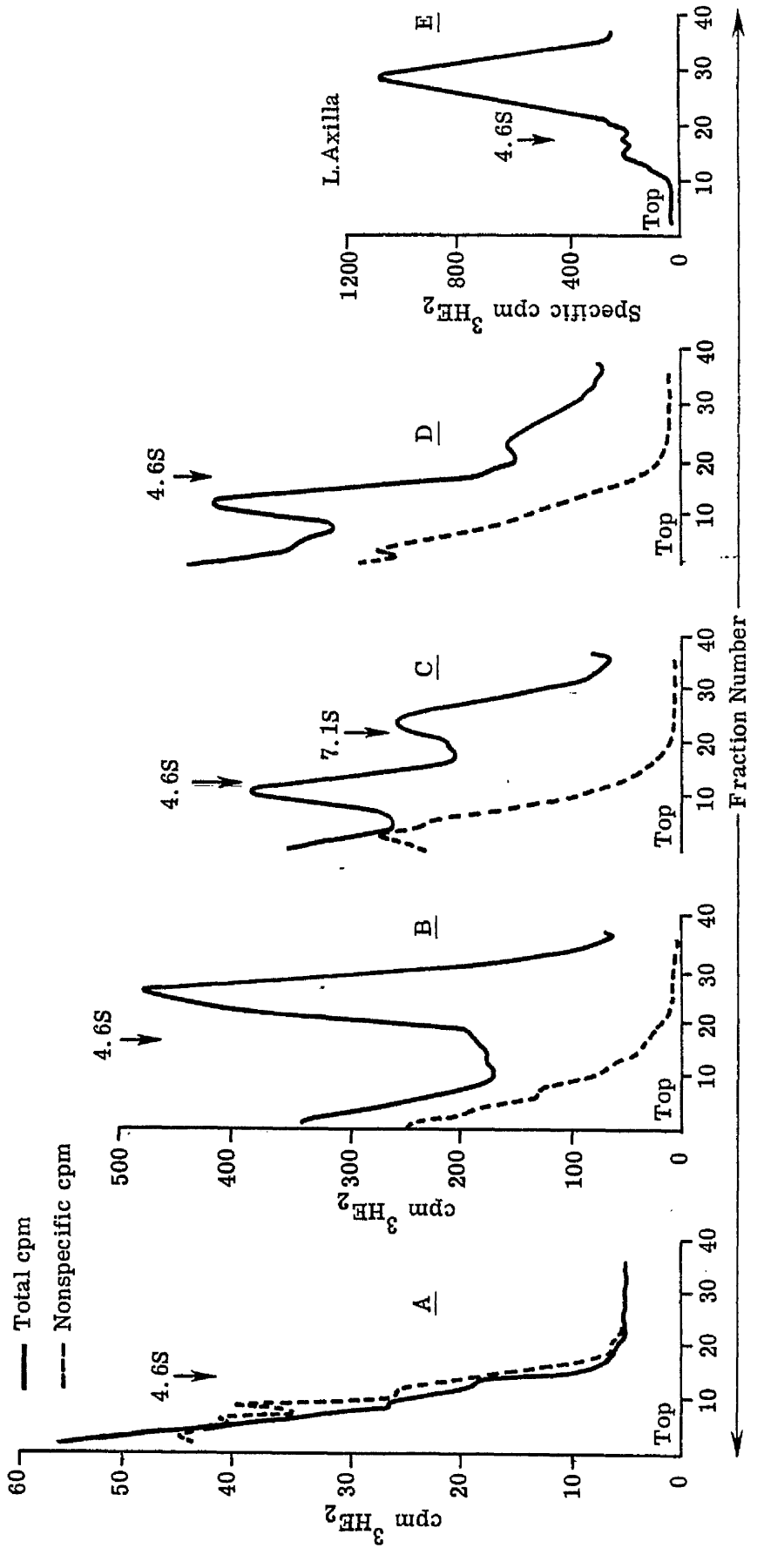


Table 3.1. Distribution of the Various Molecular Forms of Oestrogen Receptor within Human Breast Cancer

Preparation of human breast tumour cytosol and SDGA was as described in Section 2.2.2.1.

Receptor Species	Tumour Sections taken from both within a single tumour and from different tumours
1. 4S [a]	11 (sections from 8 tumours) *
2. 4S + 8S [b]	49 (sections from 36 tumours) **
3. 8S [c]	19 (sections from 6 tumours)
4. DCC^{+}, $SDG^{\pm}$	29 (sections from 20 tumours)***
5. DCC^{-}, SDG^{-}	10 (sections from 10 tumours)
	118 sections from 74 tumours

* 4 tumours showed another section in 4S + 8S class

** Excluding the tumours from (1), two tumours showed two further sections in DCC^{+}, $SDG^{\pm}$ class

*** DCC^{+}, $SDG^{\pm}$ represents tumour section exhibiting receptor in one point DCC assay but receptor could not be quantified on SDG.

a 3.70 ± 0.32 S

b 3.96 ± 0.64 S

7.64 ± 0.34 S

c 7.62 ± 0.40 S

Wittliff et al., 1978) but in agreement with others (Jensen et al., 1975; Freedman and Hawkins, 1980), the 4S receptor was predominant in only a few tumours (Table 3.1), out of the 8 tumours which displayed predominant 4S receptor in one section, 4 tumours displayed a 4S + 8S profile in another section. An example is presented in Figure 28.1 (Section 3.1.3.3). It is possible that aggregation of receptor could have resulted in a false distribution on the gradient (the 4S:8S ratio can only be calculated from the receptor sedimenting in the gradient fractions - aggregated receptor which may comprise a complex of one or both of these forms was not considered). One of the 8 tumours, with predominant 4S receptor, displayed a predominant 4S class of profile in all the four sections studied.

3.1.2.1.2 **The 4S + 8S ER_c Distribution**

A total of 49 sections from 36 tumours fell into this category (Table 3.1). From two tumours, however, further sections showed DCC competable counts but no clear sedimentation profile on the gradient (DCC^+, $SDG^{\pm}$ class). In some other tumours when two or more sections were analyzed the molecular status of the tumour remained constant but the relative distribution of the ER_c between the molecular forms differed (further discussed in Section 3.1.3.2).

3.1.2.1.3 **The Predominant 8S Profile**

Six tumours from which 19 sections were analyzed, all showed the predominant 8S profile (Table 3.1).

3.1.2.1.4 **DCC^+, $SDG^{\pm}$ and DCC^-, SDG^- ER_c Profile**

Twenty nine sections taken from 21 tumours showed DCC^+, $SDG^{\pm}$ type of sedimentation profile i.e. DCC assay showed significant competable counts, but these could not be quantitated under a defined 8S or 4S peak.

An example is shown in Figure 16 where the specific counts can be seen to be distributed from 3-4S area and extending in an aggregated manner into the 8-9S region. This class most probably results from some modification of receptor structure during storage (Section 3.1.1.7). The DCC^-, SDG^- type showed no competable counts in either one point assay or SDG.

3.1.2.2 Distribution of Molecular Forms Relative to Receptor Concentration

Table 3.2 illustrates that there does not appear to be any relationship between receptor concentration and the molecular form exhibited by the tumour cytosol. However, the mean receptor concentration for the predominant 8S profile was about 2-fold higher than predominantly 4S or 4S + 8S class. Conversely, the mean value for DCC^+, $SDG^{\pm}$ class was 3-4 fold lower than tumours exhibiting 4S or 4S + 8S forms. This could indicate a higher proteolytic damage to the receptor in the DCC^+, $SDG^{\pm}$ class, a lower tumour cell content of the biopsy or abnormal receptor. A similar form of classification, the $\pm$ profile, was reported by Iino et al. (1980).

3.1.3 Intratumoural Variation of both Oestrogen Receptor Concentration and Molecular Form in Human Breast Cancer

3.1.3.1 Variability in Soluble and Nuclear Oestrogen Receptor Within a Single Tumour (Peripheral and Adjacent Sections)

Table 4 shows the variability obtained in both soluble and nuclear oestrogen receptor across individual breast tumours. There appears to be a loss of both soluble and nuclear oestrogen receptor towards the centre of the tumour (tumour numbers 2 and 3). In tumour number 1, the section adjacent to the peripheral section showed a higher oestrogen receptor concentration and the same was found to be the case in five additional tumours, reported in Table 5. Further, tumour number 4 (Table 4) showed the peripheral section

Table 3.2. Distribution of Molecular Forms of Oestrogen Receptor Relative to Receptor Concentration

Preparation of human breast tumour cytosol and SDGA was as described in Section 2.2.2.1. The values of receptor concentration are calculated from an aliquot taken prior to gradient analysis (Section 2.2.2.1).

	RECEPTOR SPECIES				
	4S	4S + 8S	8S	$DCC^{+}, SDG^{\pm}$	DCC^{-}, SDG^{-}
No. of Sections	11	49	19	29	10
Receptor concentration fmoles/mg protein (mean ± S.D.)	152 ± 124	117 ± 98	310 ± 105	42 ± 31	-
Range	38 - 432	20 - 431	43 - 531	11 - 143	-

Table 4. **Variability of Soluble and Nuclear Oestrogen Receptor Content across the same Breast Tumour**

Scatchard analysis of Peripheral and Adjacent zones towards the centre of the tumour. The tumours were sectioned selectively as described in Figure 9. The processing of tumour and receptor content measurement was as described by Leake et al. (1981) (Section 2.2.2.3).

Variability in Soluble and Nuclear Oestrogen Receptor across the same Breast Tumour. Scatchard analysis of Peripheral and Subsequent Adjacent Zones towards the Centre of the Tumour.

$$\frac{ER_c \ \frac{\text{fmoles}}{\text{mg Pr}} \ / \ K_d \times 10^{-10}M}{ER_n \ \frac{\text{fmoles}}{\text{mgDNA}} \ / \ K_d \times 10^{-10}M}$$

Tumour	Section 1 (Peripheral)	Section 2	Section 3
1. JM	$\frac{ER_c}{ER_n} = \frac{155/1.0}{3639/2.0}$	$\frac{*\ /\ *}{4132/1.37}$	$\frac{114/0.65}{3905/1.9}$
2. SC	$\frac{308/1.3}{ND/ND}$	$\frac{174/4.0}{ND/ND}$	$\frac{131/1.3}{ND/ND}$
3. LK	$\frac{*\ /\ *}{*\ /\ *}$	$\frac{74/1.1}{4730/1.1}$	$\frac{21/1.7}{1746/2.8}$
4. MM	$\frac{45/1.4}{1754/6.6}$	$\frac{0/0}{0/0}$	$\frac{0/0}{0/0}$
5. MK	$\frac{25/3.9}{3294/3.1}$	$\frac{14/2.3}{2014/1.0}$	$\frac{19/4.6}{1882/1.4}$
6. AK	$\frac{34/3.9}{0/0}$	$\frac{46/1.5}{3205/6.1}$	$\frac{41/3.6}{1835/1.4}$
	Three additional tumors were found negative for oestrogen receptor in all sections.		

* Receptor Concn. too high to attain equilibrium.

ND - Not determined.

to be positive for ER_c and ER_n (nuclear oestrogen receptor), but other sections were uniformly negative. Both tumours 5 and 6 showed relatively constant concentration of ER_c throughout. However, in tumour number 6 only the soluble receptor was found in the peripheral section. Three additional tumours were found to be negative in all sections studied.

These results are in agreement with several published papers (Tilley et al., 1978; Silfversward et al., 1980) with respect to the soluble oestrogen receptor. The number of tumours reported in the present investigation is too small, however, to make any comments regarding the significance of dissociation constant changes observed in different sections. But the intratumoural variability of oestrogen receptor concentration (both ER_c and ER_n) and the decrease towards the centre approach (but do not achieve significance - non-parametric test (P. Boyle)). The loss of nuclear receptor towards the centre of the tumour was not observed by Silfversward et al. (1980) who reported the contrary. However, a similar loss towards the older part of the tumour has been recognised in larger endometrial cancers (Castagnetta et al., 1983).

3.1.3.2 **Variability in Soluble Oestrogen Receptor across the same Breast Tumour relative to 4S:8S Ratios (Peripheral and Adjacent Sections)**

Table 5 shows an intratumoural study of soluble oestrogen receptor concentration and the corresponding molecular forms. Where one molecular form of ER_c was detected, this was normally the predominant form found across the tumour, even when an additional random section was analyzed (tumour numbers 3 and 4). In tumour number 8, however, the peripheral section had predominantly the 4S form of ER_c, the adjacent section was negative and a third section, chosen randomly, showed predominantly 8S.

With respect to receptor concentration (Table 5), tumours

Table 5. **Variability in Soluble Receptor Content across the same Tumour.**

Sucrose Density Gradient Analysis of Sections Obtained Selectively

The tumours were sectioned selectively as described in Figure 9.

The processing of tumour and SDGA was as described in Section 2.2.2.1.

Tumour	Section 1 = Peripheral 2 & 3 = Adjacent sections towards tumour centre	ER_c $\frac{\text{f moles}}{\text{mg Protein}}$ * (from DCC one point assay)	Variation Obtained	ER_c From SDG ± = detectable, not possible to quantitate	Molecular Form 4S	Molecular Form 8S (aggregate)	ER_c $\frac{\text{fmoles}}{\text{mg DNA}}$ ND = Not determined	Variation Obtained
1. JM	1	28	1.0	±	±	±	1790	1.0
	2	335	12.0	281	82	199	8045	4.5
	3	207	7.4	195	90	105	4328	2.4
2. SM	1	166	1.0	204	171	33	10540	1.2
	2	393	2.4	432	350	82	11281	1.2
	3	170	1.0	122	±	±	9138	1.0
3. SCU	1	90	1.0	80	19	24(37)		
	2	345	3.8	291	116	116(59)		
	3	337	3.7	322	136	118(68)	ND	ND
	A Random	177	2.0	127	54	52(21)		
	B Random	625	7.0	538	249	101(188)		
4. MC	1	250	1.0	230	0	230		
	2	303	1.2	276	0	276	ND	ND
	A Random	275	1.1	231	0	231		
5. AK	1	35	1.2	33	21	12		
	2	69	2.3	36	21	15	ND	ND
	3	60	2.0	±	±	±		
	4	30	1.0	±	±	±		
6. FM	1	89	4.2	83	19	53(11)		
	2	50	2.4	44	18	23(3)	ND	ND
	3	21	1.0	23	9	11(3)		
7. AH	1	42	2.5	±	±	±	1725	2.5
	2	17	1.0	-	-	-	688	1.0
	3	17	1.0	-	-	-	702	1.0
8. CB	1	38	3.0	30	20	7(3)		
	2	13	1.0	-	-	-	ND	ND
	A Random	90	7.0	77	28	50(9)		
9. MM	1	21	2.0	-	-	-	430	1.6
	2	11	1.0	-	-	-	277	1.0
	3	24	2.2	-	-	-	384	1.4
10. EK	1	-	-	-	-	-	ND	ND
	2	-	-	-	-	-	ND	ND
	3	-	-	-	-	-	ND	ND

number 1-5 all showed the peripheral section lower in ER_c (cf. tumour number 1 in Table 4) relative to adjacent section, but a fall towards the centre was evident (see Figure 27 for tumour number 1 reported in Table 5). In Figure 27 the Protein:DNA ratio indicated that, perhaps, the cell number is lower in the outside or peripheral section and this may be the reason for low receptor values recorded in this section. Table 5 further shows that tumours number 6-8 all indicated a drop of ER_c content from the periphery to the centre as detected using DCC single point assay prior to SDGA. However, no receptor could be detected on gradient in any of the three sections for tumour number 7 and for one section of tumour number 8. Again, tumour number 9 showed low receptor concentration of ER_c throughout as judged by DCC single point assay, but no receptor could be detected on the gradient. Finally, tumour number 10 was judged negative by both DCC and gradient analysis and is illustrated as an example of control.

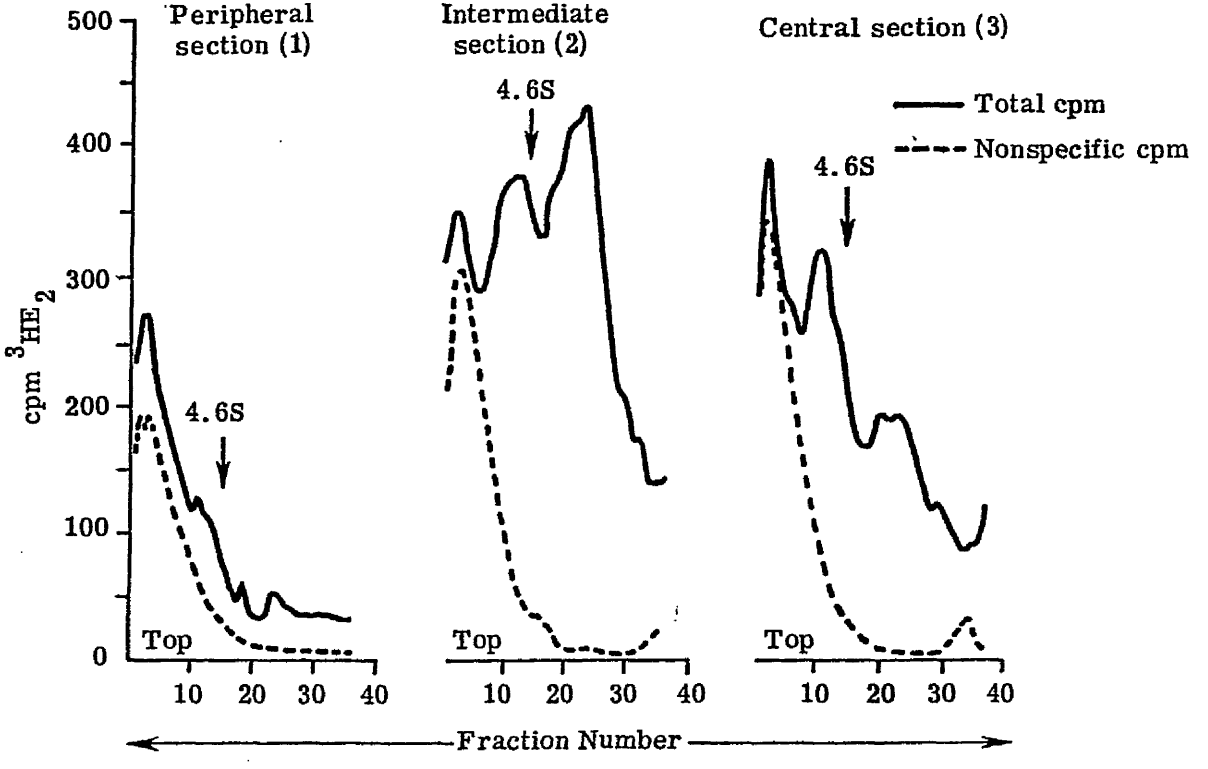
When ER_c concentration was expressed per unit DNA, rather than per unit protein, individual tumours showed a reduction in variation (see tumour number 1 in Table 5 and tumour number 6 in Table 6). However in the two axillary node tissues studied (tumour number 2 in Table 6 and the tumour reported in Table 7), the reverse was found to be the case i.e. variation increased when results were expressed per unit DNA. Once again, although the number of tumours analyzed was small, the possibility of lymph gland infiltration with leukocytes (Silfversward et al.,1980) might provide the answer for such variation. The possibility of having oestrogen receptor positive and negative cells in the same tumour should also be considered.

Wittliff and Savlov (1975) have presented evidence that two sections from the same tumour show the same distribution of molecular forms. This observation is confirmed and extended by the data in Table 5

Figure 27. **Intratumoural Variation in the Sedimentation Profile of Oestrogen Receptor from Human Breast Cancer (Selected Sections)**

Human breast tumour was sectioned selectively as described in Figure 9. Cytosol was prepared and analyzed on SDGA as described in Section 2.2.2.1. Percentage recovery of specific receptor was 81% in the intermediate section and 94% in the central section. In the periphery it is not possible to calculate percentage recovery of specific receptor as no defined peak(s) were seen.

Intratumoral variation in the sedimentation profile of ER_c from human breast tumor cytosol (selected sections)



Section	ER_c $\frac{\text{fmoles}}{\text{mg P}}$	Relative value	ER_c $\frac{\text{fmoles}}{\text{mg DNA}}$	Variation	$Pr_{/DNA}$	Relative value	Molecular Form
1	27.5	1	1790	1	65	1.0	Low 4S, 8S
2	335.2	12	8045	4.5	24	0.3	4S, 8S (29%) (71%)
3	207.0	7.5	4328	2.4	21	0.3	4S, 8S (46%) (54%)

and 6. The recently published results of Izuo et al. (1982) show data similar to those presented here. However, the sedimentation patterns and associated changes in concentration of the molecular forms were not previously documented. The observation that, in a single tumour, one section was receptor positive whilst another receptor negative confirmed the results of Poulsen (1981). Such cases however, are rare.

3.1.3.3 Variability in Soluble Oestrogen Receptor across the same Tumour (Random Sections)

Table 6 is an accumulation of data from 12 tumours in which two sections were randomized prior to analysis with respect to receptor concentration and molecular form. A very similar pattern to that reported in Table 5 was found, i.e. tumour preserves its status with respect to the molecular form. However in one case, tumour number 8, the distribution of 4S and 8S concentration in Section A was predominantly 4S (>70%) whereas Section B was classed as containing both 4S (59%) and 8S (Figure 28.1). This conclusion depends very much on the small amount of 8S recovered in Section A and may be of limited significance. Experimental artifacts, such as aggregation of receptor can result in false estimation of total receptor concentration from the gradient (see Section 3.1.1.6). However, in tumour number 8 (Table 6), there was total recovery of receptor concentration on the gradient and, therefore, no evidence of 8S being lost through aggregation.

In keeping with Table 5, Table 6 also demonstrates the variation of 4S and 8S profile from two sections of the same tumour. Figure 28.2, representing tumour number 2 in Table 6, demonstrates another case of extreme variation where Section A can be classified as 4S + 8S type and Section B as predominantly 8S type.

Table 6. Variability in Soluble Oestrogen Receptor across the same Tumour. Sucrose Density Gradient Analysis of Sections obtained Randomly

The tumours were sectioned randomly as shown in Figure 9. The processing of tumour and SDGA was as described in Section 2.2.2.1.

Variability in soluble oestrogen receptor across the same tumour. Sucrose density gradient analysis of sections obtained randomly.

Tumour	Section	ER_c $\frac{\text{fmoles}}{\text{mg Protein}}$ (one point DCC assay)	Variation Obtained A : B	ER_c from SDG	Molecular Form 4S	Molecular Form 8S (aggregate)	ER_c $\frac{\text{fmoles}}{\text{mg DNA}}$ ND = not determined	Variation Obtained
1. JD ♂	A	70	1.0	88	47	21 (20+)		
Breast	B	957	13.7	1131	203	151 (777)	ND	ND
2. MK	A	201	1.0	189	74	115 (ND)	2,111	1.0
Axilla	B	198	1.0	152	38	114 (ND)	4,236	2.0
3. AW ♂	A	189	1.0	170	13	157	15,653	1.0
R. Breast	B	231	1.2	215	36	179	22,705	1.5
4. CH	A	29	1.0	-	-	-	1,648	1.0
+/o Breast	B	28	1.0	-	-	-	1,611	1.0
5. R ♂	A	15	-	-	-	-	ND	ND
Breast	B	-	-	-	-	-		ND
6. DM ♂	A	19	1.0	20	6	14	3,412	2.8
Breast	B	76	4.0	71	15	56	1,232	1.00
7. EK	A	149	2.4	121	63	58 (+)		
L. Breast	B	62	1.0	47	26	21 (+)	ND	ND
+/o	C	151	2.4	101	59	42		
	D	107	1.7	72	27	45		
8. GM	A	76	1.0	84	61	23	ND	ND
Breast	B	115	1.5	114	67	47		
9. SCU	A	177	1.0	127	54	52 (21+)	ND	ND
Breast	B	625	3.5	538	249	101 (188+)		
10. SCR	A	337	1.0	290	0	290	ND	ND
Breast	B	388	1.2	334	0	234		
11. AP	A	409	7.9	389	0	389	ND	ND
R. Breast	B	52	1.00	43	0	43		
12. HB	A	280	1.0	291	106	171 (14)	ND	ND
L. Breast	B	292	1.0	277	136	118 (23)		

Figure 28.1. **Intratumoural Variation in the Sedimentation Profile of Oestrogen Receptor from Human Breast Tumour Cytosol (Random Sections)**

Two sections of human breast tumour were selected randomly for analysis. Cytosol was prepared, labelled, stripped of free steroid and analyzed as described in Section 2.2.2.1 (patient CB). Protein concentration was 8.2mg/ml in A and 3.2mg/ml in B. Receptor concentration in A was 76fmoles/mg protein and in B, 115fmoles/mg protein. Percentage recovery was near 100% in both A and B. Centrifugation was at $4^{o}C$ for 14h at 45,000 rev/min in a Beckman SW 50.1 rotor.

Intratumoral variation in the sedimentation profile of ER_c from human breast tumor cytosol (random selection)

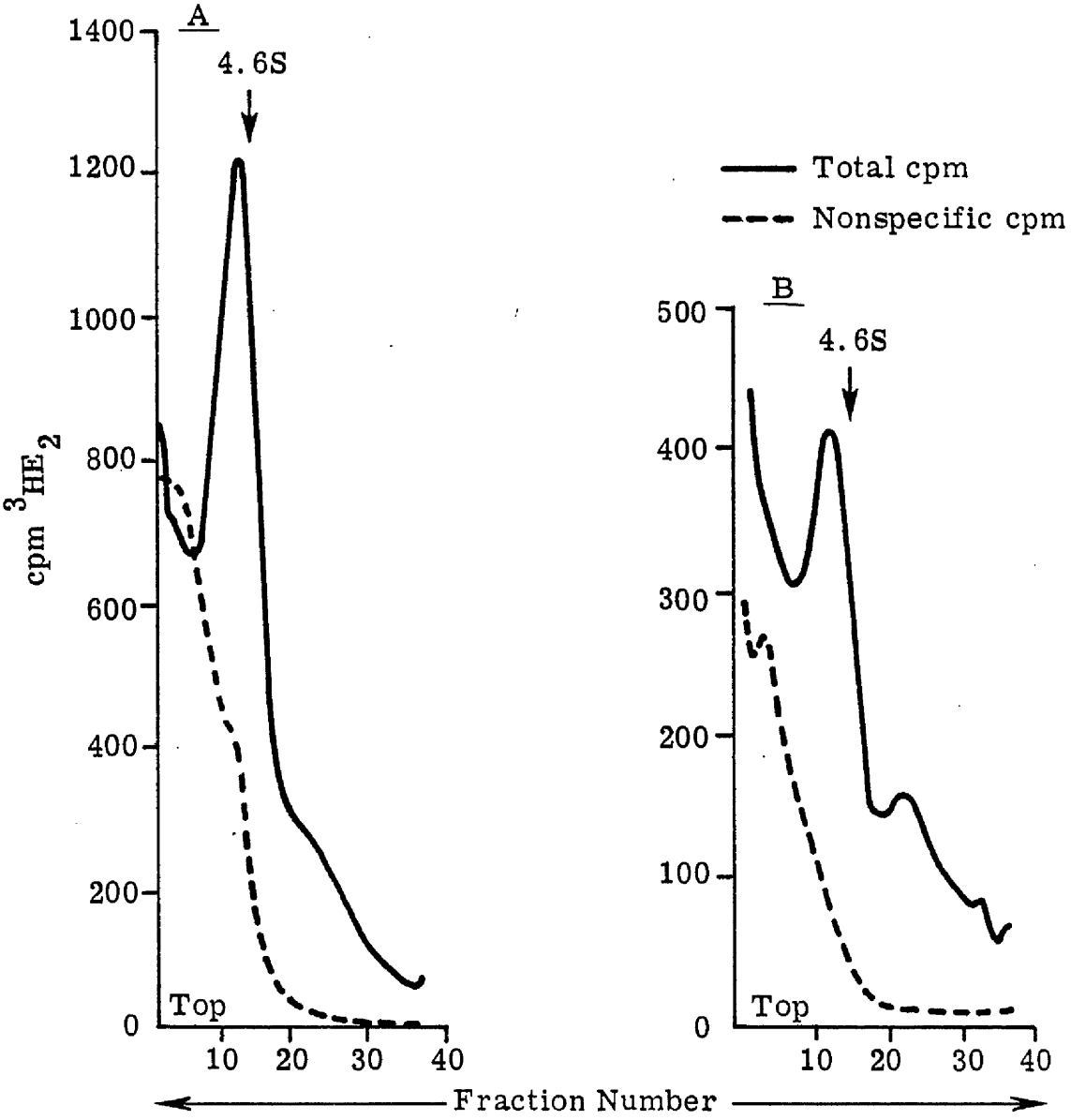
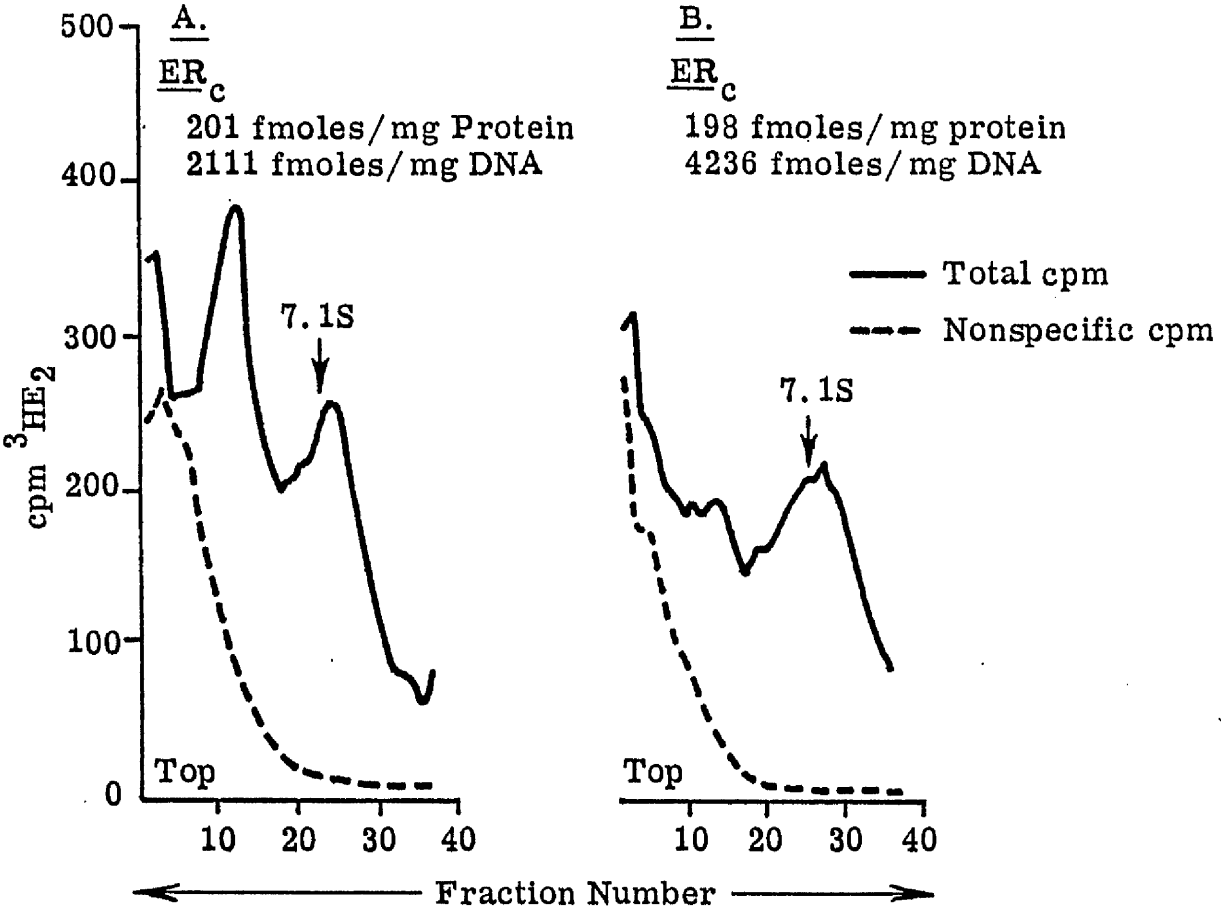


Figure 28.2. **Intratumoural Variation in the Sedimentation Profile of Oestrogen Receptor from Human Breast Tumour (Random Sections)**

Two sections of human breast tumour cytosol were selected randomly (Figure 9) for analysis. Cytosol preparation and SDGA was as described in Section 2.2.2.1. Details are given in Figure 24. Percentage recovery was 94% in A and 80% in B.

Intratumoral variation in the sedimentation profile of ER_c from human breast tumor cytosol (random selection)



Tumour number 7 in Table 6 was reported as ER_c^+/ER_n^- clinically. This was based on one section analyzed. However for a further four sections, Scatchard analysis showed that two of these could be classified into ER_c^+/ER_n^+ class (data provided by M. McMenamin). When a further three sections were analyzed by both Scatchard and gradient analysis, all sections showed 4S + 8S profile, yet one of these sections proved to be ER_c^+/ER_n^- type. This implies that 8S was present in a +/o sample. This may question the validity of the idea that the presence of the 8S form predicts patients response (Wittliff and Savlov, 1975).

As previously indicated, some tumours showed a considerable proportion of ER_c sedimenting to the bottom of the tube (tumour number 1 and 9 in Table 6). Receptor assays based only on SDGA could seriously underestimate total ER_c if the tube-bottom associated receptor were not taken into account.

Table 7 shows nine sections studied from a very large axillary node mass. The 8S form is predominant throughout. This result, in conjunction with that of tumour number 2 in Table 6, suggests that axillary nodes are more homogeneous than primary tumours in terms of malignant cell population.

3.1.3.4. Summary of Results of Intratumoural Study

Variation was found in receptor concentration across individual tumours. The peripheral and intermediate sections contained a higher proportion of receptor relative to the central sections. As judged from both selected and random sections, the molecular status of a tumour qualitatively was constant throughout. The 4S : 8S ratio, however, did show variation between sections from the same tumour. In only 4 cases out of 46 (9%) where both 4S and 8S could be detected was the intratumoural

Table 7. Variability in Soluble Oestrogen Receptor in Random Sections from an Axillary Tumoural Mass

A large axillary tumoural mass was divided into several sections and anlyzed as described in Section 2.2.2.1.

Variability in Soluble Oestrogen Receptor in Random Sections from an Axillary Tumoral Mass (BM)

Section	ER_c $\frac{\text{fmoles}}{\text{mg Protein}}$ * (from DCC one point assay)	Variation Obtained	ER_c from SDG $\frac{\text{fmoles}}{\text{mg Protein}}$	Molecular Form 4S	Molecular Form 8S	ER_c $\frac{\text{fmoles}}{\text{mg DNA}}$	Variation Obtained	$\frac{\text{Protein}}{\text{DNA}}$
A	349	1.18	293	51	242	2593	3.11	35
B	340	1.15	364	32	332	2399	2.88	35
C	350	1.19	371	43	328	1788	2.15	20
D	402	1.36	414	18	396	1573	1.89	20
E	400	1.36	412	34	378	833	1.00	7.5
F	295	1.00	304	20	284	ND	ND	ND
G	315	1.07	324	33	291	ND	ND	ND
H	516	1.75	531	23	508	ND	ND	ND
I	400	1.36	412	34	378	ND	ND	ND

$\bar{x}$ = 374 $\bar{x}$ = 380

SD = 62 SD = 69

* represents the concentration of oestrogen receptor loaded onto SDG.

ND = Not determined

change large enough to have altered the actual classification of molecular status e.g. 8S⟶8S + 4S or 8S + 4S⟶4S. This assumes that greater than 70% of one receptor species makes the tumour predominant in that species.

3.1.4 Effect of Mixing Breast Tumour Cytosol with Immature Rat Uterine Cytosol

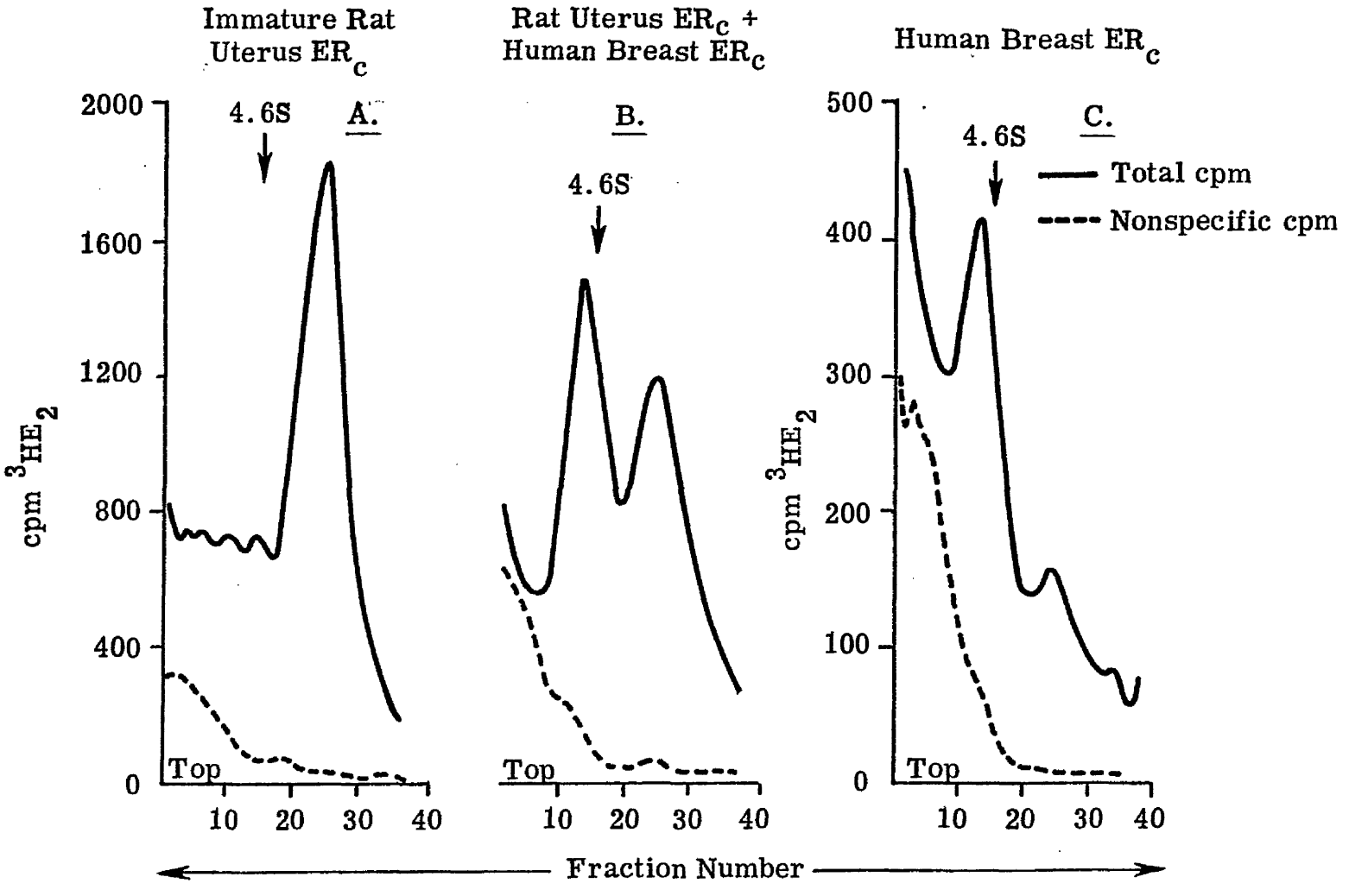
The previous section demonstrated that the 4S and 8S form of ER_c can co-exist in different proportions in different tumours and even within the same tumour. This most probably reflects an effect of variable levels of protease(s) acting on the 8S form (or the salt dissociated 4S aggregatable type). Immature rat uterine ER_c sediments primarily as an 8S complex. Advantage of this was taken to see if the human tumour protease(s) recognises and proteolyses rat receptor. Figure 29 shows that, indeed, such an effect was observed with a quantitative conversion of rat 8S receptor into the 4S form. No loss of oestrogen binding capacity was observed suggesting a very specific site(s) for the protease action (a bifunctional domain of receptor aggregation site(s) and receptor oestrogen binding site, on the same receptor molecule, with considerable distance between them). It is not known if a complete conversion of 8S into the 4S form would have taken place over a longer incubation period. Neither was the effect of a tumour cytosol containing mainly 8S receptor tested. It is quite possible that tumours showing predominantly 8S complex may have a lower proteolytic effect. The tumour cytosol used in Figure 29 was of the 4S + 8S type.

Some preliminary data (not shown) indicated that mixing human breast tumour cytosol with immature rat uterine cytosol prevents the 4S⟶5S conversion associated with immature rat uterine transformation reaction. This is in agreement with results presented by Sato *et al.* (1981a, b) and lends further support to the alteration of receptor

Figure 29. **Effect of Mixing Cytosol from Immature Rat Uterus with Cytosol Obtained from Human Breast Tumour**

Human breast tumour cytosol was prepared as described in Section 2.2.2.1. (patient GM; protein concentration 6.4mg/ml). Immature rat uterine cytosol was prepared as described in Section 2.2.2.4.1 (protein concentration 2.1mg/ml). Prior to labelling, an equal volume of human breast tumour cytosol and immature rat uterine cytosol were mixed and left standing for 1h at $4^{o}C$ (B). As control, another set of aliquots were prepared by adding equal volume of buffer to immature rat uterine cytosol (A) and similarly to human breast tumour cytosol (C). This was followed by incubating aliquots for an additional 1h at $4^{o}C$, with $5 \times 10^{-9}M$ $^{3}HE_{2}$ in the absence (——) or presence (....) of $5 \times 10^{-6}M$ DES. Free steroid was then removed with DCC and 200ul aliquots loaded on 5-20% gradients. Centrifugation was for 14h at 45,000 rev/min at $4^{o}C$ in Beckman SW 50.1 rotor.

8S to 4S ER_c transforming activity in human breast carcinoma cytosol and rat/human mixed cytosols



structure by an activity present in tumour cytosol.

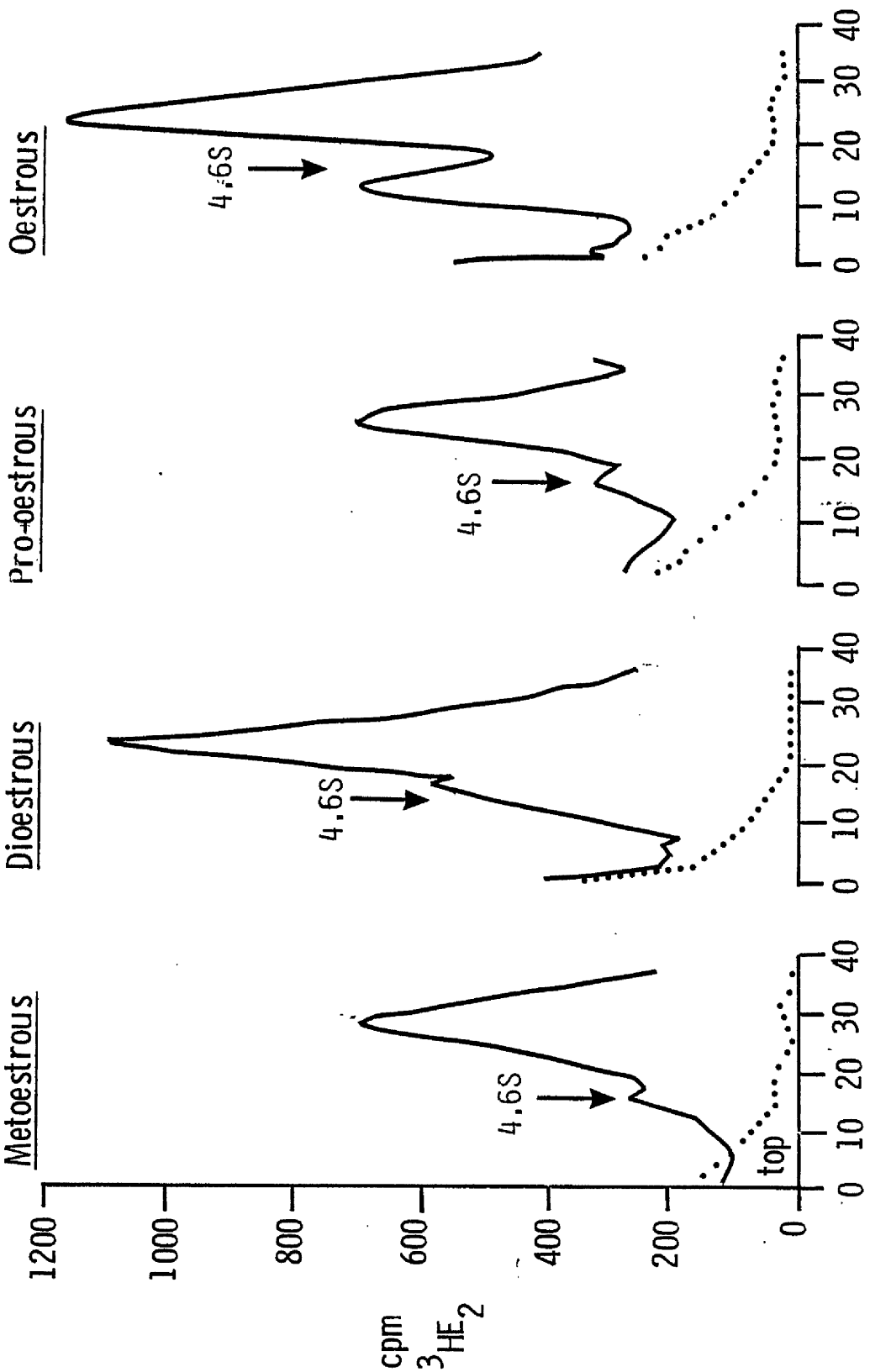
3.1.5. Sedimentation Profile of Mature Rat Uterine Oestrogen Receptor at Various Stages of Oestrous Cycle

The previous observations of 8S⟶4S conversions as a proteolytic sleavage step prompted an investigation of mature rat uterine soluble oestrogen receptor sedimentation profile at various stages of the oestrous cycle. It was possible that there might be a stage at which an exclusive 4S form could be located. This would therefore provide a rich and a convenient source of protease activity for further characterization of its significance. Previous observations have indicated that a pro-oestrous, the DMBA induced mammary carcinoma lack the 8S form (Freedman and Hawkins, 1980). At pro-oestrous plasma oestradiol concentration is highest. Two possible explanations could, therefore, be that (i) all functional (8S) receptor is now present in the nuclear compartment leaving the non-functional receptor (4S) in the cytoplasm or (ii) there is an abundance of oestrogen induced protease-like activity (cytosol or nuclear) promoting 8S⟶4S conversion. An oestrogen induced trypsin-like activity has been reported (Katz et al., 1976). Muldoon (1977) has also related plasma oestradiol concentration to the 4S and 8S forms in mouse mammary tissue. Interconversion of 4S and 8S forms of oestrogen receptor is also found in normal rat mammary gland during pregnancy and lactation (Mohla et al., 1981).

Figure 30 shows that in the rats, undergoing a 4 day oestrous cycle, uterine cytosols show the presence of 8S receptor at all stages. The 4S form can also be detected at every stage but it seems to be minimal at dioestrous, the time at which oestrogen concentration is lowest, and highest at about oestrous, the time at which the uterus has just passed

Figure 30. **Sedimentation Profile of Oestrogen Receptor from Mature Rat Uterus at Various Stages of the Oestrous Cycle**

Preparation of mature rat uterine cytosol is described in Section 2.2.2.4.2. Cytosol was labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 1h at 4^{o}C. Unbound steroid was removed with DCC. Aliquots were then loaded onto 5-20% gradients which were centrifuged for 14-16h at 45,000-50,000 rev/min in SW 50.1 rotor at 4^{o}C. Arrow indicates the position of ^{14}C-BSA marker protein. The receptor concentrations in fmoles/mg protein [mean $\pm$ SD (number of analysis)] were (i) metoestrous, 194 $\pm$ 40 (3), (ii) dioestrous, 275 $\pm$ 50 (3), (iii) pro-oestrous, 158 $\pm$ 13 (3) and (iv) oestrous, 292 $\pm$ 8 (3).


Metoestrous
Dioestrous
Pro-oestrous
Oestrous
4.6S
top
1200
1000
800
600
400
200
0
cpm
$^3HE_2$
0 10 20 30 40
Fraction number

the maximal exposure to oestrogen. Once again this would favour the view that there is an oestrogen-induced enzyme capable of regulating receptor molecular form *in vitro*.

3.2 ACTIVATION/TRANSFORMATION STUDY

3.2.1 Oestrogen Receptor Binding to DNA-Cellulose as a Measure of Extent of Activation

3.2.1.1 Conditions for DNA-Cellulose Binding Assay

3.2.1.1.1 Influence of Non-Specific Proteins on Extraction of Steroid from DNA-Cellulose

To measure the non-specific retention of tritiated oestradiol-17β ($^3HE_2$) on the DNA-cellulose slurry, $HDK._{15}$ buffer was made 5×10^{-9}M with respect to $^3HE_2$ in the presence or absence of receptor-free cytosol. This cytosol was prepared from an ER_c^-/ER_n^- tumour, thus providing approximately the same protein environment as when ER_c^+/ER_n^+ cytosol was analyzed for receptor activation. The exact procedence described in Section 2.2.4.1.1 to measure receptor activation was then followed. After cooling to 4^oC, 200ul aliquots of activated cytosol (or control) were introduced into a series of tubes containing increasing concentration of DNA-cellulose (DNA range 20-200ug). Table 8 demonstrates that, when using ER_c^-/ER_n^- tumour cytosol in $HDK_{.15}$, there is complete recovery of total counts. A small but insignificant retention of counts was noted with increasing DNA-cellulose concentration. Similar results were obtained if $HDK_{.15}$ was used in place of buffered cytosol. DNA-cellulose, under these conditions, does not retain free steroid.

3.2.1.2 Studies with Immature Rat Uterine Soluble Oestrogen Receptor

3.2.1.2.1 The Effect of Washing DNA-Cellulose after Incubation with Cytosol Containing Activated and Non-activated Receptor

The effect of washing on binding of steroid-receptor complexes to DNA-cellulose was tested using both heated (30^o, 30') and control

Table 8. Influence of non-specific Protein on Extraction of Steroid from DNA-Cellulose

Human breast tumour cytosol was prepared from a receptor negative tumour (Section 2.2.3.2) (protein concentration 6.6mg/ml) and was made 5 x 10^{-9}M with $^{3}HE_{2}$ ± 1000 fold excess DES. After a 1h incubation at 4°C the cytosol was transferred to 30°C for 30 min and then incubated with increasing concentration of DNA-Cellulose in duplicate. The binding assay was as described in Section 2.2.4.1 and 2.2.4.1.1 and the washes were analyzed for radioactivity. Values represent mean of two determinations and the variation did not exceed more than 3% of the quoted values.

μg DNA in DNA-Cellulose Slurry	Wash cpm					Salt Extracted cpm	Alcohol Extracted cpm	cpm Recovery (%)
	1	2	3	4	5			
20	62319	3623	561	107	42	30	96	66778 (102)
40	59924	5315	894	206	52	57	127	66575 (102)
60	57334	6459	1240	328	126	69	161	65717 (101)
80	56509	7072	1597	439	132	99	195	66043 (101)
100	54370	8269	1979	528	175	127	209	65657 (101)
150	50587	9308	2563	824	291	183	299	64055 (98)
200	50509	10758	3216	1058	384	225	370	66520 (102)

cytosol (Section 2.2.4.1.1). After each wash the supernatant obtained was checked for specific receptors, using the DCC assay. This was the method used by Park and Wittliff (1977) in their DNA-cellulose study of activation. However, Table 9.1 shows that the DCC treatment probably results in a gross underestimation of the total receptor bound to DNA. (It was shown in Table 8 that free steroid is not retained by DNA-cellulose, therefore the 25,383 counts obtained from DNA cellulose slurry (Table 9.1) after incubation with activated cytosol most probably represent specific receptor. After incubation with DCC the salt extracted supernatant only registered 3,726 counts). Even so, activation does result in a greater proportion of receptor bound to DNA. As has been previously indicated both ionic strength and protein concentration can influence the DCC assay (Section 2.2.4.2). Table 9.1 also shows that most of the free counts were removed by Wash 5. Further washing was therefore not carried out, although employing an analogous procedure, Sato et al.(1981a) concluded that no loss of DNA-bound receptor occurs even after extensive washing.

Table 9.2 shows that, when using DES competitor, no significant counts are retained by the DNA cellulose slurry and, therefore, the salt extracted counts from incubation in the absence of competitor do represent specific receptor. Similarly alcohol failed to extract any further counts from incubation tubes containing competitor. However, there were alcohol extractable counts present in tubes incubated in the absence of DES competitor. This implies that a single extraction with 0.6M KCl-containing buffers fails to extract total receptor.

3.2.1.2.2 **Sedimentation Properties of Receptor Recovered from DNA-Cellulose**

To check that DNA-bound receptor did not represent aggregated material or non-specific retention of $^{3}HE_{2}$, SDGA was carried out. After salt extraction of receptor from DNA-cellulose an aliquot (200ul from

Table 9.1 Effect of DCC Treatment on Supernatant Obtained from DNA-celulose Washings and Salt Extraction

Immature rat uterine cytosol (protein concentration 3.8mg/ml) was prepared, labelled and taken through the DNA-cellulose binding assay in duplicate as described in Section 2.2.4.1 and 2.2.4.1.1. The supernatants obtained on washing DNA-cellulose or on salt extraction (0.6M KCl) was divided into two halves. One of these was treated with DCC at 0.25% w/v (final concnetration) as described in Section 2.2.4.1. Values represent mean of two determinations and the variation did not exceed 3% of the quoted values.

	4^{o}C (non activated)				30^{o}, 30' (activated)			
	Total cpm		Non specific cpm		Total cpm		Non specific cpm	
	-DCC	+DCC	-DCC	+DCC	-DCC	+DCC	-DCC	+DCC
Wash 1	57943	32138	62393	4847	24755	11629	60866	2558
Wash 2	4906	894	8241	-	3505	816	9118	-
Wash 3	1148	-	1686	-	1223	-	1946	-
Wash 4	693	-	491	-	1003	-	1201	-
Wash 5	512	-	226	-	958	-	260	-
Salt Extracted	6833	818	383	-	25383	3726	370	-
Alcohol Extracted	2103	-	405	-	7591	-	413	-
Total	74138		73825		64418*		74124	

*total cpm recovery was near 100% except in this case where total cpm recovery was only 73%. The fate of remaining counts was not determined but this is not due to cellulose retention as further experiments confirmed. An experimental error in aliquoting radioactivity is suspected.

Table 9.2. **Analysis of Salt Extracted DNA-cellulose Bound Counts in the Presence of Competitor and in the Absence of any DCC Treatment of the Supernatant**

The procedure was the same as that described for Table 9.1 except that there was no DCC treatment of supernatant obtained from washings or salt extraction. Aliquots from salt extracted receptor (40% of the counts shown) were loaded onto SDG (Figure 31).

	Non-activated Cytosol 4^{o}, 30'		Activated Cytosol 30^{o}, 30'	
	$^{3}HE_{2}$	$^{3}HE_{2}$ + DES	$^{3}HE_{2}$	$^{3}HE_{2}$ + DES
Wash 1	39,652	53,540	22,050	53,318
Wash 2	3,857	7,585	3,135	8,230
Wash 3	1,392	1,915	1,315	1,872
Wash 4	978	560	1,018	597
Wash 5	782	208	997	256
Salt Extract	9,673	405	25,634	487
Alcohol Extract	4,766	393	10,340	435
Total	61,100	64,606	64,489	65,195

500ul total extraction volume - Table 9.2) was loaded onto a 5-20% linear sucrose density gradient prepared in either HED or $HEDK_{.4}$ (Figure 31). Salt extraction represents a relatively purified form of receptor. The purified receptor is known to aggregate (Puca et al., 1980) and this was seen when the receptor was loaded onto the gradient prepared in HED (Figure 31A). There was also a trailing effect of free steroid towards the top of the gradient. However when a high salt gradient was used (Figure 31B), the aggregation was retarded, although there was still a trailing effect. In the high salt gradient, the bulk of the receptor sedimented after the ^{14}C-BSA marker (4.6S) as a broad peak. No sedimentation peak was seen in the gradient containing salt extracted material from tubes containing the competitor DES. The counts observed at the top of the gradient could not be free $^{3}HE_{2}$ which had been trapped in the DNA-cellulose since, if this was the case, similar counts would have been observed in the gradient of the DES competition control (.....) in Figure 31A and B . Further as is shown in Figure 32A and B, the counts extracted from DNA-cellulose after incubation with activated cytosol and washing (Section 2.2.4.1.1), will sediment as a sharp peak (after the ^{14}C-BSA marker) if ovalbumin (Figure 32A) or BSA (Figure 32B) is included in the KCl extraction buffer (4mg/ml) and the centrifugation buffer (200ug/ml). The best results were obtained when BSA was included as the carrier protein both in the extraction and gradient buffers (Figure 32B - sedimentation peak 5.5S). This is probably because BSA provides the best protection of receptor in the 4-5S area, being a molecule of similar size. The recovery of counts from the gradient was low in the absence of added proteins but with the carrier protein in the gradient, recovery was increased to approximately 60%. If the whole centrifuge tube was extracted with alcohol after removal of the sucrose gradient, recovery could be increased to 75%.

Figure 31. **Analysis of Salt-extracted Receptor from DNA-cellulose on Low and High Salt Gradients**

The DNA-cellulose binding assay of immature rat uterine cytosol receptor was as described in Section 2.2.4.1 and 2.2.4.1.1. After the binding assay, the receptor was extracted with $HDK._6$ from DNA-cellulose and 200ul aliquots were loaded onto 5-20% gradients prepared in either HED(A) or $HDK._4$ (B). Centrifugation was for 16h at 4^oC at 45,000 rev/min in a Beckman SW 50.1 rotor. Profiles in the absence (——) or presence (....) of DES are shown. The percentage recovery of salt extracted counts in A (——) was 77% (comprising 34% within the gradient, 10% at the bottom of the tube and 33% from the walls of the tube extracted with alcohol). The percentage recovery in B (——) was 85% (comprising 44% within the gradient, 3% at the bottom of the tube and 38% attached to the walls of the tube). The sedimentation of receptor in B was as a broad 5S peak.

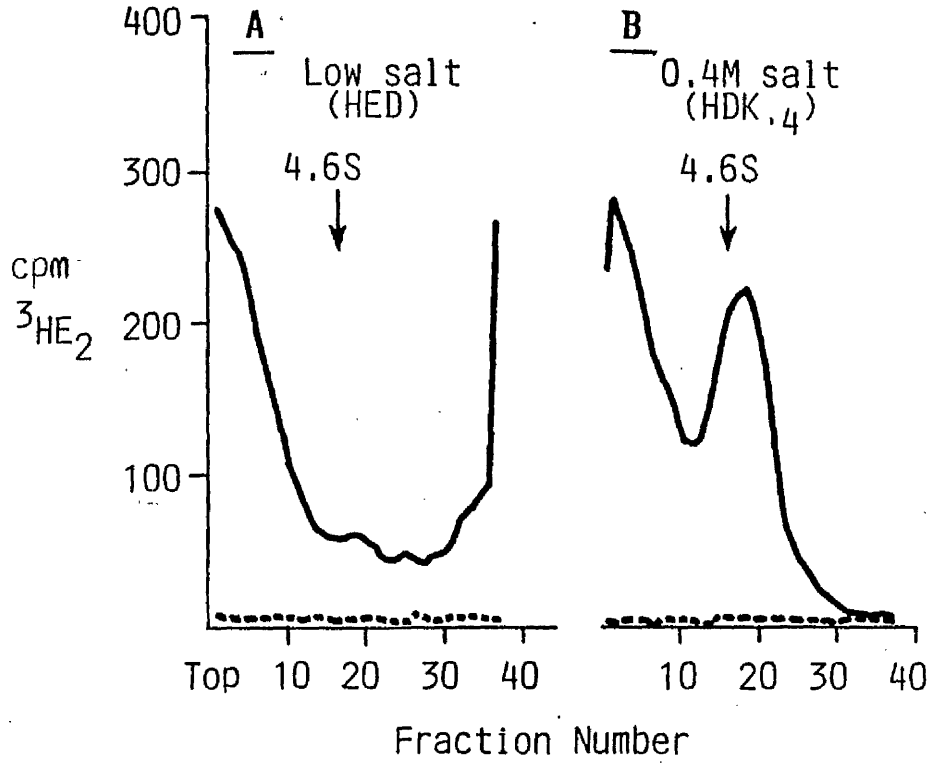
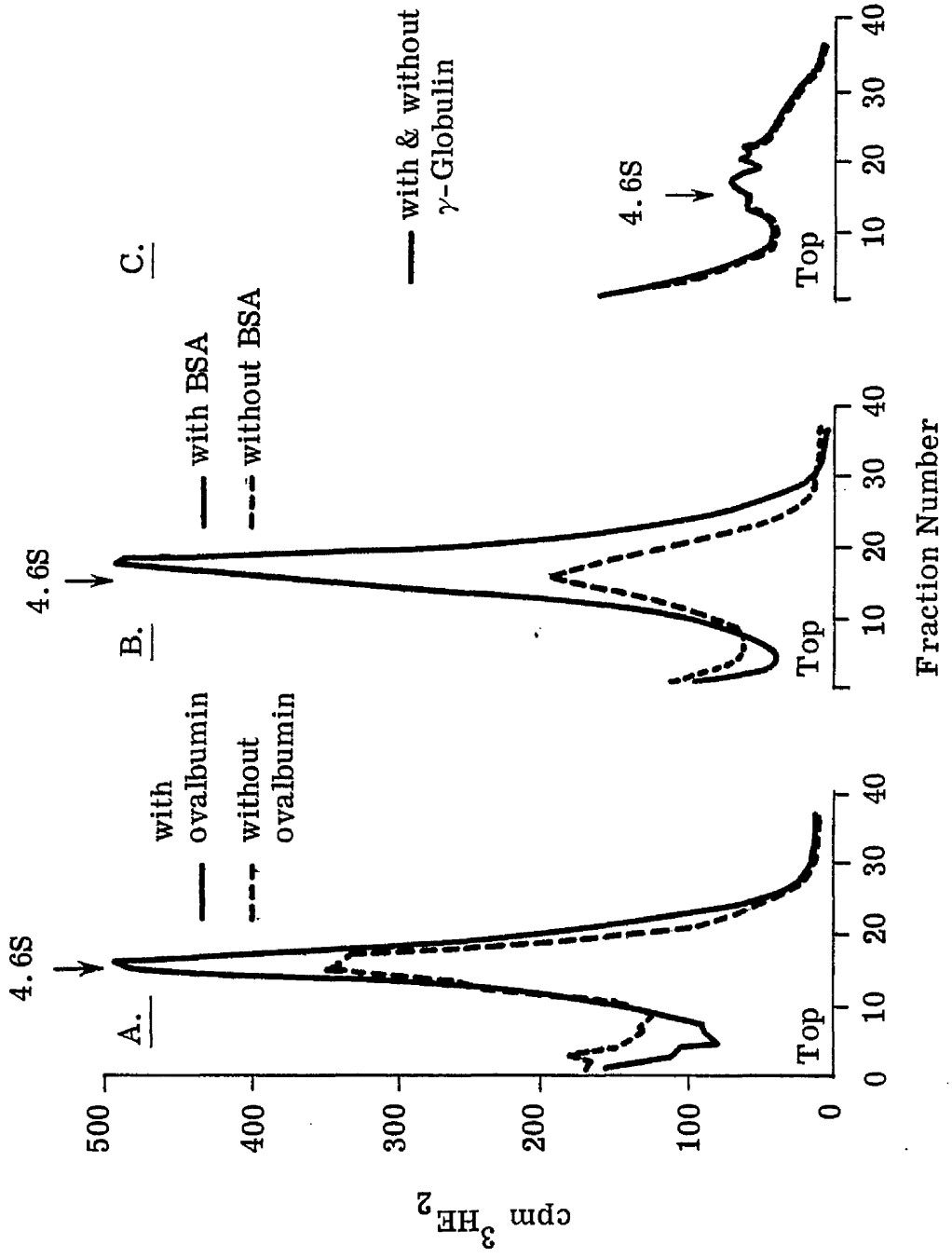

400
300
200
100
cpm
$^3HE_2$
A
Low salt
(HED)
4.6S
B
0.4M salt
($HDK_{.4}$)
4.6S
Top 10 20 30 40
10 20 30 40
Fraction Number

Figure 32. **Influence of Addition of Selected Proteins on the Sedimentation Profile of Salt Extracted Receptor from DNA-cellulose.**

The DNA-cellulose binding assay of immature rat uterine cytosol receptor was as described in Section 2.2.4.1 and 2.2.4.1.1. After the binding assay, the receptor was extracted with $HDK._6$ containing 4mg/ml of (A) ovalbumin, followed by analysis on gradient in the presence (——) or absence (....) of 200ug/ml ovalbumin or (B) BSA, followed by analysis on gradient in the presence (——) or absence (....) of 200ug/ml BSA, or (C) γ-globulin, followed by analysis on gradient in the presence (——) or absence (....) of γ-globulin (200ug/ml). Gradients were 5-20% (w/w) prepared in $HDK._{15}$, and centrifugation was at 4^oC for 15h at 45,000 rev/min in a Beckman SW 50.1 rotor. Arrow indicates the position of ^{14}C-labelled marker protein. There was no difference in the DNA bound receptor extraction efficiency of $HDK._6$ in the absence or presence of 4mg/ml protein. However, extraction in the presence of γ-globulin was only 50% that of extraction in ovalbumin or BSA. Percentage recovery of total counts (gradient + alcohol extraction of the whole tube) was (A) 60% (....) and 79% (——), (B) 55% (....) and 72% (——) and (C) 47% (....) and 58% (——). The sedimentation peak in the presence of protein in the gradient is 5.0S (A) and 5.5S (B).


A.
B.
C.
4.6S
4.6S
4.6S
with ovalbumin
without ovalbumin
with BSA
without BSA
with & without γ-Globulin
cpm $^3HE_2$
500
400
300
200
100
0
Top
10
20
30
40
Fraction Number

An unexpected result was obtained when using human-γ-globulins in the KCl extraction buffer and the gradient buffer. When the same cytosol incubation as that shown in Figure 32A and B was extracted with KCl buffer containing 4mg/ml of human-γ-globulins, a very low concentration of receptor was solubilized (Figure 32C). The reason for this is not clear.

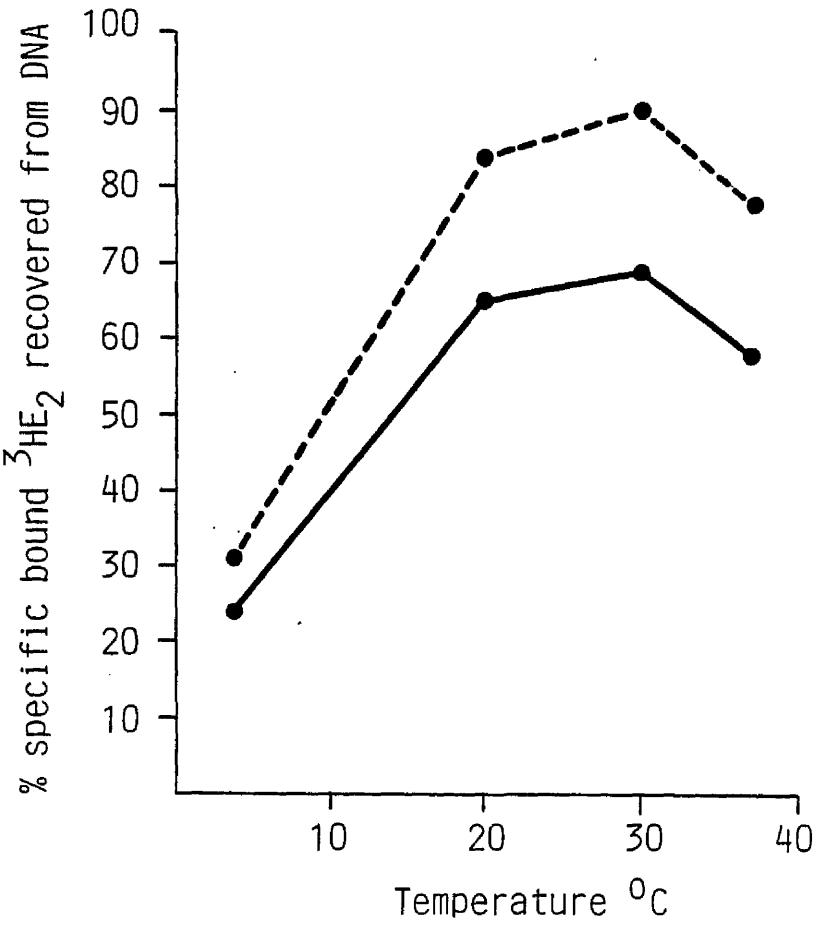
Thus the molecular form of receptor bound to DNA-cellulose is 5S. Further, inclusion of non-specific (DES) values is unnecessary in DNA-cellulose binding experiments when only the level of activation is to be determined. However, an estimate of total and non-specific binding of the cytosol used for the assay is needed to quantitate the specific receptor present, bound to DNA. Exclusion of a DES value from the binding assay only applies when quantitating activated receptor from immature rat uterine cytosol. In the case of human breast tumour ER_c activation assay, the total DNA bound counts are relatively low (Section 3.2.1.3) and, therefore, DES values must be included.

3.2.1.2.3 Temperature Dependence of Activation of Oestradiol-Receptor Complexes

Using a constant time of 30 min, the optimum temperature for maximum activation was determined, as measured by binding of activated $^3HE_2$-receptor complexes to DNA cellulose (Figure 33). It can be seen that when the cytosol was heated at 30°C for 30 min (30°, 30') in the presence of $^3HE_2$, maximum binding to DNA-cellulose was obtained. Under these conditions approximately 90% of the specific counts were bound to DNA cellulose. Slight variation in maximum binding was obtained, as discussed later (3.2.1.2.7). A significant proportion (∿30%) of receptor was bound to DNA even from the non-heated control. This presumably reflects activation in $HDK_{.15}$ at 4°C. If the labelled

Figure 33. **Effect of Temperature on Activation of Immature Rat Uterine Oestrogen Receptor**

Immature rat uterine cytosol was prepared and labelled at 4°C for 1h as described in Section 2.2.4.1 (protein concentration 3.6mg/ml, receptor concentration 3.1nM). Aliquots of cytosol were then incubated at the indicated temperatures for 30 min. These were then returned to 4°C and kept for a further 5 minutes. The DNA-cellulose binding assay was then carried out as described in Section 2.2.4.1 and 2.2.4.1.1. (●—●) salt extracted receptor, (●...●) alcohol extracted receptor + salt extracted receptor.


% specific bound $^3HE_2$ recovered from DNA
100
90
80
70
60
50
40
30
20
10
10
20
30
40
Temperature ^oC

cytosol was heated to 37°C for 30 min. rather than 30°C for 30 min, then there was a fall in the number of receptor complexes bound to DNA cellulose. This most probably resulted from aggregation (37°C promotes rapid aggregation of receptor, data not shown) or destruction of receptor by masking or destroying the DNA binding site.

3.2.1.2.4 **Time Course of Binding of Activated Receptor to DNA-Cellulose**

Figure 34 shows that after activation (30°, 30'), maximum binding of activated receptor to DNA-cellulose was observed within 1h. The alcohol extractable receptor binding population did not show time dependence indicating that certain sites on the DNA were rapidly filled. These may be the highest affinity binding sites (Spelsberg, 1976). The saturation of salt extractable counts possibly demonstrates the occupation of the lower affinity sites. Its quite possible that the lowest affinity receptor complex binding sites may dissociate during washing and perhaps explains why binding of 100% of receptor to DNA was not observed. Alternatively, there could be (i) an equilibrium between the activated and non-activated receptor (Atger and Milgrom, 1976) such that there is always a proportion of non-activated receptor, (ii) loss of some DNA during washings, (iii) dissociation of activated into non-activated form during washing, (iv) lack of activation factor(s) limiting the extent of activated receptor and (v) presence of a form of receptor which cannot undergo activation. Further, heating the cytosol invariably leads to some aggregation which could mask the DNA binding site on individual receptor molecules (Nishizawa et al., 1981, Sakly and Koch, 1982).

3.2.1.2.5 **Binding of a Constant Amount of Receptor to an Increasing Concentration of DNA-Cellulose**

It can be seen (Figure 35) that the use of 100ug DNA provided

Figure 34. Time course of Activated Receptor Binding to DNA-cellulose (Immature Rat Uterus)

Immature rat uterine cytosol was prepared and labelled at $4^{\circ}C$ for 1h as described in Section 2.2.4.1 (Protein concentration 3,8 mg/ml; receptor concentration 3.3 nM). Cytosol was then warmed to $30^{\circ}C$ for 30 min, cooled to $4^{\circ}C$ for 5 min. and DNA-cellulose binding assay performed, for the indicated length of time, as described in Section 2.2.4.1 and Section 2.2.4.1.1. (●—●) salt extracted receptor, (O—O) alcohol extracted receptor.

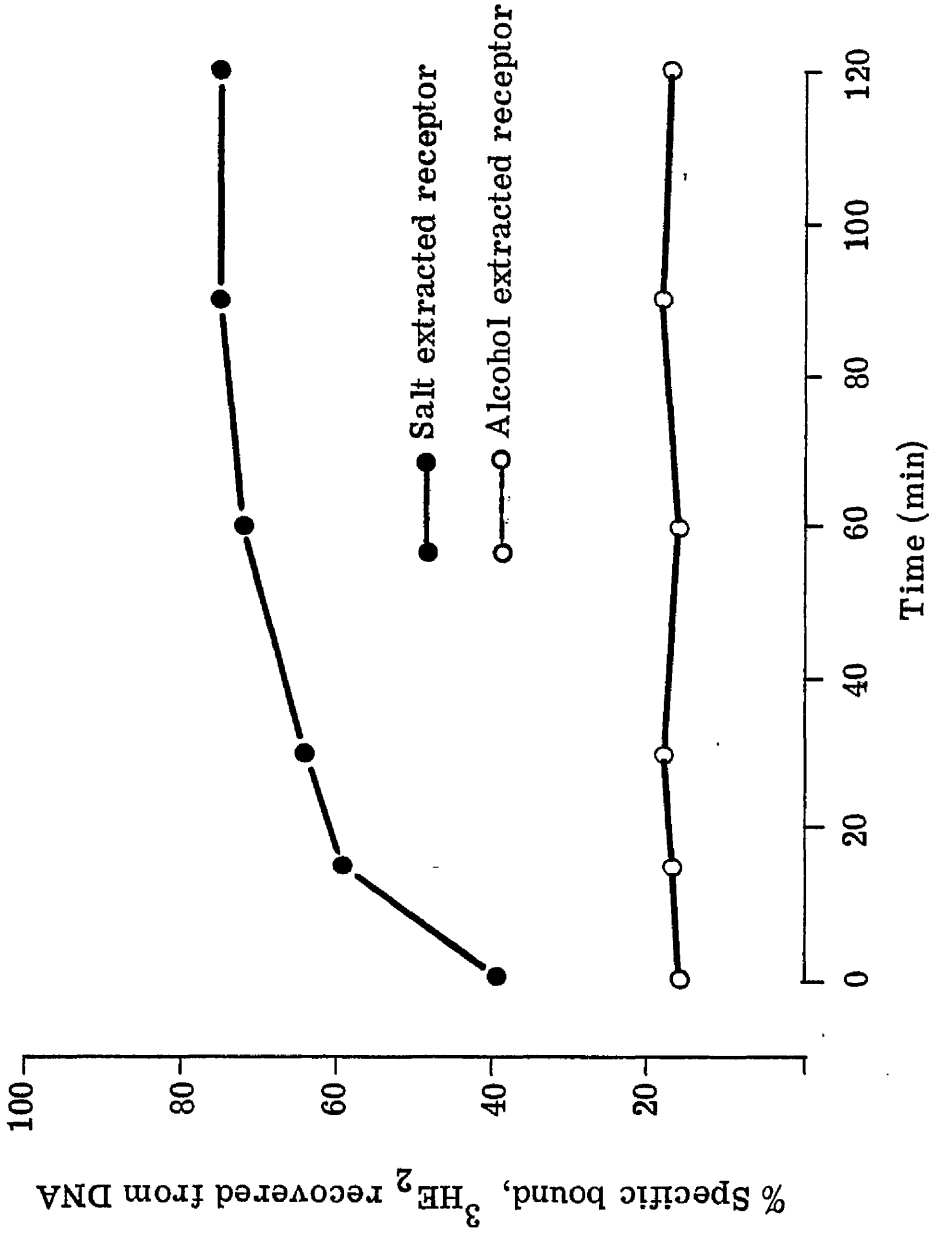
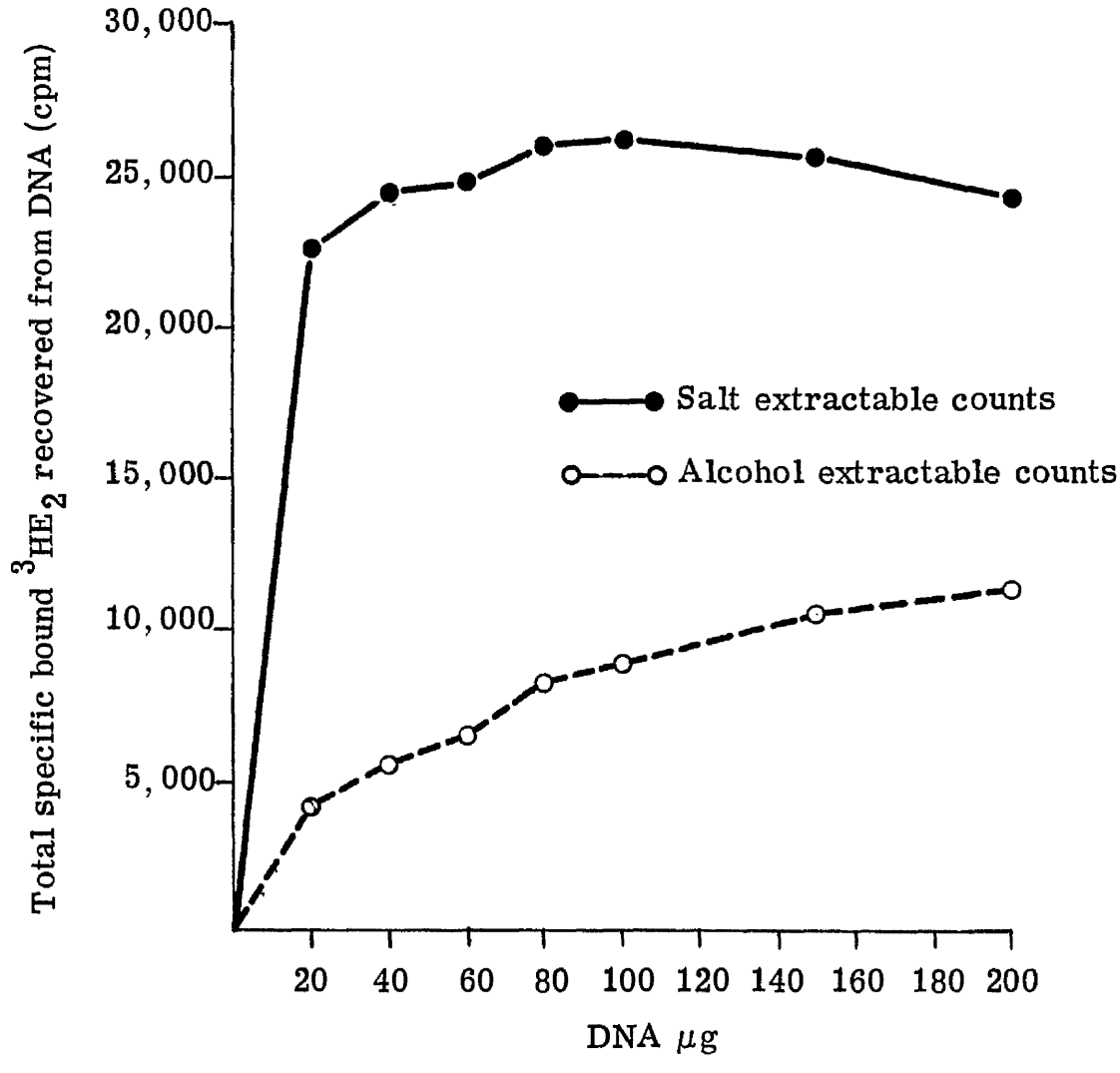

% Specific bound, $^3HE_2$ recovered from DNA
100
80
60
40
20
0
20
40
60
80
100
120
Time (min)
Salt extracted receptor
Alcohol extracted receptor

Figure 35. **Influence of Increasing DNA Concentration on Binding of Activated Oestrogen Receptor from Immature Rat Uterine Cytosol**

Immature rat uterine cytosol was prepared and labelled at 4°C for 1h as described in Section 2.2.4.1 (protein concentration 3.4mg/ml; receptor concentration 2.5nM). Cytosol was then warmed to 30°C for 30 min, cooled to 4°C for 5 min. and then incubated with increasing concentrations of DNA-cellulose (DNA 20-200ug). The DNA-cellulose binding assay was carried out as described in Section 2.2.4.1 and 2.2.4.1.1 (●——●) salt extracted receptor, (o...o) alcohol extractable receptor.


30,000
25,000
20,000
15,000
10,000
5,000
Total specific bound $^3HE_2$ recovered from DNA (cpm)
Salt extractable counts
Alcohol extractable counts
20
40
60
80
100
120
140
160
180
200
DNA μg

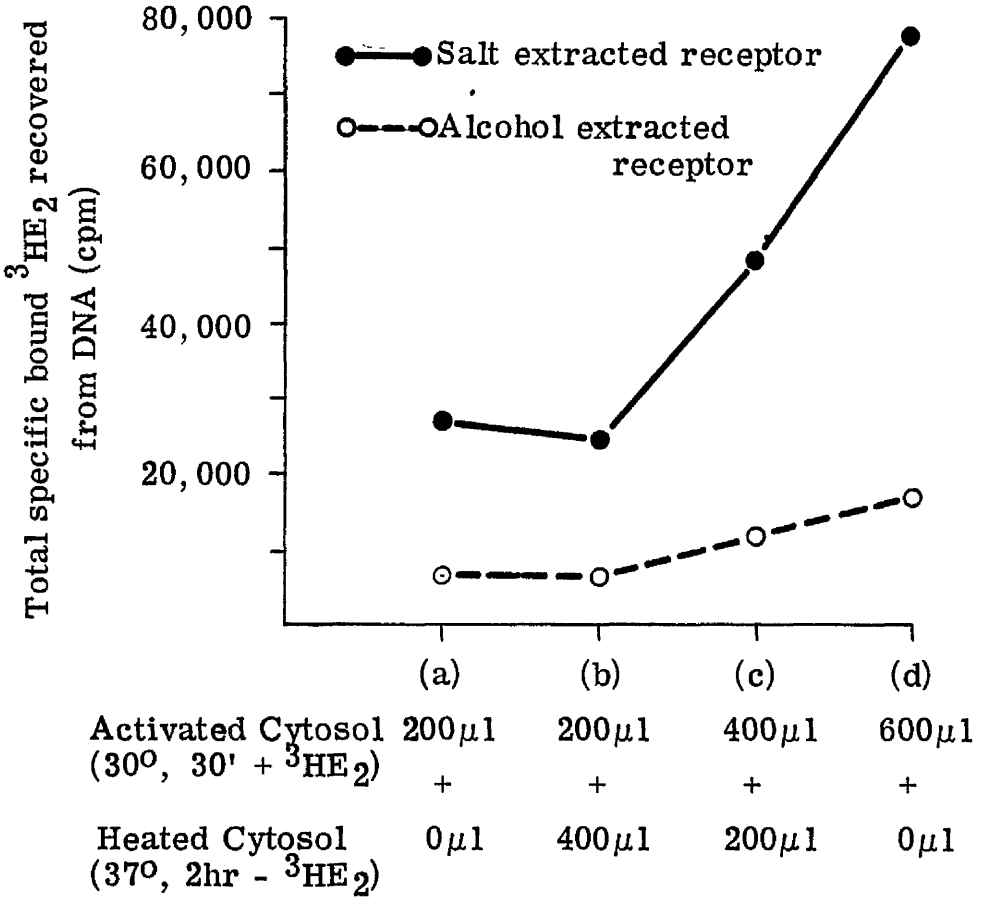
the highest level of bound $^{3}HE_{2}$-receptor complex after activation. Lower values of DNA also showed values close to maximum binding. The standard deviation obtained for each point within a single experiment was very low in all experiments using DNA-cellulose. Figure 35 further shows that saturation was obtained with the salt extractable counts, although binding began to drop when higher concentrations of DNA were used suggesting that the receptor was limiting. The alcohol extractable counts, however, seemed to increase with increasing concentration of DNA. The significance of this was not clear. However, if this was a genuine increase then it might further lend support to the concept that the high affinity receptor binding sites on the DNA are not salt extractable (Clark and Peck, 1979; the concept of salt resistant receptor is mainly derived from studies with whole nuclei).

3.2.1.2.6 **Binding of an Increasing Concentration of Activated Receptor to a Fixed Amount of DNA**

The optimum ratio of activated receptor to DNA-cellulose, should be the same whether it is the DNA or receptor concentration that is being varied. This was found to be indeed the case (Figure 36). In Figure 36(b) and (c), the concentration of activated receptor was adjusted with inactivated immature rat uterine cytosol (heated in the absence of steroid for 2h at 37°C) to give the same final volume and protein concentration. In Figure 36(a), however, there was no dilution and the activated cytosol was simply mixed with the DNA-cellulose slurry. The binding in Figure 36(a) was of the same order of magnitude as in Figure 36(b). The heated (37°C, 2h) immature rat uterine cytosol still showed $\sim$50% of the original counts intact. This, however, did not influence the linear relationship observed in Figure 36((b), (c) and (d)). If the heated cytosol was exchanging any steroid for that from the activated cytosol then there

Figure 36. **Influence of Increasing Activated Receptor Concentration on DNA-celulose Binding. (Immature Rat Uterine Cytosol)**

Immature rat uterine cytosol was prepared and labelled at $4^{o}C$ for 1h as described in Section 2.2.4.1 (protein concentration 3.4mg/ml; receptor concentration 3nM). Cytosol was then warmed to $30^{o}C$ for 30 min, cooled to $4^{o}C$ for 5 min. and then increasing concentrations of cytosol were incubated with 100ug DNA cellulose. In (a) there was no dilution of cytosol while in (b), (c) and (d) the volume was adjusted to 600ul with either the same cytosol which had been previously heated to $37^{o}C$ for 2h in the absence of steroid, or with buffer, as shown. The DNA-cellulose binding assay was carried out as described in Sections 2.2.4.1 and 2.2.4.1.1. (●—●) salt extracted receptor, (O---O) alcohol extracted receptor.


Total specific bound $^3HE_2$ recovered from DNA (cpm)
80,000
60,000
40,000
20,000
Salt extracted receptor
Alcohol extracted receptor
(a)
(b)
(c)
(d)
Activated Cytosol (30°, 30' + $^3HE_2$) 200µl + 0µl
200µl + 400µl
400µl + 200µl
600µl + 0µl
Heated Cytosol (37°, 2hr - $^3HE_2$)

should have been a difference in the extent of binding between Figure 36(a) and (b). This would have been the case if the heated cytosol contained any activated receptor (it should not since the heating was done in the absence of steroid). In addition, heating at 37°C leads to receptor aggregation (data not shown) and most probably loss or masking of the DNA binding site (Sakly and Koch, 1982). Thus heated cytosol (37°C, 2h) is inactive and not influencing DNA-binding of activated receptor (activated cytosol - 30°, 30'), when the two are mixed. It is further shown in Figure 36 that alcohol extractable counts also increase with increasing activated cytosol incubation with a fixed amount of DNA-cellulose (cf. Fig.35).

3.2.1.2.7 **Percentage Binding of Soluble Receptor from Activated and Non-activated Cytosol and Extent of Salt and Alcohol Extraction**

Table 10 shows the total binding obtainable with the activated receptor from three different experiments. A higher proportion (~80%) of receptor from the activated cytosol binds to DNA when compared to control (28%). Approximately 70% of the DNA-bound receptor was salt extractable, using a single extraction. This is in agreement with published results of Katzenellenbogen et al. (1980) using intact immature rat uteri nuclei. Variation between experiments was obtained in the total amount of receptor bound to DNA-cellulose from the activated and non-activated cytosol, yet the ratio of salt and alcohol extractable receptor was always similar for both conditions. This implies that non-activated cytosol contains a proportion of activated receptor which can bind DNA without prior warming. This observation is documented (Le Fevre et al., 1979) and could be the result of inclusion of 0.15M KCl and exclusion of EDTA from the homogenization and incubation buffers. Salt is known to induce activation (Notides, 1978). Table 11 shows accumulated results

Table 10. Percent Recovery of DNA-cellulose-bound Receptor after Incubation with Activated and Non-activated Cytosol

Immature rat uterine cytosol was prepared as described in Section 2.2.2.4.1. After warming the cytosol to 30°C for 30 min to activate the receptor while keeping another set at 4°C as control, the DNA binding assay, salt and alcohol extraction of DNA-bound receptor was carried out as described in Section 2.2.4.1 and 2.2.4.1.1.

Expt.	Total Binding		Salt Extractable		Alcohol Extractable	
	4^{o}	30^{o}	4^{o}	30^{o}	4^{o}	30^{o}
1	31	73	70	72	30	28
2	32	88	68	63	22	37
3	20	78	79	78	21	22
	28 ± 5	80 ± 6	72 ± 5	71 ± 6	28 ± 5	29 ± 6

Table 11. Percent Binding to DNA-cellulose after Receptor Activation

Immature rat uterine cytosol was prepared as described in Section 2.2.2.4.1. After heat activation the cytosol was incubated with DNA-cellulose, and salt and alcohol extraction carried out as described in Section 2.2.4.1 and 2.2.4.1.1.

	Total	Salt Extractable	Alcohol Extractable
$\bar{x} \pm$ S.D.	83 ± 6	77 ± 6	23 ± 6
n = 9			
Range	76 - 90	63 - 82	18 - 37

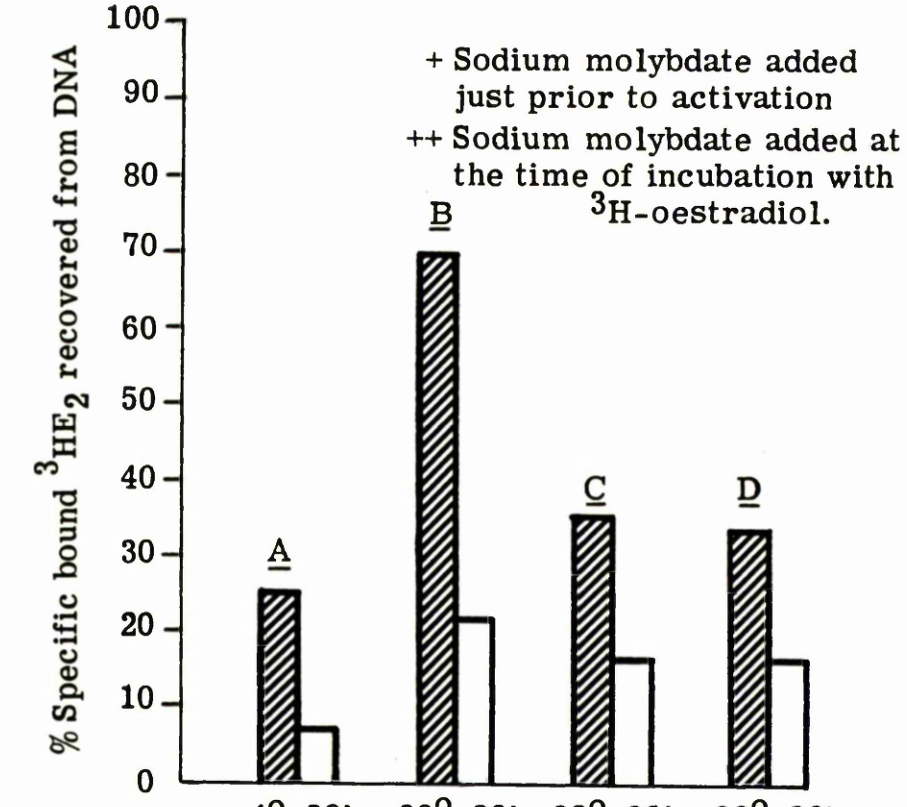
from 9 experiments with $HDK_{.15}$ buffer. It confirms the data in Table 10 showing that approximately 80% of the total receptor from activated cytosol is capable of binding to DNA-cellulose and about 70-80% of this is released on one salt extraction.

3.2.1.2.8 **The Effect of Sodium Molybdate on Receptor Activation**

The effect of sodium molybdate in inhibiting receptor activation is well documented (Section 1.1.5.3.2). This effect was confirmed (Figure 37). However, inclusion of 20mM sodium molybdate, still left ~20% of the receptor whose activation could not be inhibited when compared to the $4^{o}C$ control. A similar level of DNA-bound receptor was observed whether sodium molybdate was added just prior to activation or at the start of incubation with $^{3}HE_{2}$. The same cytosol without sodium molybdate, showed a much greater proportion (90%) of receptor bound to DNA cellulose after activation (Figure 37). In addition, if sodium molybdate was added to the homogenization buffer ($HDK_{.15}$ + 20mM sodium molybdate) and the incubation and activation performed as before, again ~ 20% of the receptor was bound to the DNA cellulose (Figure 38). However, interestingly, in this experiment, activation in the 4^{o} control was completely absent (Figure 38). It appears, therefore, that ~20% of the receptor population normally activated by 30^{o}, 30' incubation is not sensitive to sodium molybdate under the present experimental conditions. The optimum time of exposure to sodium molybdate was not specifically established. Data in Figure 37 might suggest that exposure time to sodium molybdate was inadequate but Figure 38 was drawn from experiments in which the sodium molybdate was present throughout. This shows the same level of molybdate-resistant binding suggesting that the observation is real.

Figure 37. **Effect of Sodium Molybdate on Activation of Immature Rat Uterine Cytosol Oestrogen Receptor**

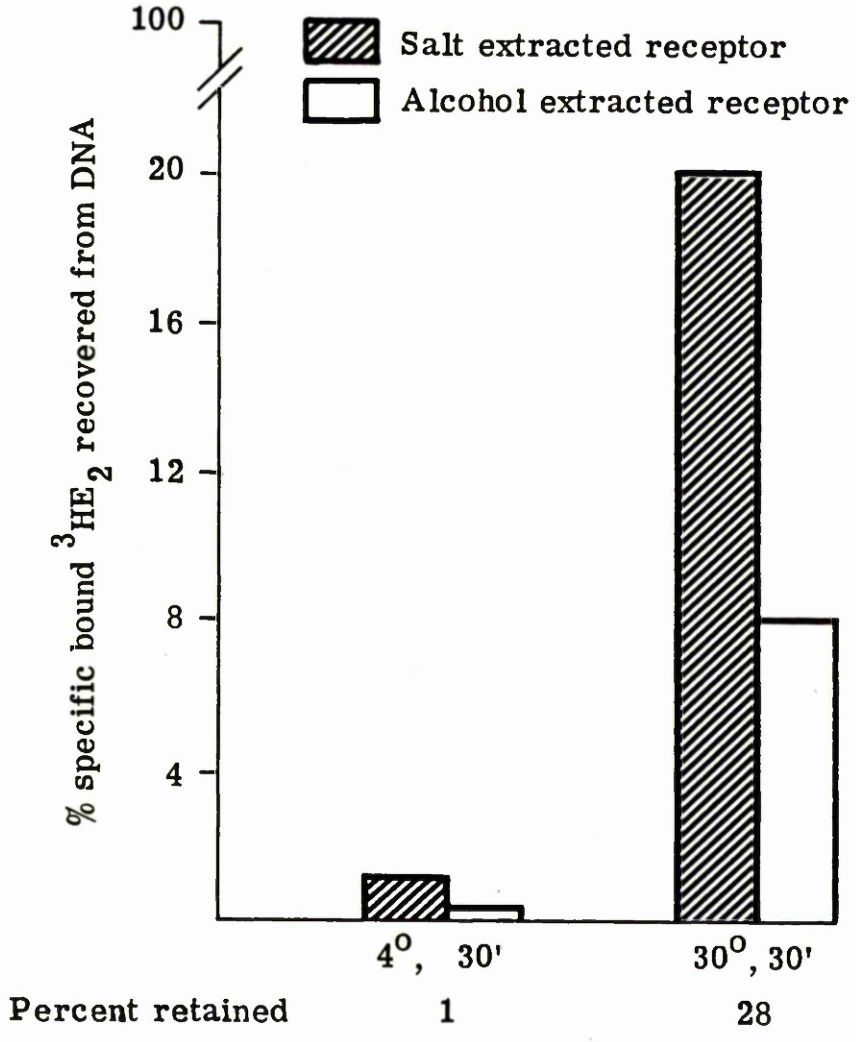
Immature rat uterine cytosol was prepared and labelled at 4°C for 1h as described in Section 2.2.4.1 (protein concentration 3.6mg/ml; receptor concentration 3.1nM). At the start of incubation with $^{3}HE_{2}$ one set of aliquots was made 20mM with respect to sodium molybdate (D). A second set of aliquots were similarly treated with sodium molybdate just before the heat activation step (C). Two further sets were kept sodium molybdate free. One set of sodium molybdate free cytosol (A) was retained at 4°C, all other cytosols were warmed to 30°C for 30 min. including a sodium molybdate free cytysol (B). The warmed cytosol aliquots were cooled to 4°C for 5 min. DNA-cellulose binding assay of all the aliquots was as described in Section 2.2.4.1 and 2.2.4.1.1 (▨) salt extracted receptor, (□) alcohol extracted receptor.



Total Bound (%) 31 90 51 49

Figure 38. **Influence of addition of Sodium Molybdate to the Homogenization Medium on Receptor Activation**

Immature rat uterine cytosol was prepared in $HDK._{15}$ containing 20mM sodium molybdate and labelled for 1h at $4^{o}C$ as described in Section 2.2.4.1 (protein concentration 3.4mg/ml; receptor concentration 2.5nM). While keeping one set of aliquots at $4^{o}C$, another set was warmed to $30^{o}C$ for 30 min. Warmed cytosol was then cooled to $4^{o}C$ for 5 min. Both sets were then incubated with DNA-cellulose and the binding assay carried out as described in Section 2.2.4.1 and 2.2.4.1.1 (▨) salt extracted receptor, (□) alcohol extracted receptor. Figure represents the percent specifically bound steroid retained by the DNA (Salt soluble + salt resistant counts) at the end of the extraction procedure. After a second experiment corresponding mean values were 2 and 25 respectively.


100
20
16
12
8
4
Salt extracted receptor
Alcohol extracted receptor
% specific bound $^{3}HE_{2}$ recovered from DNA
4°, 30'
30°, 30'
Percent retained
1
28

3.2.1.3 DNA-Cellulose Binding of Soluble Oestrogen Receptor from Human Breast Cancer

Early attempts to show binding of the activated receptor (ER_c) from human breast tumour to crude nuclear pellets, derived from the same tumour, were unsuccessful (data not shown). Park and Wittliff (1977) have commented on similar problems associated with nuclear preparations from mammary tissues. The failure of the earlier reconstitution experiments was probably due to the following reasons:-

a) the human breast tumour cytosol, when heated for activation, resulted in the degradation of receptor to the 4S form (Figure 15) probably through the proteolysis of the 4S form (the form which can aggregate to 8S form in low salt)

b) this protease effect can be inhibited with DFP (Figure 51) which was not used in earlier experiments

c) the proteolyzed 4S form probably does not bind to DNA (Figure 43).

3.2.1.3.1 Activation and DNA-cellulose Binding of Human Breast Tumour Soluble Oestrogen Receptor in the Absence or Presence of DFP

Figure 39 shows that the human breast tumour receptor, like that from the rat, showed temperature dependent binding to DNA-cellulose. However, the maximum binding obtained (only 18% of total receptor) was strikingly in contrast to the 90% or so binding obtained with immature rat uterine activated receptor (Figure 33). The results presented in Figure 40 and Table 12 show that, in the presence of DFP, significantly more receptor was bound to DNA-cellulose after activation compared to control. Comparison of Figure 39 and 40 shows the variability that can be obtained in DNA-cellulose binding of human activated receptor in the

Figure 39. **Effect of Temperature on Human Breast Carcinoma Cytosol Oestrogen Receptor Activation**

Human breast tumour cytosol was prepared in $HDK._{15}$ as described in Section 2.2.3.2 (patient SN; protein concentration 8.2mg/ml). The DNA-cellulose binding assay was as described in Section 2.2.4.2. Receptor concentration of the cytosol preparation was 416fmoles/mg protein. (□) Salt extractable receptor, (■) alcohol extractable receptor.

Effect of temperature on activation and DNA binding of Human Tumour ER_C

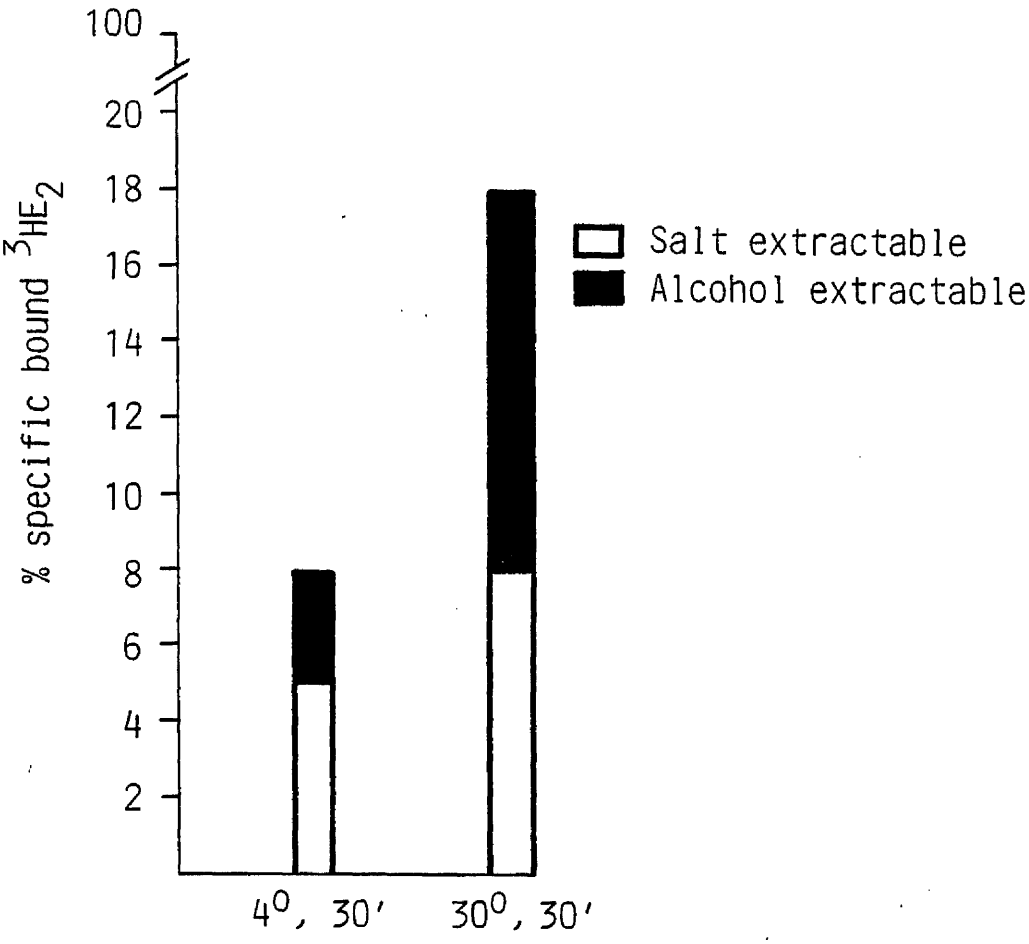


Figure 40. **Effect of DFP on Termperature-dependent Activation of Receptor from Human Breast Cancer**

After homogenization of tumour cytosol in $HDK._{15}$ (Section 2.2.3.2) one portion of the homogenate was made 10mM with respect to DFP. Cytosol was prepared as described in Section 2.2.2.1 and DNA-cellulose binding was as described in Section 2.2.4.2 except that both the cytosols in the absence and presence of DFP were heat activated at 30°C for 30 min. (□) receptor bound in the absence of DFP, (■) receptor bound in the presence of DFP. The recoverable receptor shown is the sum of salt extracted and alcohol extracted receptor. The receptor concentration for Tumour 1 (patient SC), was 146fmoles/mg protein (protein concentration 2.2mg/ml) in the absence of DFP and 123 fmoles/mg protein (protein concentration 2.6mg/ml) in the presence of DFP. The receptor concentration for Tumour 2 (patient FM) was 136fmoles/mg protein (protein concentration 8.6mg/ml) in the absence of DFP and 101fmoles/mg protein(protein concentration 9.2mg/ml) in the presence of DFP .

Effect of DFP on Human Tumour
ER_C activation and DNA Binding

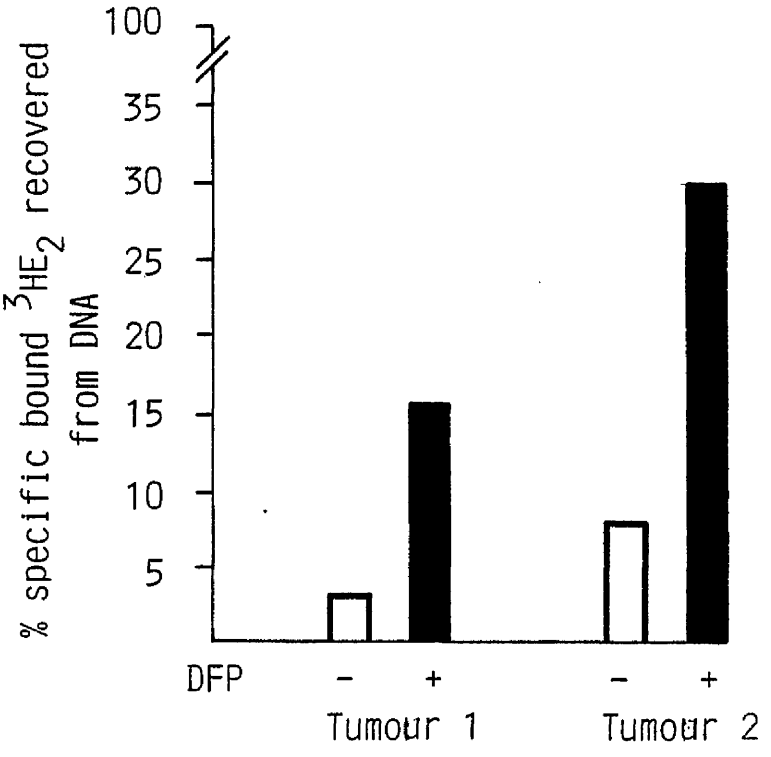


Table 12. Influence of DFP on Recovery of Receptor from DNA-Cellulose

Human breast tumour was homogenized in $HDK._{15}$ and immediately divided into two halves. To one half DFP was added to a final concentration of 10mM and both DFP containing and DFP free cytosols prepared as described in Section 2.2.3.2. After heat activation at $30^{o}C$ for 30 min, the cytosol was incubated with DNA-cellulose and the binding assay and salt and alcohol extraction carried out as described in Section 2.2.4.2.

	Activated Cytosol 30°, 30'			
	- DFP		+ DFP	
	$^3HE_2$	$^3HE_2$ + DES	$^3HE_2$	$^3HE_2$ + DES
Wash 1	39635	44970	43440	42535
Wash 2	4700	6300	5915	6345
Wash 3	832	1487	1122	1322
Wash 4	222	412	382	372
Wash 5	65	95	115	120
Salt Extracted	186	133	581	124
Alcohol Extracted	532	197	1477	248
Total	46172*	53594	53032	51069

* Recovery of cpm throughout was ~90% except in this case (recovery 84%).

absence of DFP (3-18%), confirming the data of Sato et al. (1981a), Table 12 shows the effect of washing DNA-cellulose after the receptor binding reaction, indicating that the problem is not due to loss of counts during the experiment. DFP was used in these experiments since it is known to be the most potent inhibitor of the trypsin-like activity encountered in human tissue (Notides et al., 1976; Lukola et al., 1980; Lukola and Punnonen, 1982). The results presented in Figure 40 show that a greater proportion of receptor (about 5-fold more) bound to DNA-cellulose in the presence of DFP. When Park and Wittliff (1977) noted the lower binding obtained with human breast tumour ER_c, their experiments, like those of Sato et al. (1981a),were conducted in the absence of protease inhibitors. However, even in the presence of DFP the extent of binding of human breast tumour activated ER_c was much lower than that obtained with immature rat uterine activated ER_c in the absence of DFP. Nevertheless, the binding was more in the presence than in the absence of protease inhibitor in human tumour cytosol. It was also noted that in the presence of DFP, the total specific counts obtained (by DCC method) were lower than from the same cytosol in the absence of DFP. This is in agreement with results published by Notides et al. (1976) and Lukola et al. (1979).

Finally, Table 13 shows that, in contrast to the immature rat uterine activated ER_c binding (Table 11), the human breast tumour receptor is less easily extracted with salt from the DNA-cellulose and a greater proportion is, correspondingly, only extractable in alcohol. However, the actual counts obtained were too low (see Table 12) to be resolved on SDGA. The difference in salt extractability of human and rat receptor may indicate different types of interaction of receptor with DNA from two different sources. Such differences in salt

Table 13. Percent Recovery of Activated Human Breast Tumour Oestrogen Receptor from DNA-cellulose

Human breast tumour was homogenized in $HDK._{15}$. DFP was added to homogenate and cytosol prepared as described in Section 2.2.3.2. After heat activation at $30^{\circ}C$ for 30 min, the cytosol was incubated with DNA-cellulose and the binding assay and salt and alcohol extraction was as described in Section 2.2.4.2.

Recovery of activated human tumour ER_C from DNA-cellulose

	Total	Salt Extractable	Alcohol Extractable
$\bar{x} \pm SD$ n = 3	21 ± 6	32 ± 9	68 ± 9
Range	16 - 29	25 - 44	56 - 75

extractability of receptor are documented (Katzenellenbogen et al., 1980 where tissue under investigation was human endometrium and immature rat uteri).

3.2.2 Analysis of DNA Oestrogen-receptor Interaction using Low Salt SDGA

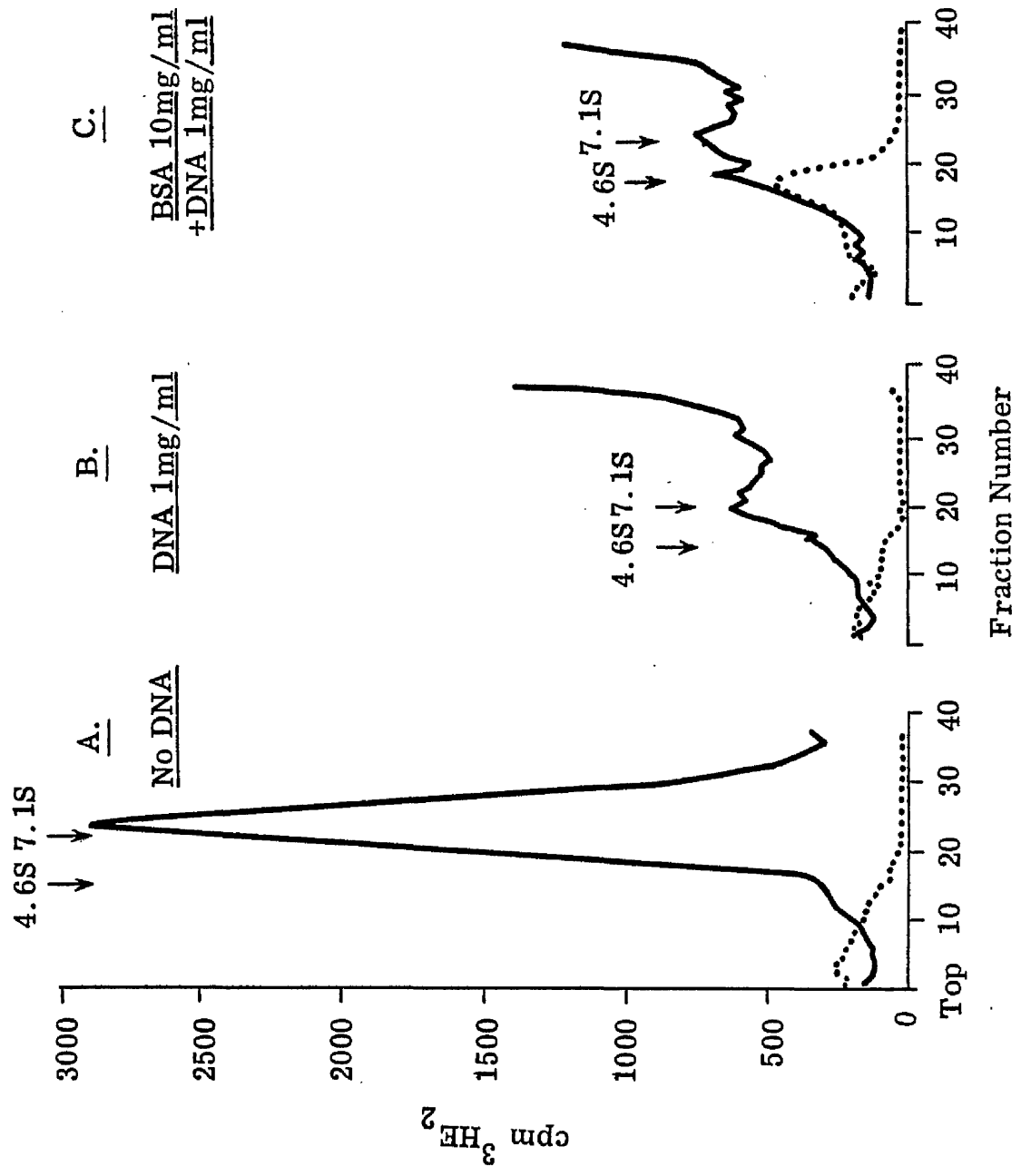
It has been suggested that the proteolyzed receptor, which sediments at 3-4S, fails to bind to DNA (Andre and Rochefort, 1973; Rochefort et al., 1980). Nevertheless, the appearance of 4S receptor in low salt cannot be ascribed solely to proteolysis since, it is known that the 8S form may dissociate reversibly into the 4S form (Wittliff et al., 1972 - cited in Leake, 1981b). However, it has recently been suggested (Park and Wittliff, 1980) that human breast tumour 4S receptor fails to bind to DNA, reflecting the proposal that patients, whose tumours contained only the 4S form of receptor, failed to show subsequent response to endocrine therapy (Wittliff and Savlov, 1975). In contrast, the results reported below show that, for some tumours at least, a large proportion of 4S as well as 8S was removed from the gradient by the soluble calf thymus DNA.

3.2.2.1 Interaction of Immature Rat Uterine Soluble Oestrogen Receptor with Calf Thymus DNA

To check the validity of the assay system (Section 2.2.5), immature rat uterine soluble receptor, known to sediment at 8S under low salt conditions was used. In accordance with published results of Toft (1973), the 8S receptor complex was shown (Figure 41A and B) to bind to calf thymus DNA and sediment to the bottom of the tube. This result confirmed that the 20 minute incubation with DNA prior to sedimentation, removed most activated receptor from the cytosol. One

Figure 41. **Influence of Addition of Calf Thymus DNA on the Sedimentation Profile of Oestrogen Receptor from Immature Rat Uterine Cytosol**

Immature rat uterine cytosol was prepared as described in Section 2.2.2.4.1 (protein concentration 2.9mg/ml). Aliquots were then labelled with 5 x 10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 1000-fold excess DES for 55 min. at 4^{o}C. One set was then made 10mg/ml with respect to BSA (C). After 5 min, DNA was added to a final concentration of 1mg/ml (B and C), while keeping one set of aliquots DNA free (A). Incubation was then continued for a further 20 min. at 4^{o}C. Following the removal of unbound steroid with DCC, oestrogen receptor was separated on linear 5-20% gradients at 45,000 rev/min for 14h at 4^{o}C in Beckman SW 50.1 rotor. Arrows indicate the position of ^{14}C-labelled marker proteins.


Influence of DNA on Sedimentation Profile
Immature Rat Uterine ERC
A.
No DNA
B.
DNA 1mg/ml
C.
BSA 10mg/ml
+DNA 1mg/ml
4.6S 7.1S
4.6S 7.1S
4.6S 7.1S
cpm 3HE2
3000
2500
2000
1500
1000
500
0
Top 10 20 30 40
10 20 30 40
10 20 30 40
Fraction Number

further effect, reported in Figure 41(C) was that in the presence of excess protein (10mg/ml BSA added to the cytosol at the time of incubation with $^{3}HE_{2}$) the profile was effectively unaltered, although a small additional peak at 4.6S was observed. This most probably represents the $^{3}HE_{2}$ non-specifically associated with the BSA. This latter experiment was included to check the effect of protein concentration on DNA binding since breast tumour cytosol preparations had a higher protein concentration range than that generally used with the immature rat uterine cytosol. This does not exclude the possibility that the breast tumour cytosol may provide additional DNA binding proteins.

3.2.2.2 Interaction of Human Breast Tumour Soluble Oestrogen Receptor with Calf Thymus DNA

Analogous to the results reported in Figure 41, Figure 42 shows that the 8S form of human breast tumour ER_{c} bound to DNA and sedimented to the bottom of the tube in association with it. The small but sharp 4S peak observed in this low salt gradient became more disperse when the incubation was carried out in the presence of DNA. However, this is unlikely to represent a specific effect of the DNA. In addition Figure 43 shows that in accordance with the proposed model of Wittliff, most of the 8S form of the receptor bound to the DNA, leaving behind most of the 4S form. i.e. the 4S form is less efficient in binding to DNA than is the 8S form. However, Figure 44 clearly demonstrates that in certain tissues 4S receptor bound to DNA equally efficiently. This latter observation indicates that some of the 4S form can exist with an intact DNA binding site. Most probably this 4S represents the disaggregated 8S receptor rather than the proteolyzed form detected in Figure 43. It should be mentioned that these studies did not involve the warming

Figure 42. **Influence of Addition of DNA on the Sedimentation Profile of Oestrogen Receptor from Human Breast Tumour Cytosol**

Cytosol was prepared as described in Section 2.2.5 (patient DM ♂ ; protein concentration 4.4mg/ml). Aliquots were then incubated for 3h at 4°C with 5 x 10^{-9}M $^{3}HE_2$ in the absence (——) or presence of DES (....). One set was then made 1mg/ml (final concentration) with respect to DNA. Aliquots were further incubated for 20 min at 4°C. Following removal of unbound steroid with DCC, oestrogen receptor was separated on linear 5-20% sucrose density gradients at 50,000 rev/min for 14h at 4°C in a Beckman SW 50.1 rotor.

Influence of addition of DNA on sedimentation profile
of oestrogen receptor from human breast tumor cytosol

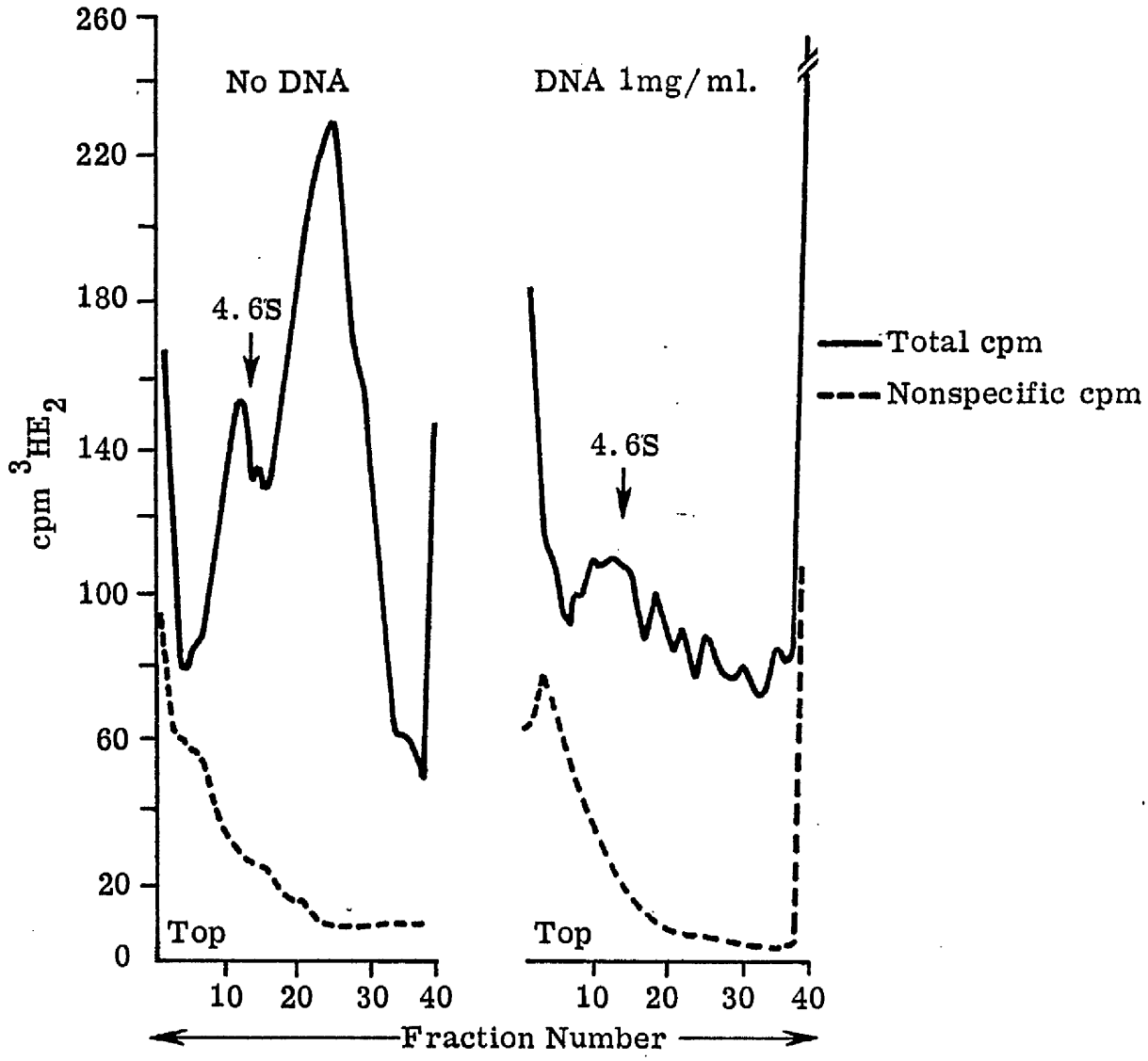


Figure 43. **Selective Binding of Human Breast Tumour Cytosol 8S Receptor to Calf Thymus DNA.**

Human breast tumour cytosol was prepared as described in Section 2.2.5. (protein concentration 8.2mg/ml; patient IH). Aliquots were then labelled with 5 x 10^{-9}M $^{3}HE_2$ ± 1000 fold excess DES for 1h at 4°C. This was followed by the addition of DNA to a final concentration of 1mg/ml to one set (....) while keeping the other set DNA free (——). Incubation was then continued for a further 20 min. at 4°C. Following removal of unbound steroid by DCC, receptor was separated on linear 5-20% gradients at 45,000 rev/min for 14.5h in a SW 50.1 rotor at 4°C. Arrow indicates the position of ^{14}C-labelled marker protein. The receptor concentration, as determined by DCC analysis, was 93fmoles/mg protein . Percentage recovery in the absence of DNA was 95%. In the presence of DNA total recovery of receptor including the counts determined at the bottom of the tube approximated to 96%.

Human 8S Receptor alone binding to DNA

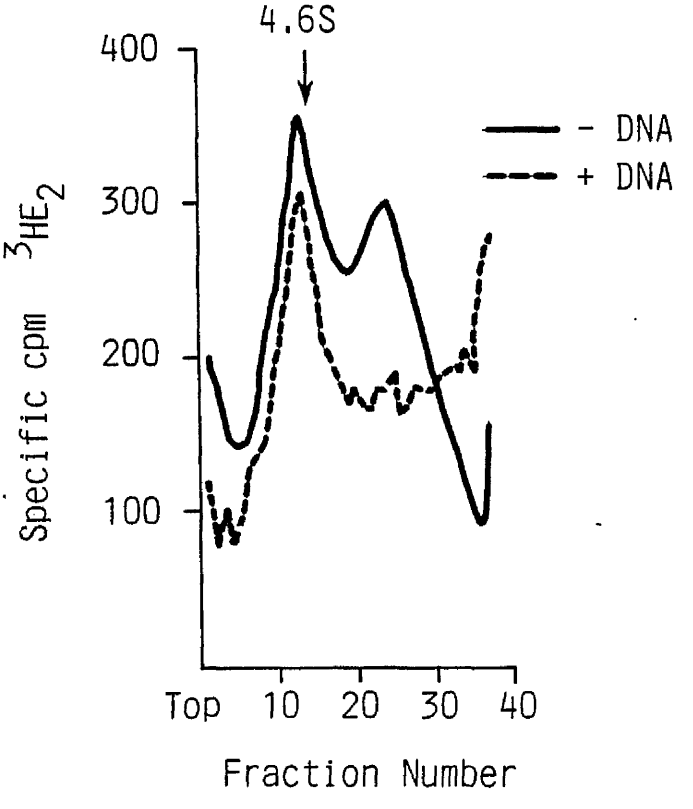
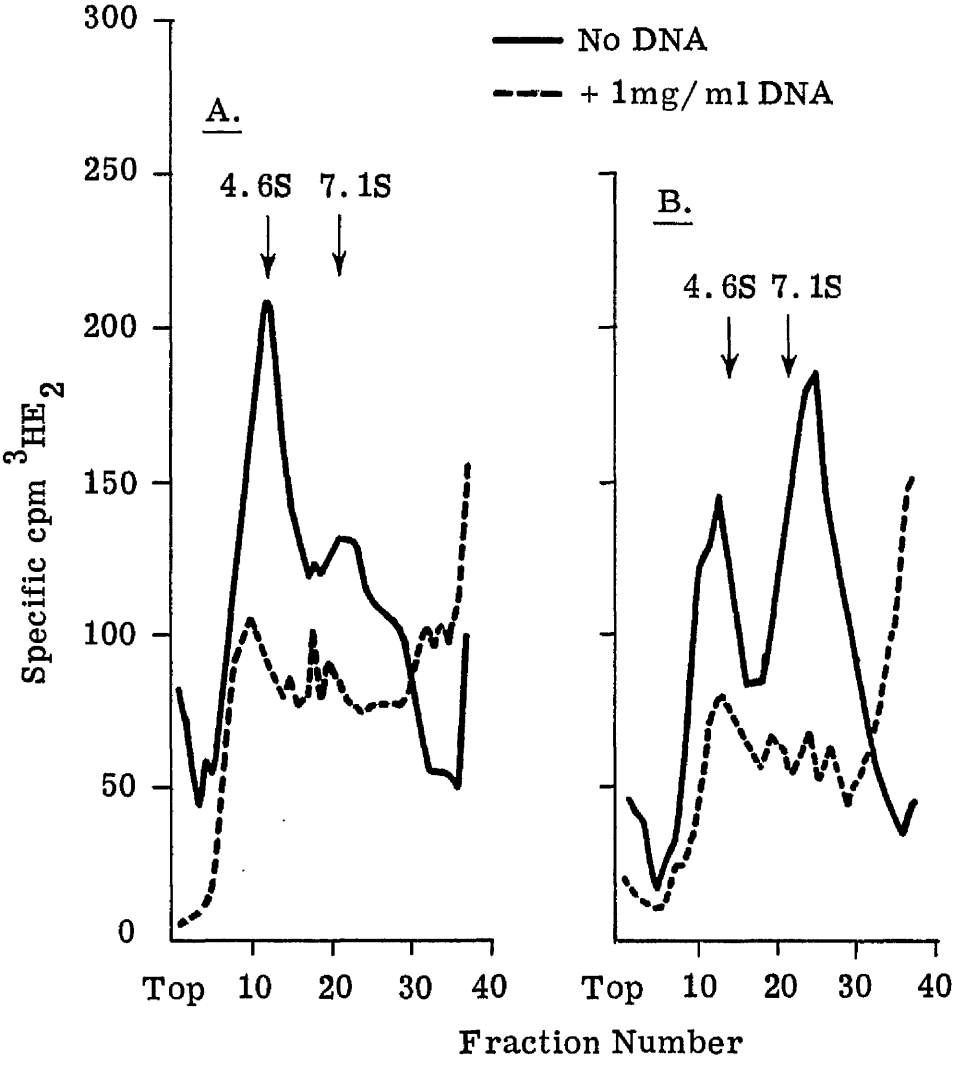


Figure 44. **Binding of both 8S and 4S Oestrogen Receptor to Calf Thymus DNA**

Cytosol was prepared in HED as described in Section 2.2.5 (A, patient CM, protein concentration 6.2mg/ml; B, patient IH, protein concentration 6.2mg/ml). All procedures were as described in the legend to Figure 43. The receptor concentration in fmoles/mg protein as determined by DCC was 70 (——) and 65 (....) in A and 116 (——) and 112 (....) in B. Percentage recovery of specific counts in A was 95% in the absence of exogenous DNA and 103% in the presence of DNA including the counts sedimenting to the bottom. Percentage recovery in B was 84% in the absence of exogenous DNA. In B, counts at the bottom of the tube were not determined but recovery in the gradient in the presence of DNA was 49%.


No DNA
+ 1mg/ml DNA
A.
B.
4.6S 7.1S
4.6S 7.1S
300
250
200
150
100
50
0
Specific cpm $^3HE_2$
Top 10 20 30 40
Top 10 20 30 40
Fraction Number

procedure for activation (30^{o}, 30') since the heating process resulted in either degradation of receptor to 4S form or formation of high molecular weight aggregates (Section 3.2.3.2.7).

The DNA binding observed in low salt conditions may represent non-specific interactions but when these studies were repeated in the presence of 0.15M KCl the similar proportion of binding was obtained (data not shown) although under these conditions 4S and 8S forms do not exist as separate entities.

Finally, one tumour was obtained from which both 4S and 8S ER_c bound to the DNA (Figure 45) but, in each case, to only a limited extent. The high protein concentration (10.4mg/ml) of the cytosol might account for such a result, since the acceptor sites on the DNA may be masked by other DNA Binding proteins or that an excess of an inhibitor(s) (Section 1.1.5.3.2), may reduce activation of ER_c. As shown in Figure 41C where 10mg/ml BSA was added to block receptor binding to DNA, this blocking effect was not a non-specific effect of increased protein content.

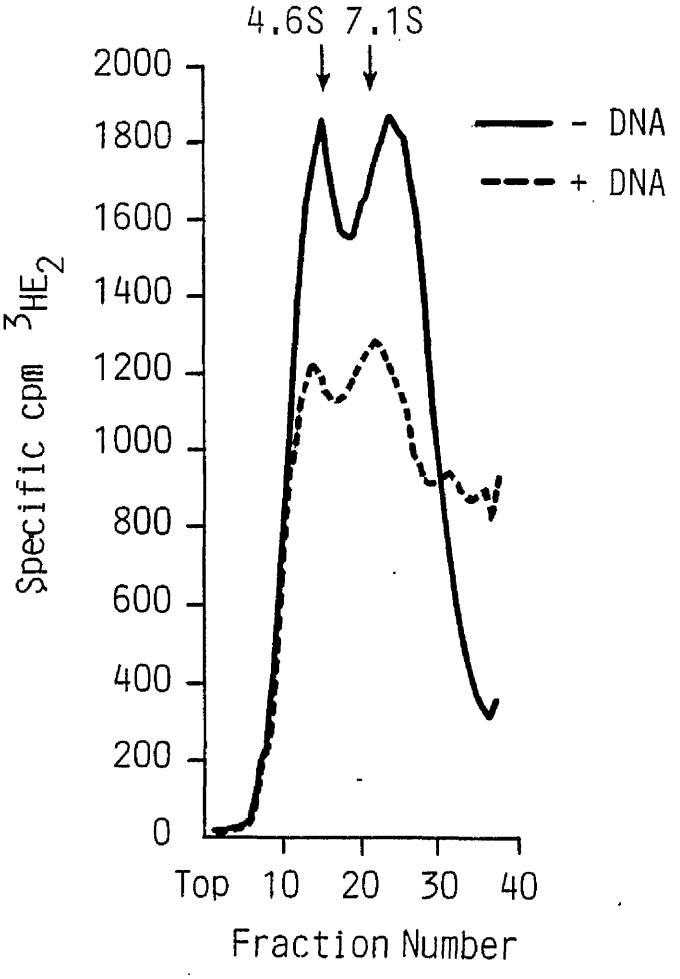
3.2.2.3 **Conclusions Drawn from the DNA-binding Studies**

The 4S form of receptor, seen at $4^{o}C$ in low salt conditions, is generally less efficient in DNA binding than the 8S form. The 4S population of receptor may comprise not only proteolyzed 8S complex (or proteolyzed 4S receptor not able to form 8S complex) but also reversibly dissociated 8S complex (or intact 4S receptor which under optimum conditions will form 8S complex). Thus 4S receptor observed *in vitro* is probably a heterogeneous population. It appears that by heating the cytosol or during the storage period (Section 3.1.1.4 and Section 3.1.1.7 respectively), some of the receptor is proteolyzed to the inactive 4S form, since even the immature rat uterus stored for

Figure 45. **Partial Binding of both 8S and 4S Receptor from Human Breast Tumour Cytosol to Calf Thymus DNA**

Cytosol was prepared in HED as described in Section 2.2.5 (patient HB; protein concentration 10.4mg/ml). All procedures were as described in the legend to Figure 43. Receptor concentration as determined by DCC prior to SDGA was 280fmoles/mg protein. Percentage recovery of specific counts in DNA free tube was 104% and in DNA containing tube, including the receptor sedimenting to the bottom of the tube was 91%.

Limited Tumour ER_C Binding to DNA



24h in sucrose/glycerol buffer possesses receptor which has lost its ability to transform to 5S (Figure 57C). Thus presence of intact 4S seems essential for formation of 8S complex and also for binding to DNA.

3.2.3 In Vitro Transformation Studies

Transformation is the hormone and temperature dependent event reflected by an increase in the sedimentation value from 4S→5S for soluble immature rat uterine oestrogen receptor on SDGA containing 0.4M KCl (Section 1.1.5.3).

3.2.3.1 The 4S⟶5S Transition of Soluble Oestrogen Receptor from Immature Rat Uterine Tissue

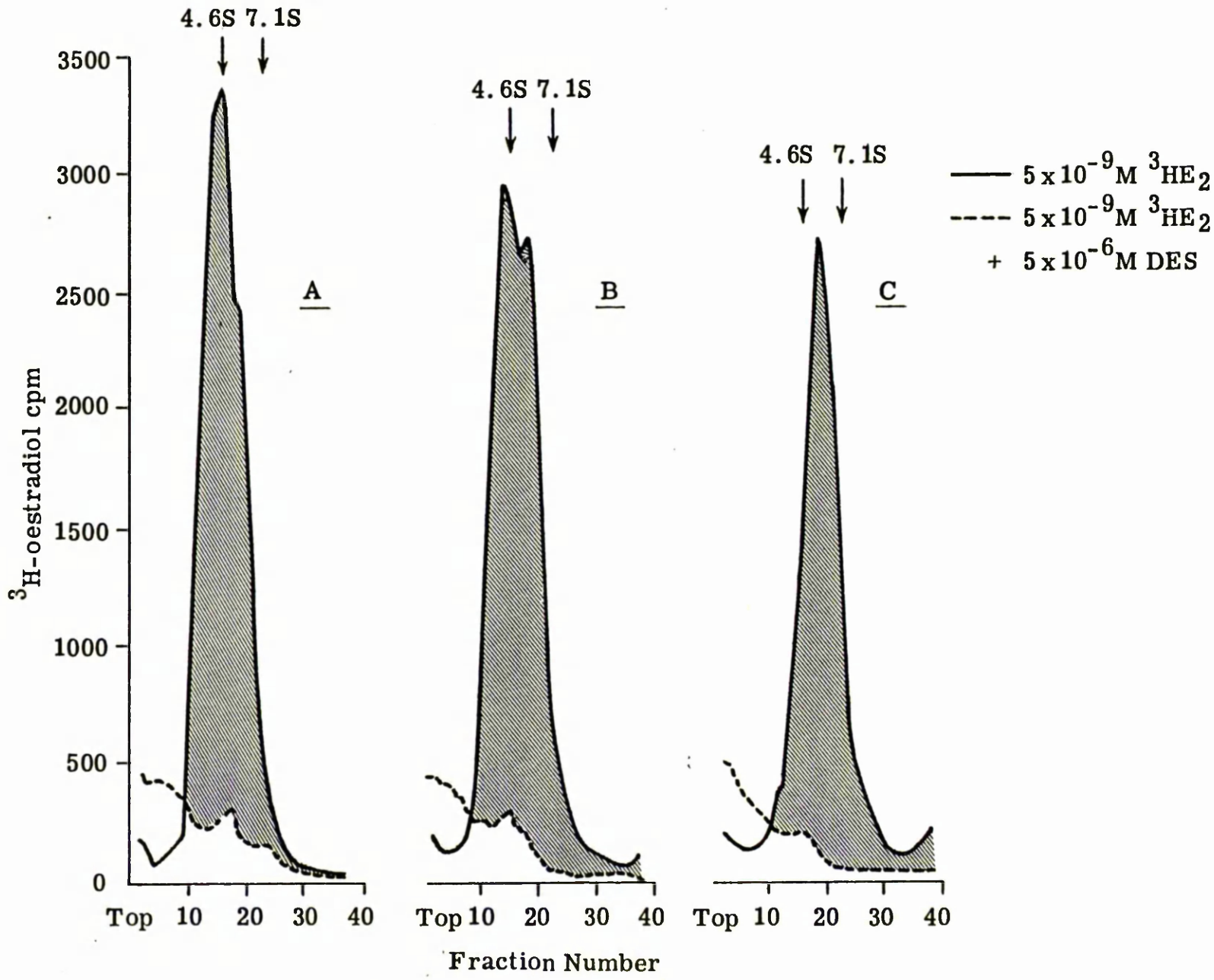
3.2.3.1.1 The Effect of Warming at 30°C for 30 min. prior to Analysis

The 4S→5S transition observed with immature rat uterine ER_c is shown in Figure 46. The receptor from non-activated cytosol sedimented at 4.6S (Figure 46A). After activation only the 5.6S peak was observed (Figure 46C). The latter has been presumed to be the form binding to DNA (Section 1.1.5.3). Figure 46B shows that the appearance of the 5.6S peak was a time and temperature dependent process. Warming the cytosol at 30°C for 5 min. leads to only a minimal increase in the 5.6S peak (Figure 46B) whilst complete conversion to 5.6S was seen after 30°C for 30 min. warming (Figure 46C). In fact the complete conversion to 5.6S form may occur earlier followed by the formation of salt resistant aggregation as indicated by only an 89% recovery of specific counts on the gradient in Figure 46C. It can also be seen that there was a slight shoulder present in the 5.6S area even in the absence of any warming procedure (non-activated cytosol - Figure 46A). This probably accounts for the 20-30% receptor bound to DNA-cellulose in the absence of any warming procedure (Section 3.2.1.2) indicating that some activated

Figure 46. **Transformation of Immature Rat Uterine Cytosol Oestrogen Receptor**

Immature rat uterine cytosol (5 uteri/ml) was prepared in $HDK._{15}$, labelled with $^{3}HE_{2}$ in the absence (——) or presence (....) of DES for 1h at $4^{o}C$ and DCC stripped as described in Section 2.2.3.1 (protein concentration 3.4mg/ml; receptor concentration 581fmoles/mg protein). One set of aliquots was then kept at $4^{o}C$ (A) while another set was warmed at $30^{o}C$ for 5 min and then cooled to $4^{o}C$ (B). A further set was warmed to $30^{o}C$ for 30 min and then cooled to $4^{o}C$ for 5 min. (C). 200ul aliquots from each set were loaded onto 5-20% linear gradients prepared in $HDK._{4}$. Centrifugation was for 16h at 45,000 rev/min in a Beckman SW 50.1 rotor at $4^{o}C$. Arrows indicate the position of ^{14}C-labelled marker proteins. The $^{3}HE_{2}$-receptor sedimentation peak shown between the ^{14}C-labelled marker proteins is at 5.6S.

Transformation of Immature Rat Uterine Oestrogen Receptor



receptor can be found even at $4^{o}C$ in the present experimental conditions.

It was also observed that the presence of EDTA retarded the 4S⟶5S transition (data not shown), supporting the observation of Sato et al. (1978a). It should also be mentioned that 4S → 5S transition was observed in $HDK_{.4}$ whether the initial homogenization buffer was HD or $HDK_{.15}$.

3.2.3.1.2 **The Effect of Dilution on 4S⟶5S Transition**

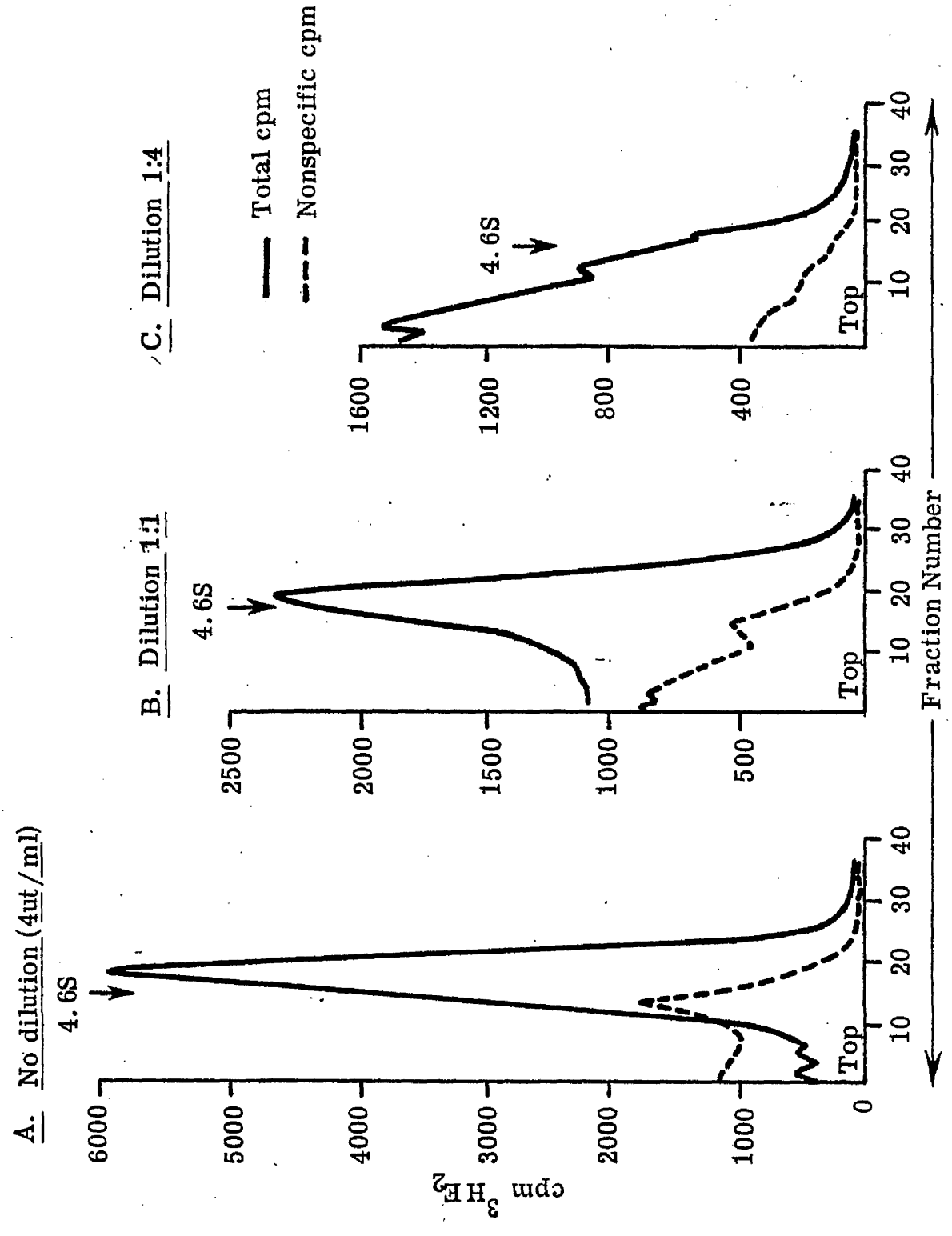
Figure 47A shows that, in agreement with results presented in Figure 46, the heat activated receptor sediments at 5S. Figure 47B further shows the effect of dilution of the same cytosol after activation. With increasing dilution, an increasing tendency for the $^{3}HE_{2}$ to dissociate during the centrifugation run was observed. There was also a slight shift of the 5.6S form towards the BSA marker. This most probably resulted from the relatively low concentration of (bound) receptor and its effect on the 4S⟶5S equilibrium. Figure 47C illustrates that on further dilution 5S could no longer be detected. However, since the $^{3}HE_{2}$ had all dissociated to the top of the gradient, it is only possible to say that steroid was no longer bound to receptor. The molecular size of the empty receptor under these conditions was unknown.

The reason for investigating the effect of dilution resulted from the observations that this process resulted in little or no temperature dependent binding to either DNA-cellulose or to nuclei prepared from crude pellets after several (X 3) pellet washings (data not shown). It was subsequently found that dilution followed by heat activation also prevented the formation of the 5S peak giving instead a broad peak at 4.6S, with loss of radioactivity towards the top of the gradient. This contrasted with the reported increase in activation on dilution

Figure 47. **Effect of Dilution on Transformed Oestrogen Receptor from Immature Rat Uterine Cytosol**

Immature rat uterine cytosol was prepared as described in Figure 46 (4 uteri/ml; protein concentration 3.6mg/ml). After warming the cytosol to 30°C for 30 min and cooling it to 4°C for 5 min aliquots were either undiluted (A), diluted 1:1 (B) or diluted 1:4 (C) and loaded onto 5-20% sucrose density gradients prepared in HDK_4. Centrifugation was at 45,000 rev/min for 14h at 4°C in a Beckman SW 50.1 rotor. ^{14}C-BSA (4.6S) was included as protein marker. The receptor peak shown in A is at 5.5S and that shown in B is at 5.2S.

Effect of dilution on the immature rat uterus ER_c transformation. centrifugation temp. 4°C. SDG containing 0.4M Kcl.



(Notides, 1978). However, it must be emphasized that in the results reported above, the dilution process was followed by warming. The sedimentation profile of diluted cytosol, in the absence of heating to promote activation, was not determined. Dilution may simply be affecting the association of receptor activation factor (Thampan and Clark, 1981) or might involve some protease activity. As a result of such experiments it was concluded that at least 2mg/ml cytosol protein concentration is essential for studying 4S⟶5S transformation. The effect of receptor concentration was not investigated but results in the literature suggest that at least 1nM receptor concentration is required for efficient activation (Notides et al., 1981). The receptor concentration effect however, may be different in test tube assays and in non-equilibrium sucrose density gradients.

3.2.3.2 The 4S⟶5S Transition of the Soluble Oestrogen Receptor from Human Breast Tumours

3.2.3.2.1 The Effect of Warming at 30°C for 30 min. prior to Analysis

Figure 48.1 and 48.2 show that, in contrast to the 4S⟶5S change generated in immature rat uterine cytosol, no such conversion was observed with human breast soluble oestrogen receptor. The receptor sedimented in the 4S area either at 4°C or after activation at 30°, 30' (Figure 48.1 A and B, Figure 48.2A and B). Further, activation resulted in a decrease of total binding capacity. Dilution of the activated cytosol, to reduce receptor concentration showed the presence of a 3-4S peak (Figure 48.1C). In another tumour cytosol a split in the equivalent peak was evident (Figure 48.2C). The latter observation suggested the presence of mero-receptor (Sherman et al., 1980) arising as a result of receptor degradation.

Figure 48.1. **Transformation of Oestrogen Receptor from Human Breast Cancer Cytosol**

Human breast tumour was homogenized in HD and cytosol prepared as described in Section 2.2.3.2 (protein concentration 3.6mg/ml; receptor concentration 165fmoles/mg protein). Aliquots were labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 2h at 4^{o}C. An aliquot was then kept at 4^{o}C (A) and a second aliquot heated to 30^{o}C for 30 min (B). The warmed cytosol was cooled to 4^{o}C for 5 min. and both cytosols were then DCC stripped (Section 2.2.3.2). The warmed cytosol was then diluted 1:2 (C). 200ul aliquots were then loaded onto 10-30% linear sucrose density gradients prepared in $HDK._4$ and centrifuged at 50,000 rev/min for 14.5h in a Beckman SW 50.1 rotor. Arrow indicates the position of ^{14}C-labelled marker proteins. The precentage recovery of specific receptor was 94% in A and 71% in B.

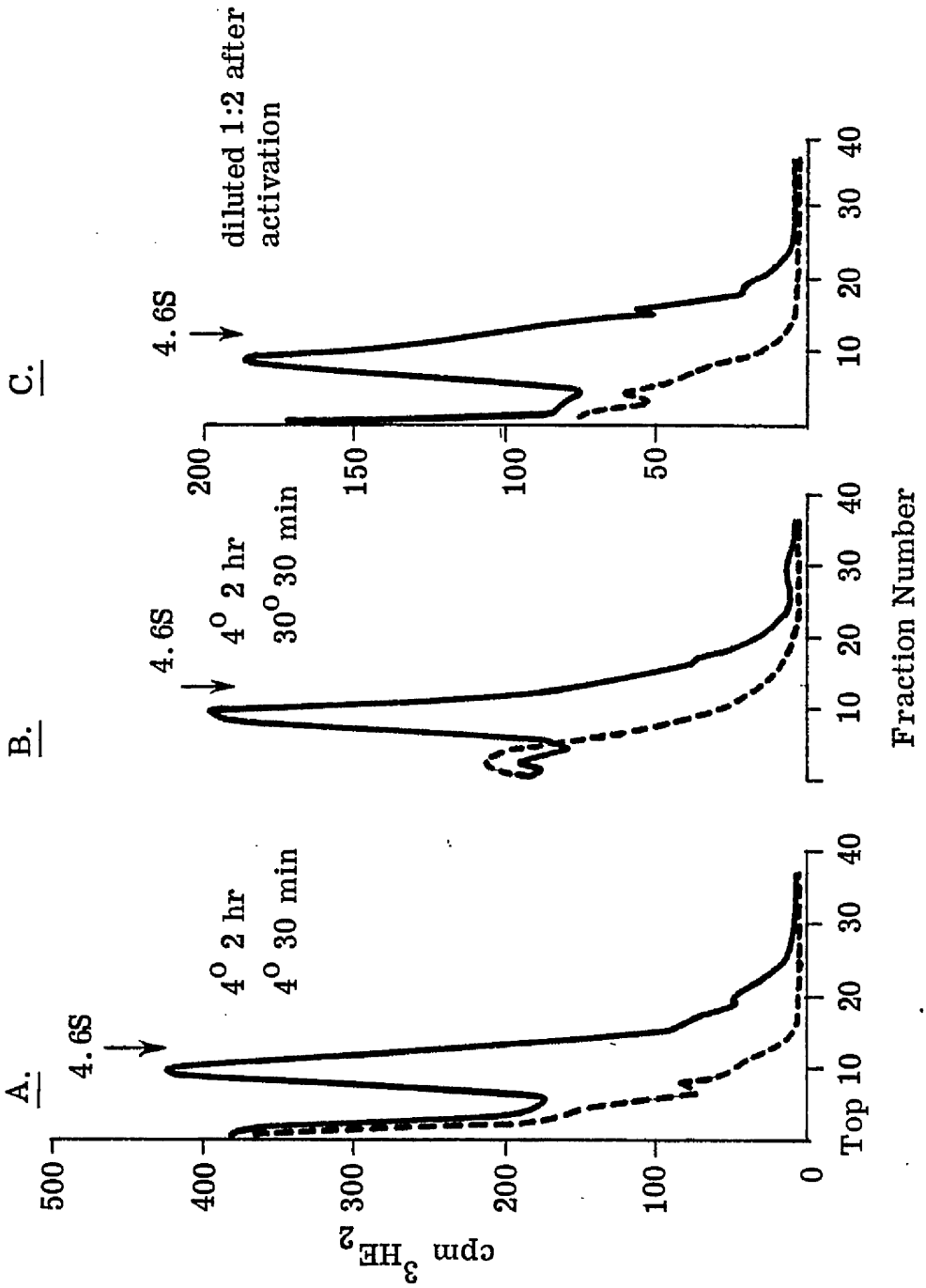
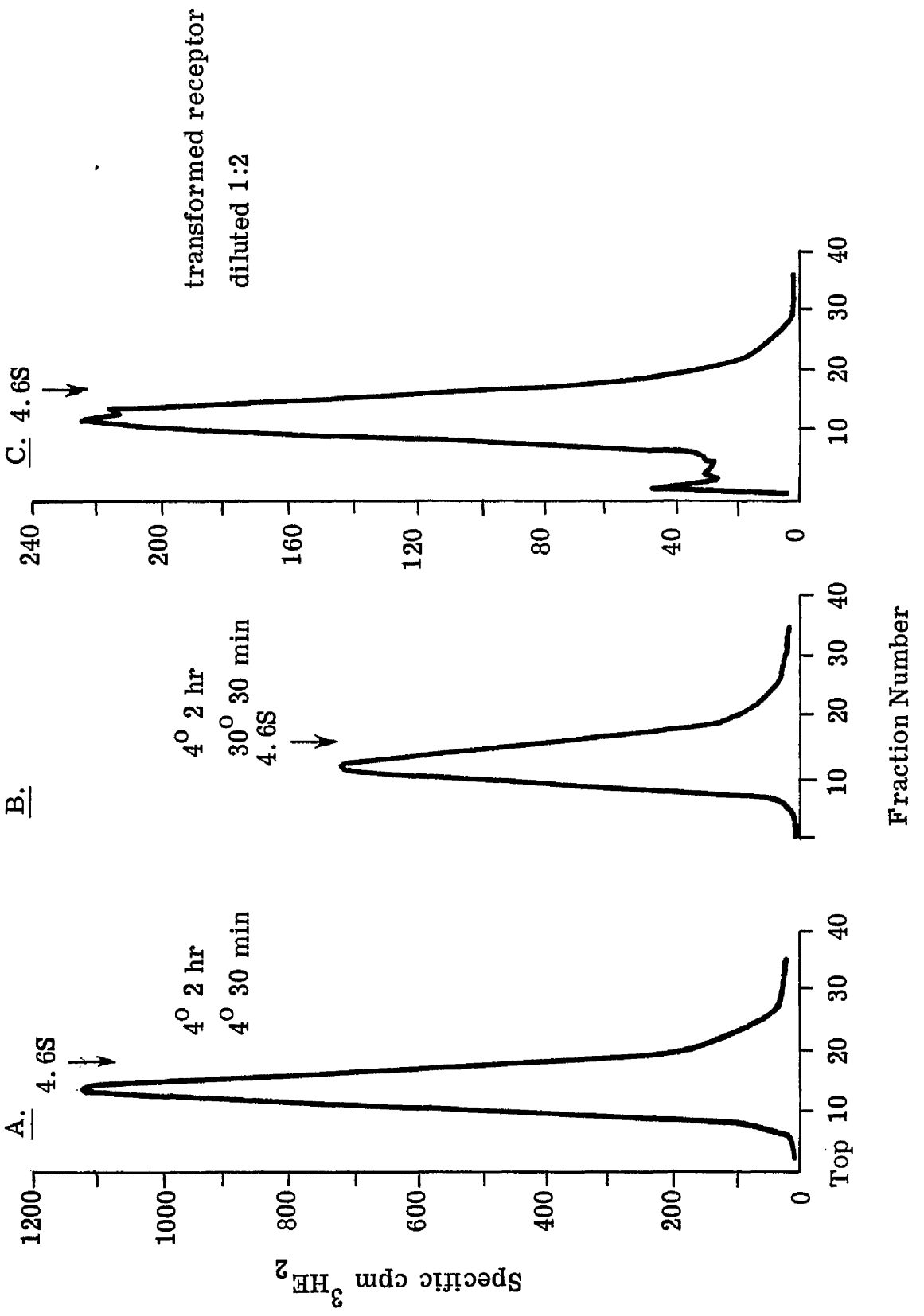

A.
4.6S
4° 2 hr
4° 30 min
B.
4.6S
4° 2 hr
30° 30 min
C.
4.6S
diluted 1:2 after activation
cpm $^3H_{E_2}$
500
400
300
200
100
0
200
150
100
50
Top 10 20 30 40
10 20 30 40
10 20 30 40
Fraction Number

Figure 48.2. **Transformation of Oestrogen Receptor from Human Breast Cancer Cytosol**

The procedure followed was as described for Figure 48.1. Protein concentration of the cytosol was 3.0mg/ml (patient BM). Centrifugation was for 14h at 50,000 rev/min at $4^{o}C$ in a Beckman SW 50.1 rotor. Receptor concentration was 269fmoles/mg protein with 100% recovery in the gradient in A and 186fmoles/mg protein with 94% recovery in B.


A.
4.6S
4° 2 hr
4° 30 min
B.
4° 2 hr
30° 30 min
4.6S
C. 4.6S
transformed receptor
diluted 1:2
Specific cpm $^3HE_2$
Top 10 20 30 40
Fraction Number

The above results were from ER_c^+/ER_n^+ tissues which should possess the functional receptor. The object of these experiments was to show whether ER_c^+/ER_n^- receptor was different from the ER_c^+/ER_n^+ receptor in terms of transformation. Three ER_c^+/ER_n^- tumours were analyzed, one of which showed results analogous to those shown in Figure 48.1 and 48.2, (i.e. no 4S → 5S transition) but the other two tumour cytosols showed complete loss of binding activity on activation (data not shown). This loss could result from the high proteolytic content of ER_c^+/ER_n^- tumours or, less likely, the absence of other subunit(s) involved in stabilization of receptor during transformation.

3.2.3.2.2 **The Influence of Salt Concentration**

The transformation experiments were performed in gradients containing 0.4M KCl. It was, therefore, possible that salt shifted the 4S⟶5S equilibrium in favour of the 4S form of human breast tumour ER_c. However, similar results were obtained in 0.15M KCl containing gradients (data not shown) suggesting that salt was not responsible for the failure to detect the 5S form.

3.2.3.2.3 **The Influence of Time and Equilibration**

It was possible that in the previous experiments the 4S⟶ 5S reaction did not reach equilibrium within the incubation time period. It was therefore decided to label the cytosol overnight at 4^oC with $5 \times 10^{-9}M$ $^3HE_2 \pm 5 \times 10^{-6}M$ DES. This should promote 5S promotion if the 4S⟶ 5S transition resembled the rat uterine ER_c reaction (Traish et al., 1979). However the results obtained were identical to those shown in Figure 48.1 and 48.2 (data not shown). In fact it is probable that such a pre-incubation caused 8S⟶ 4S conversion through a proteolytic effect (see Figures 12.1 and 12.2). Proteolytic activity within the gradient may

also occur.

3.2.3.2.4 **Effect of Centrifugation Temperature**

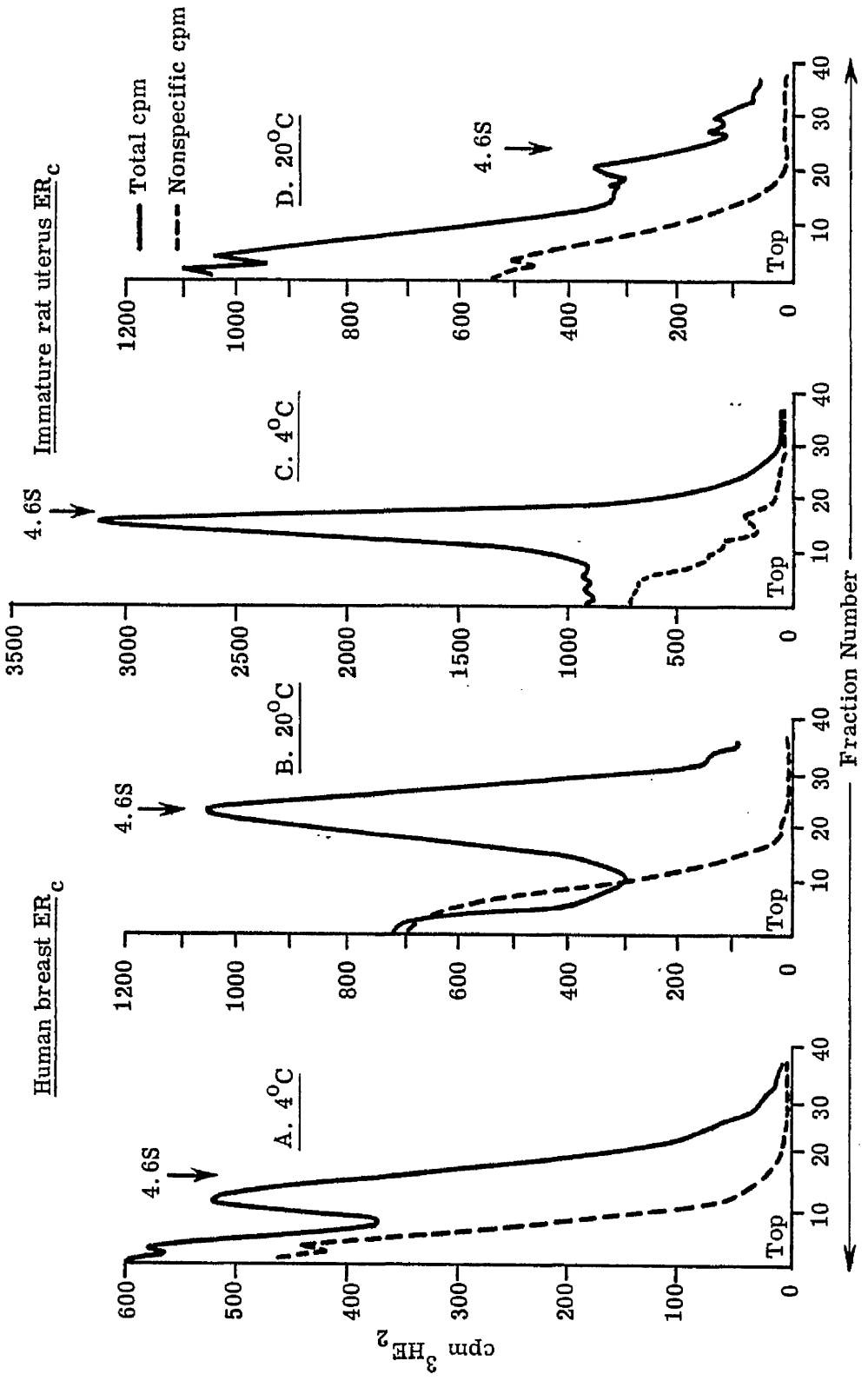
The 4S→5S transformation might take place but temperature and ionic strength might promote dissociation back to the 4S form during the centrifugation run. To test this hypothesis the following experiments were run.

The results shown in Figure 49 permit a direct comparison of immature rat uterine and human breast tumour ER_c sedimentation profile at different temperatures. In the human breast tumour experiment (Figure 49A and B) the receptor, when centrifuged at 4°C without a previous warming step, sedimented as a 3-4S peak. This was shifted to 4.6S when centrifugation was performed at 20°C (without prior warming of the cytosol). The same 4.6S peak was dominant in other breast tumour cytosols run under these conditions. This 4.6S molecule may represent a specific interaction of the receptor with another molecule(s) or a conformational change in receptor protein, increasing its sedimentation value. A change in sedimentation value of ^{14}C-BSA marker itself is ruled out since a linear relationship between it and the ^{14}C γ-globulins marker was observed under all conditions used. Further, in all the human breast ER_c analysis, there was almost 100% recovery of radioactivity indicating that receptor was not lost through aggregation or proteolysis. Immature rat uterine ER_c, while showing a sharp peak at approximately 4.4S during centrifugation at 4°C, failed to retain bound steroid during the 20°C run (Figure 49C and D) either due to protease activity and/or due to lack of protein environment in the 4-5S area (homogenization was at 2 uteri/ml giving the indicated protein concentration). Up to 50% of the receptor aggregated to the bottom of the tube suggesting that both proteolysis of receptor to non-aggregating form and aggregation of receptor must have

Figure 49. **Sedimentation Profile of Oestrogen Receptor from Human Breast Tumour and Immature Rat Uterus at Low (4°C) and Elevated (20°C) Centrifugation Temperature**

Human breast tumour cytosol was prepared in HD from two different sections (shown in A and B) as described in Section 2.2.3.2. Immature rat uterine cytosol was also prepared on two different occasions (2 uteri/ml) as described in Section 2.2.3.1 (shown in C and D). In each case, cytosol was labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 1h at 4°C. After removal of free steroid with DCC (0.25% w/v, final concentration) 200ul aliquots were loaded onto 5-20% linear sucrose density gradients and centrifuged at either 4°C (A and C) or at 20°C (B and D). Centrifugation was for 12h at 50,000 rev/min in a Beckman SW 50.1 rotor. Arrows indicate the position of ^{14}C-labelled marker protein. The protein concentration (mg/ml), receptor concentration (fmoles/mg protein) and percentage recovery (under the peak in A, B and C and total specific counts in D) were: A 2.3, 254, 90%; B 4.2, 225, 102%; C 1.9, 1168, 91%; D 1.4, 1684, 54%.

Sedimentation profile as a function of centrifugation temp. SDG containing 0.15M Kcl.



occurred at the start of the centrifugation. These results were most unexpected since human breast tumour tissues is more rich in protease activity (Sherman et al., 1980). One possibility is that the rat and human receptor degrading proteolytic enzyme(s) (or cofactors) are separated from the receptor to differing extents during centrifugation.

Figure 50 shows that DFP failed to sustain the 5S form of immature rat uterine ER_c when centrifugation was performed at 20°C. In addition ~50% of the receptor still sedimented to the bottom of the tube. Interestingly, there was no dissociation of counts towards the top of the gradient under these conditions (cf Figure 49D). In the experiments shown in Figure 50, rat uterine cytosol was prepared from homogenization of 4 uteri/ml, whereas for results shown in Figure 49C and D, homogenization was at 2 uteri/ml (it was decided only later to perform experiments at 4-5 uteri/ml of buffer since such preparations yielded optimum transformation, See Section 3.2.3.1.2). It therefore seems that the protection provided by the protein environment in Figure 50 prevented the dissociation of steroid seen in Figure 49C and D. It is concluded that DFP fails to protect the rat uterine 5S receptor form during centrifugation at elevated temperature, the 5S form being aggregating to high molecular weight complex and/or dissociating to the 4.6S form.

3.2.3.2.5 **Effect of Receptor Concentration**

It is possible that tumour cytosol receptor concentration could play an important role in the final conformation. Table 14 shows that, in the absence of protease inhibitors, receptor concentration up to ~ 1000 f moles/ml failed to induce formation of 5S receptor although increasing receptor concentration did lead to a small increase in the sedimentation value of the 4S moiety. This increase appears to be

Figure 50. **Effect of DFP on Immature Rat Uterine Oestrogen Receptor Transformation ($20^{o}C$ Centrifugation)**

Immature rat uteri were homogenized in $HDK._{15}$ as described in Section 2.2.3.1 (4 uteri/ml; protein concentration 4.0mg/ml). One portion of the homogenate was then made 10mM with respect to DFP. Homogenate was then centrifuged at 50,000 rev/min for 30 min in a Beckman 50 Ti rotor at $4^{o}C$. Aliquots of cytosol were then labelled with $5 \times 10^{-9}M$ $^{3}HE_{2}$ in the absence (——) or presence (....) of $5 \times 10^{-6}M$ DES at $4^{o}C$. Unbound steroid was then stripped with DCC (0.25% w/v, final concentration) and 200ul aliquots were then loaded onto 5-20% linear sucrose density gradients prepared in $HDK._{15}$. Centrifugation was performed at 45,000 rev/min for 7h in a Beckman SW 50.1 rotor at $20^{o}C$. ^{14}C-BSA (4.6S) used as marker protein is shown as the pointed arrow. The receptor concentration in the absence of DFP was 480fmoles/mg protein with 52% recovery in the gradient and the receptor concentration in the presence of DFP was 531fmoles/mg protein with 54% recovery in the gradient.

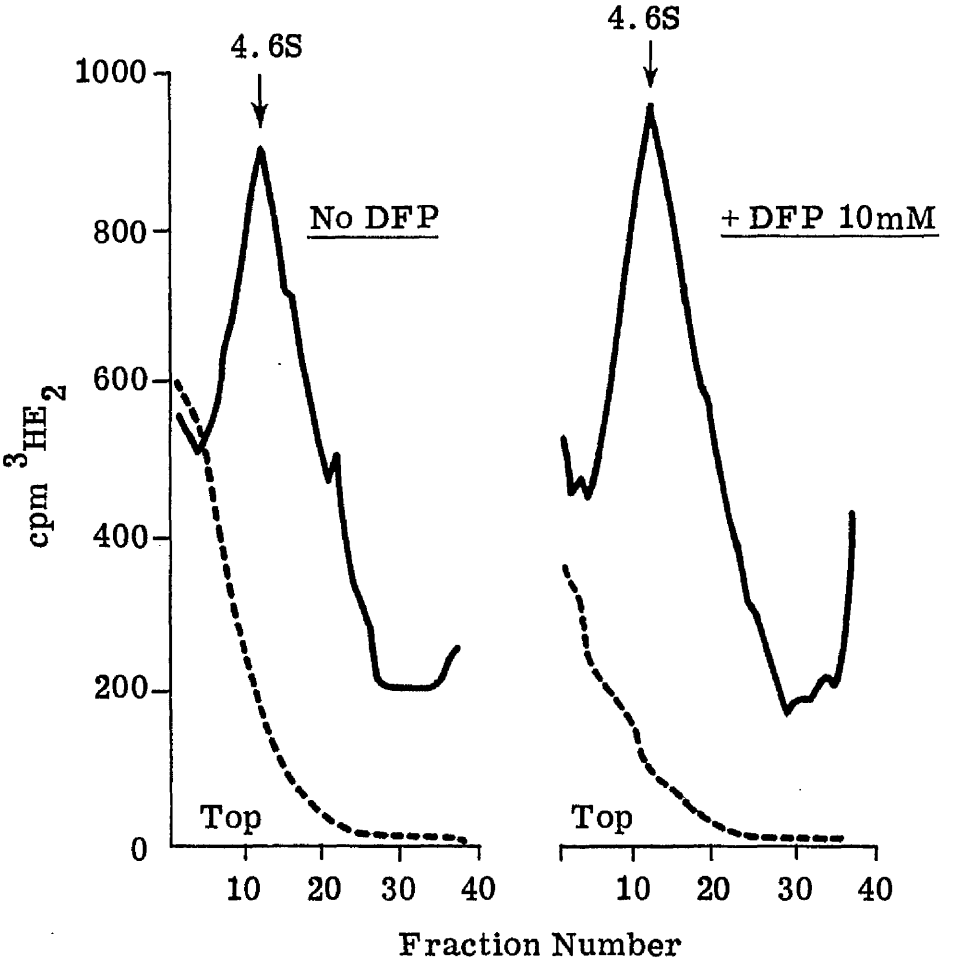

4.6S
4.6S
1000
800
600
400
200
0
cpm $^{3}HE_{2}$
No DFP
+ DFP 10mM
Top
Top
10
20
30
40
10
20
30
40
Fraction Number

Table 14. **Sedimetation Profile of Oestrogen Receptor from Human Breast Tumours in the Absence of Protease Inhibitors - Centrifugation Temperature 20°C**

Human breast tumour cytosol (100-200 mg/ml) was prepared in $HDK._{15}$ as described in Section 2.2.3.2. After incubation with $5 \times 10^{-9}M$ $^3HE_2$ ± 1000 fold excess DES for 1h at 4°C unbound steroid was removed with DCC (0.25% w/v final concentration). Cytosol was loaded on 5-20% sucrose density gradients prepared in $HDK._{15}$ and centrifuged as described in Section 2.2.3.2 at 20°C.

Tumour	Protein Concentration mg/ml	ER_c fmoles/ml	Sedimentation Coefficient
1	2.8	261	Not detectable
2	4.1	341	3.5S
3	2.3	585	3.2S
4	4.0	656	4.0S
5	4.2	946	4.6S

independant of total protein concentration, although the number of tumours studied is small. The maximum sedimentation value obtained under these conditions with cytosol from any tumour was 4.8S.

3.2.3.2.6 **Effect of Protease Inhibitors and Salt Concentration on Receptor Sedimentation Profile at a Centrifugation Temperature of 20°C**

It was possible that in the previous experiments either the receptor was undergoing mild proteolysis or that the other factor(s) required for promoting the higher sedimentation form (analogous perhaps to the activated receptor of the rat uterus), was being degraded. Inclusion of protease inhibitors in tumour cytosols was therefore investigated. The ineffectiveness of PMSF in the analysis of receptors from human sources is recognized (Lukola and Punnonen, 1982). Therefore, another potent inhibitor of serine proteases, DFP, was used. Notides et al. (1976) have already established the use of DFP in elevated temperature centrifugation studies of human ER_c from myometrial tissue.

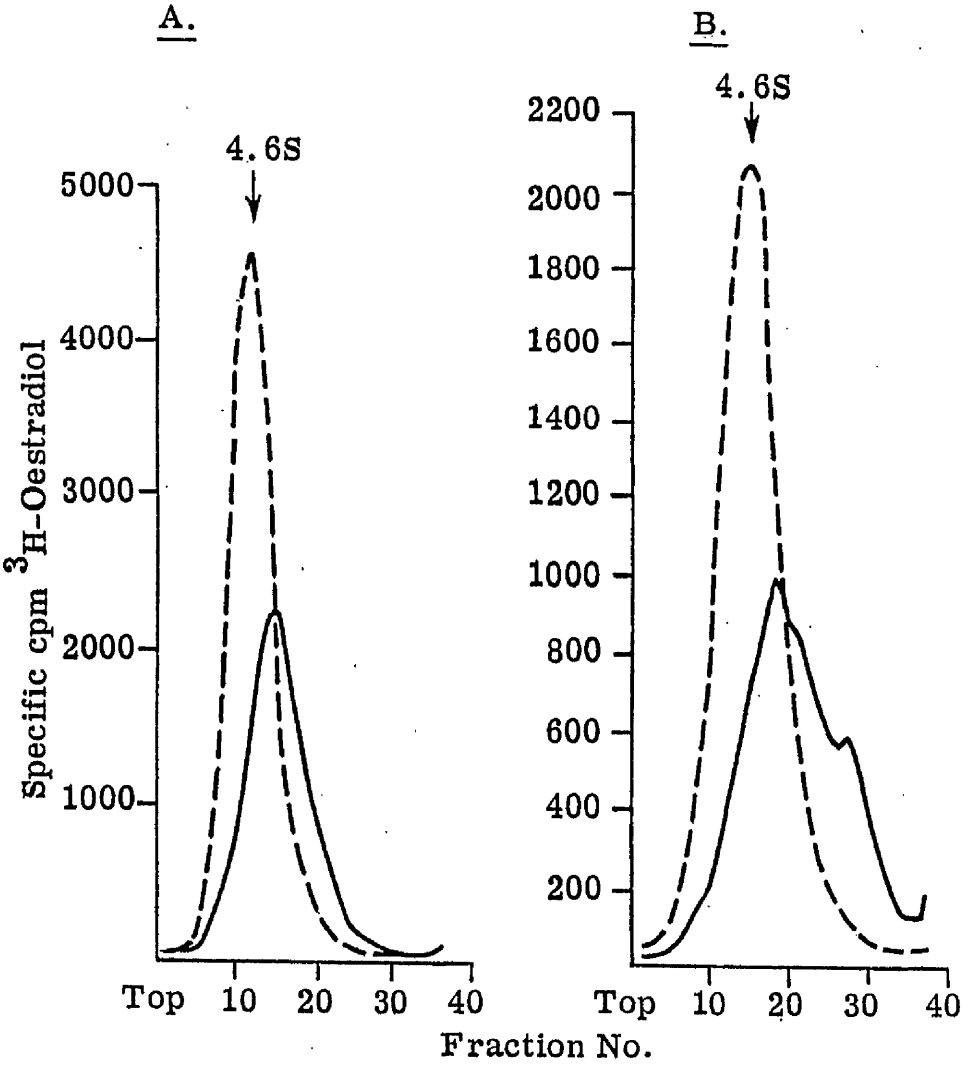
Figure 51A shows that in the presence of DFP the receptor sedimented at ~6S (5.7S) at 20°C centrifugation in physiological ionic strength buffer (0.15M KCl). This is in contrast to the 4.6S value seen for the same cytosol preparation in the absence of DFP [Figure 51 A(...)]. Thus DFP prevents proteolysis of the receptor (or some other factor(s) required for the formation of transformed ER_c). Almost 100% recovery of radioactivity was seen in the absence of DFP, whereas in the presence of DFP the recovery was only 66% with receptor showing a tendency to aggregate and sediment to the bottom of the tube. Thus, factors involved in the aggregation of the intact receptor must be very protease sensitive. In five tumour cytosols studied in duplicate for transformation at 20°C in the presence

Figure 51. **Effect of DFP and Salt Concentration on the Transformation of Oestrogen Receptor from Human Breast Cancer**

In (A) cytosol was prepared in the absence (....) or presence (——) of 10mM DFP as described in Section 2.2.3.2 (patient AP; protein concentration was 7.3mg/ml in the absence of DFP and 7.9mg/ml in the presence of DFP). Cytosol was labelled with 5×10^{-9}M $^{3}HE_2$ ± 1000-fold excess DES at 4°C for 1h. After removal of free steroid with DCC (Section 2.2.3.2) 200ul aliquots were loaded onto 5-20% gradients prepared in HDK.$_{15}$. Centrifugation was for 7h at 45,000 rev/min in Beckman SW 50.1 rotor at 20°C. ^{14}C-labelled BSA was added as marker protein. The receptor concentration in the absence of DFP was 347fmoles/mg protein with 93% recovery in the gradient. In the presence of DFP, the receptor concentration was 314fmoles/mg protein with 66% recovery of receptor in the gradient.

In (B) cytosol was prepared in the presence of 10mM DFP as described in Section 2.2.3.2 (patient SC, protein concentration 7.1mg/ml). The labelling of cytosol and DCC treatment were as above. 200ul aliquots were then loaded onto 5-20% gradients prepared in either HDK.$_{15}$ (——) or HDK.$_{4}$ (....). Centrifugation was for 11h at 43,000 rev/min in Beckman SW 50.1 rotor at 20°C. ^{14}C-labelled BSA was added as marker protein. The receptor concentration was 281fmoles/mg protein. The recovery of receptor in 0.15M KCl gradient was 61% and in 0.4M KCl gradient was 93%.

The sedimentation peak after ^{14}C-BSA marker is at 5.7S (~6S) in both A and B.


A.
B.
4.6S
4.6S
5000
4000
3000
2000
1000
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
Specific cpm ^{3}H-Oestradiol
Top 10 20 30 40
Top 10 20 30 40
Fraction No.

of DFP and in gradients containing 0.15M KCl, the average sedimentation value obtained was 5.9S $\pm$ 0.2S (n = 10).

Formation of 4.6S receptor, seen in the absence of DFP, can also be induced by the presence of 0.4M KCl in the gradients in the presence of DFP (Figure 51B) suggesting that the 4S$\longrightarrow$6S transition was salt sensitive. As in Figure 51A (profile in the absence of DFP), the presence of 0.4M KCl also leads to the loss of aggregation and full recovery of all radioactivity on the gradients. It is not known if 0.4M salt may in fact make DFP ineffective.

Both tumours used for the experiments illustrated in Figure 51A and B had been analyzed previously in low salt gradients (cytosol prepared from different sections) and were found to contain principally the 8S ER_c.

Table 15 relates the sedimentation profile obtained at 20°C centrifugation with various parameters. A comparison with Table 14 shows that the protein concentration used was higher. As seen from Table 15, tumours with only an 8S peak at low ionic strength (centrifugation conducted at 4°C) yielded a single peak at $\sim$6S in 0.15M KCl gradients in the presence of DFP (centrifugation conducted at 20°C). The presence of both 4S and 8S peaks in low ionic strength led to a broad peak sedimenting after the 4.6S marker in gradients run at 20°C at physiological ionic strength. The formation of the broad peak (after 4.6S) apparently depended on the 4S + 8S distribution, obtained in low salt and may also depend on the 8S receptor concentration since tumour number 1 in Table 15 failed to show a distinct, 6S peak in 0.15M KCl gradients although containing 8S form (at a relatively low concentration) in low salt conditions. Figure 52 on the other hand shows the sedimentation profiles obtained from tumour number 5 (Table 15). It can be seen that (Figure 52A) both 4.6S and 6S peaks are resolved. The low salt gradient of the same tumour

Table 15. **Sedimentation Profile of Oestrogen Receptor from Human Breast Tumour in the Presence of Protease Inhibitors - Centrifugation Temperature 20°C**

Preparation of tumour cytosol (400-500 mg/ml) was as described in legend 14 except that DFP was introduced immediately on homogenization (see Section 2.2.3.2). Centrifugation was in 5-20% gradients prepared in HDK.$_{15}$ and centrifugation was as described in Section 2.2.3.2 at 20°C. Also included is the low salt profile of receptor obtained from another section of the tumour which was processed and analyzed as described in Section 2.2.2.1.

Tumour	Protein Concn. mg/ml	ER_c concn. fmole/ml	Sedimentation Coefficient (0.15M KCl, 20°C)	Low Salt Profile (4°C)
1	8.4	124	BPA 4.6S*	8S
2	6.4	273	BPA 4.6S*	4S + 8S
3	6.0	455	BPA 4.6S*	4S + 8S
4	8.3	688	BPA 4.6S*	4S + 8S
5	6.2	1028	4S + 6S	4S + 8S
6	7.0	1996	5.7S	8S
7	8.0	2478	5.7S	8S
8	11.4	2829	4S - 8S	4S + 8S
9	7.6	2977	6S	8S

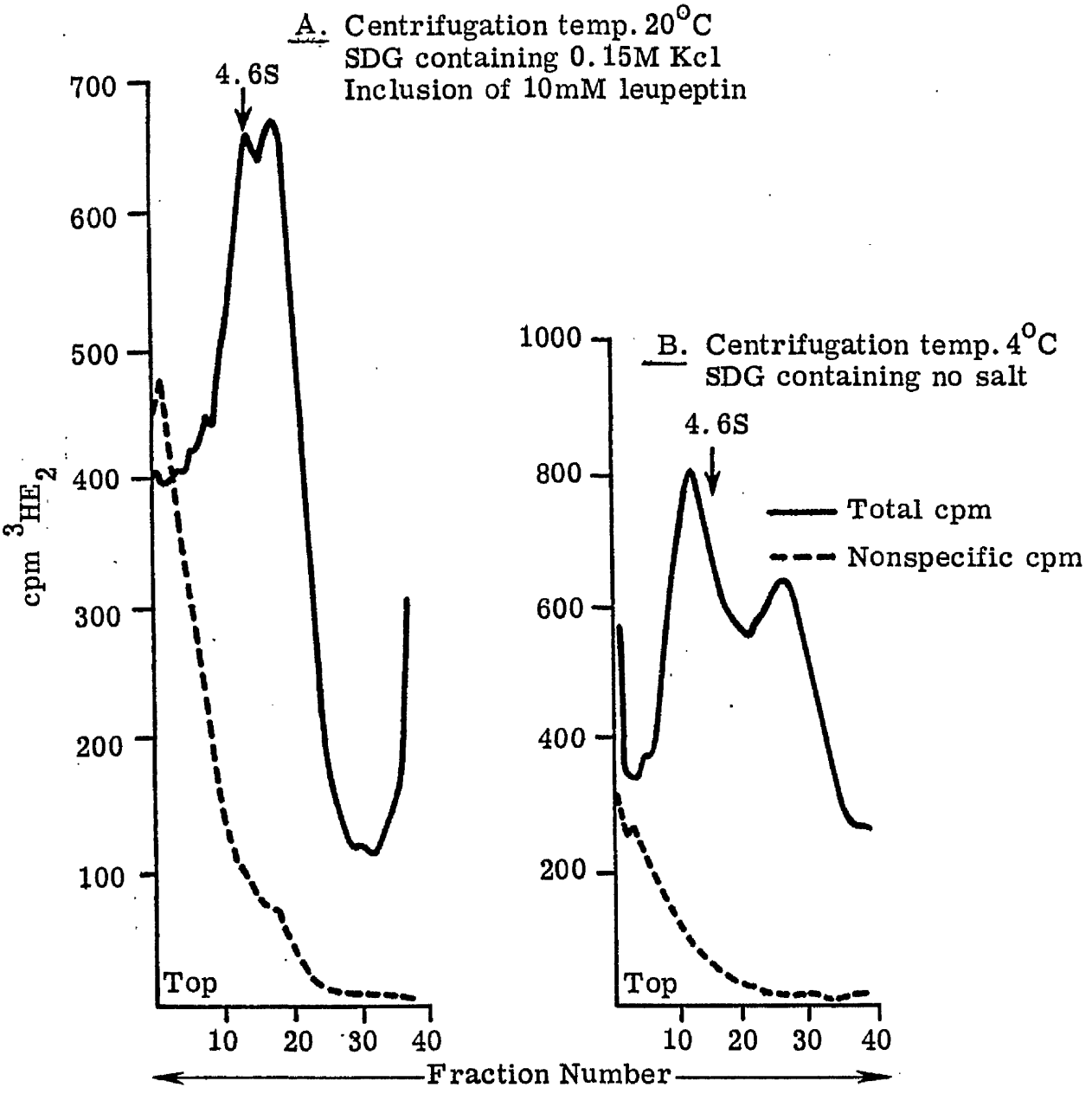
* BPA 4.6S = Broad peak after 4.6S marker

Figure 52. **Effect of Protease Inhibitor (Leupeptin) on the Sedimentation Profile of Human Breast Tumour Cytosol Oestrogen Receptor**

The profiles shown are from two different sections of a single tumour (patient IT). Leupeptin was only used in A. In (A) tumour was homogenized in $HDK._{15}$ as described in Section 2.2.3.2. Immediately on homogenization, Leupeptin was added to a final concentration of 10mM. Cytosol prepared (protein concentration 6.2mg/ml) was then labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 1h at 4^{o}C. Free steroid was then removed with 0.25% w/v final concnetration of DCC and 200ul aliquots loaded onto 5-20% linear sucrose density gradients prepared in $HDK._{15}$. Centrifugation was for 8h at 45,000 rev/min at 20^{o}C in a Beckman SW 50.1 rotor. Arrow indicates the position of ^{14}C-labelled marker protein. The receptor concentration was 166fmoles/mg protein and the recovery in the gradient was 78%. The two peaks shown represent 4.6S and $\sim$6S values.

In B cytosol (protein concentration 4.8mg/ml) was prepared in low salt, then analyzed as described in Section 2.2.2.1. Receptor concentration was 330fmoles/mg protein with 79% recovery in the gradient. The gradients were centrifuged for 14h at 45,000 rev/min in a Beckman SW 50.1 rotor at 4^{o}C.

Effect of protease inhibitor (Leupeptin) on the sedimentation profile of human breast ER_c



(a different section) showed the 4S + 8S profile (Figure 52B). It therefore seems that the receptor obtained from 8S peak in low salt yielded the 6S form in 0.15M KCl containing gradients. The 4.6 value was most probably derived from the low salt 4S peak. A direct comparison of low salt gradients and 0.15M KCl containing gradients cannot be made since these represent profiles from two different sections of the tumour and analysis at two different temperatures.

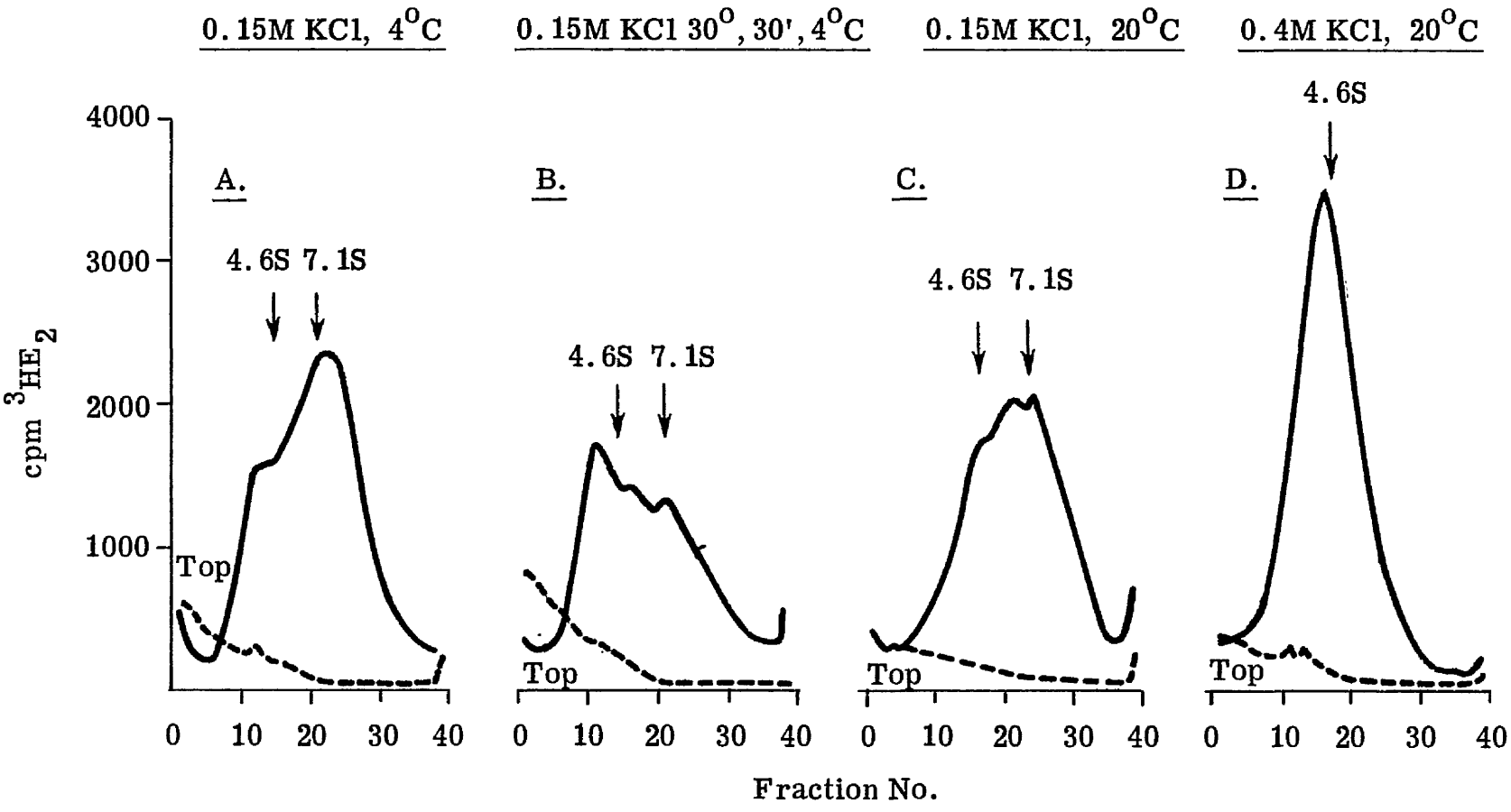
Tumour number 8 in Table 15 showed a 4S + 8S profile in low salt. In 0.15M KCl gradients (and homogenization buffer $HDK._{15}$), this profile did not change when the cytosol was centrifuged at $4^{o}C$ (Figure 53A). There was an additional peak at 6S when the centrifugation was performed at $20^{o}C$ (Figure 53C). This most probably represents a protein concentration effect (protein concentration 11.4mg/ml). This same tumour (number 8 in Table 15) had failed to show any significant DNA binding (Figure 45). Perhaps this lack of disaggregation of the receptor was responsible for such an effect since sodium molybdate is also thought to prevent DNA binding by preventing disaggregation of receptor (Redeuilh et al., 1981). Thus the DNA binding site could be masked whether in low salt or in the presence of 0.15M KCl.

3.2.3.2.7 Effect of Warming the Cytosol in the Presence of Protease Inhibitors Prior to the Analysis of Receptor at $4^{o}C$

Earlier experiments showed that activation (30 , 30') in the presence of the protease inhibitor DFP resulted in aggregation of receptor. To test whether 0.4M KCl containing gradients would promote disaggregation of the aggregated receptor to a form distinct from

Figure 53. **Transformation of Oestradiol Receptor from Human Breast Cancer**

Human breast tumour cytosol was homogenized in $HDK._{15}$ as described in Section 2.2.3.2 (patient HB). Immediately after homogenization, DFP was added to a final concentration of 10mM. Cytosol (protein concentration 11.4mg/ml) was then labelled with 5×10^{-9}M $^{3}HE_{2}$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 1h at 4^{o}C. Then in (A) cytosol was further incubated for 30 min. at 4^{o}C while in (B) cytosol was warmed to 30^{o}C for 30 min. The warmed cytosol was cooled to 4^{o}C for 5 min. and free steroid in both cases removed by DCC (0.25% w/v final concentration). 200ul aliquots were loaded onto 5-20% gradients prepared in $HDK._{15}$ which were centrifuged at 50,000 rev/min in Beckman SW 50.1 rotor at 4^{o}C. For (C) and (D), after the 1h incubation with steroid at 4^{o}C, free steroid was removed as described above and 200ul aliquots were loaded onto linear 5-20% gradients prepared in either $HDK._{15}$ (C) or $HDK._{4}$ (D). These were centrifuged at 42,000 rev/min in Beckman SW 50.1 rotor at 4^{o}C. Cytosol receptor concentration was 248fmoles/mg protein and recovery of specific counts in the gradient was 100% in A, 72% in B, 101% in C and 106% in D.

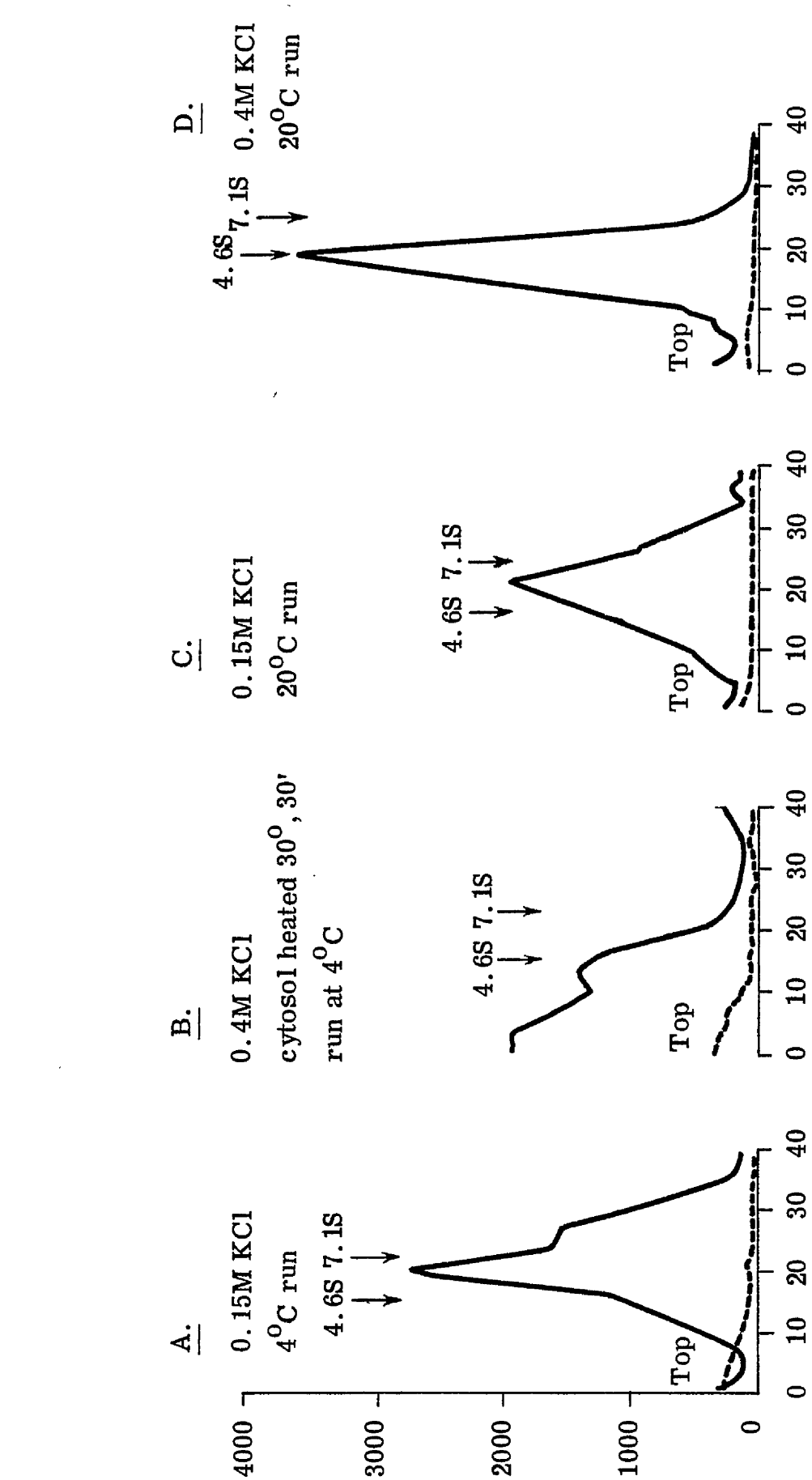

0.15M KCl, 4°C
0.15M KCl 30°, 30', 4°C
0.15M KCl, 20°C
0.4M KCl, 20°C
A.
B.
C.
D.
4.6S 7.1S
4.6S 7.1S
4.6S 7.1S
4.6S
Top
Top
Top
Top
4000
3000
2000
1000
cpm $^3HE_2$
0 10 20 30 40
0 10 20 30 40
0 10 20 30 40
0 10 20 30 40
Fraction No.

non-activated receptor, the following experiment was conducted. Tumour cytosol was heat activated and loaded onto 0.4M KCl gradients (Figure 54B) and centrifuged at $4^{o}C$.

In addition non-activated cytosol was loaded onto 0.15M KCl gradient and centrifuged at $4^{o}C$ (Figure 54A). In Figure 54A, the 6S peak can be seen in addition to the 4S and 8S shoulders. It should be pointed out that homogenization was performed in $HDK_{.15}$, rather than HED as in Figure 22 where only a 4S peak was demonstrated. Figure 54B shows the effect of heating the cytosol to $30^{o}C$ for 30 min. followed by centrifugation at $4^{o}C$ in 0.4M KCl containing gradients, an experimental situation analogous to that showing the transformed 5S receptor from rat uteri. This experiment was conducted in the presence of protease inhibitor DFP. There appears to be a considerable loss of radioactive counts towards the top of the gradient. The specific counts on the gradient (not necessarily receptor bound) only account for 78% of the total counts loaded. It is probable that aggregation (as a result of warming in the presence of protease inhibitor) followed by disaggregation on gradients is releasing a form of receptor with a modified structure giving a lower K_d value and, as a result, loss of free steroid, seen towards the top of the gradient. This possibility is further strengthened by the result shown in Figure 54C where centrifugation of the cytosol at $20^{o}C$ (without previous heating step) resulted in 78% recovery of counts on the gradient with a 6S peak. When the centrifugation of the same cytosol was performed at $20^{o}C$ in a 0.4M KCl containing gradient (Figure 54D), only the 4.6S form of the receptor was observed with 96% recovery of specific counts under the peak. The most likely explanations, therefore, for the situation observed in Figure 54B are that either, when once aggregated, the receptor cannot revert to the original 4S form in 0.4M KCl or that certain enzymes

Figure 54. **Human Breast Tumour Cytosol Oestrogen Receptor: Analysis under Various Experimental Conditions used for Transformation Study**

Human breast tumour cytosol was homogenized in $HDK._{15}$ as described in Section 2.2.3.2 (patient SN). Immediately after homogenization DFP was added to a final concentration of 10mM. Cytosol (protein concentration 7.6mg/ml) was labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 1h at 4^{o}C. Free steroid was then removed with DCC (0.25% w/v, final concentration). Two sets were then immediately loaded onto 5-20% linear sucrose density gradients prepared in either $HDK._{15}$ (C) or $HDK._{4}$ (D) and centrifuged at 42,000 rev/min for 11h at 20^{o}C in a Beckman SW 50.1 rotor. Another set of aliquots was heat activated at 30^{o}C for 30 min, cooled to 4^{o}C for 5 min and then loaded onto a gradient prepared in $HDK._{4}$ (B). A further set of aliquots which was kept at 4^{o}C during this period was loaded onto an $HDK._{15}$ gradient (A). Samples in (A) and (B) were centrifuged at 4^{o}C at 50,000 rev/min for 11h in a Beckman SW 50.1 rotor. The sedimentation peak in A and C, between the marker proteins was at $\sim$6S while the peak in D co-sedimented with 4.6S marker. The receptor concentration of the cytosol preparation was 392fmoles/mg protein and recovery of specific counts was 99% in A, 73% in B, 78% in C and 95% in D.


A.
0.15M KCl
4°C run
4.6S 7.1S
B.
0.4M KCl
cytosol heated 30°, 30'
run at 4°C
4.6S 7.1S
C.
0.15M KCl
20°C run
4.6S 7.1S
D.
4.6S 7.1S
0.4M KCl
20°C run
Top
0
10
20
30
40
1000
2000
3000
4000
cpm $^3HE_2$
Fraction Number

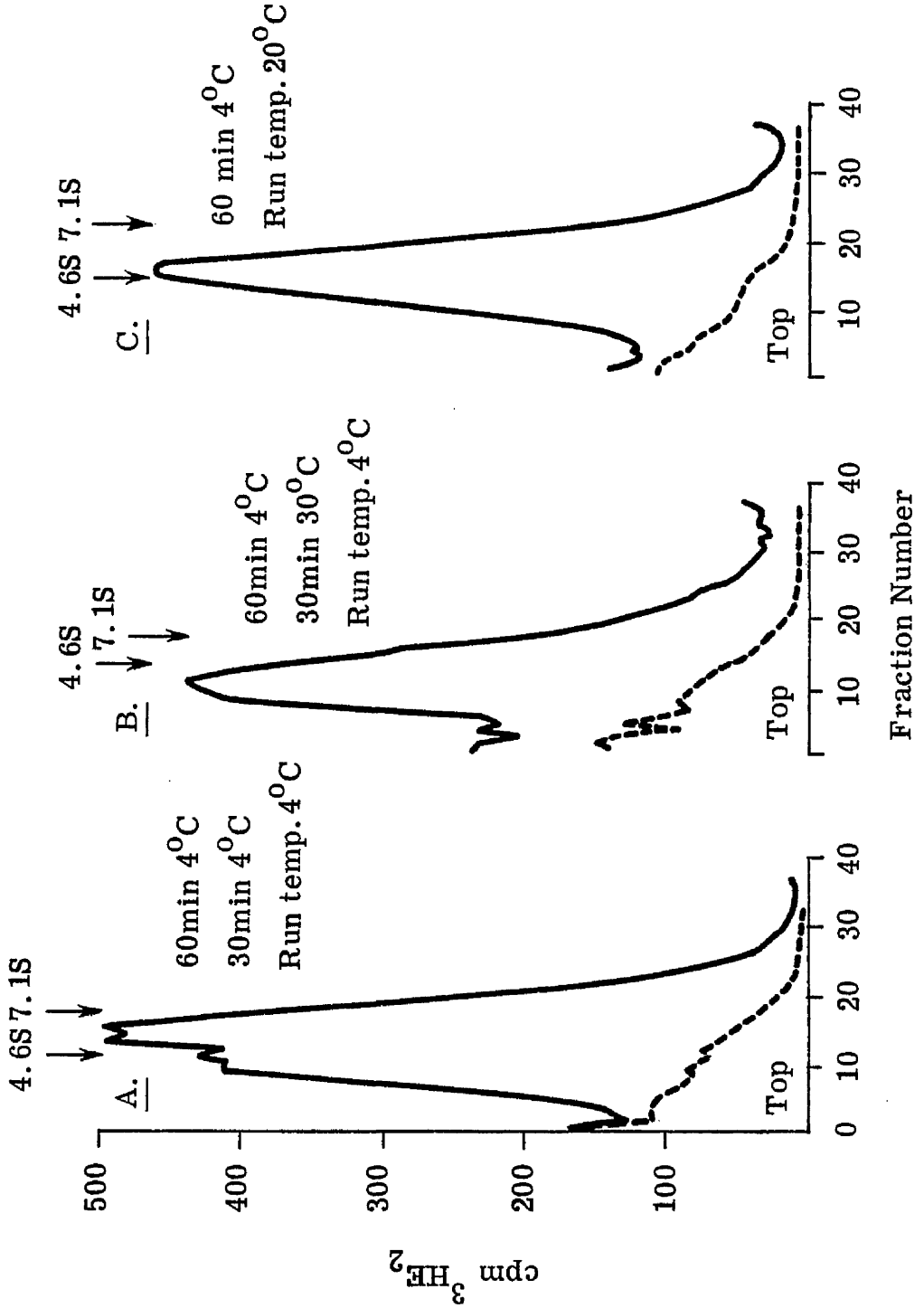
present in some tumour cytosols may hydrolyze DFP at high temperatures. Perhaps during the 20°C centrifugation, an early separation of such an enzyme has taken place or else that the enzyme has preferential activity against the aggregated protein.

Heating the cytosol to 30°, 30' followed by centrifugation in 0.15M KCl at 4°C (Figure 53B) resulted in a very broad sedimentation profile extending from 4S to 8S with only 72% receptor recovery on the gradient. 26% of the specific counts were located at the bottom of the tube and, therefore, represented aggregation even in the presence of 0.15M KCl. The unusual properties of the cytosol from this tumour have already been noted in the previous section. The low salt gradient of this tumour showed a 4S + 8S profile. Again, another tumour which showed the low salt profile of 4S + 8S forms also showed both 4S + 6S forms on 0.15M KCl gradients at 4°C (Figure 55A). Heating at 30°C, 30' of the same cytosol resulted in conversion of the 6S form to the 4S form (Figure 55B) with some aggregation to the bottom of the tube (% recovery of specific counts was 85% in Figure 55B compared to 100% in Figure 55A). The 20°C centrifugation of the same cytosol in 0.15M KCl (Figure 55C) showed complete recovery of counts (98%) but the broadness of the peak after the 4.6S marker is characteristic of 4S + 8S low salt type tumour cytosol (Table 15).

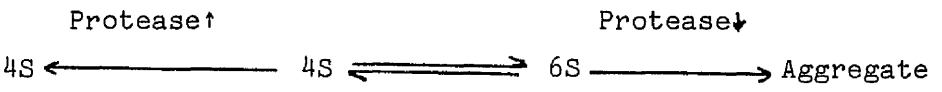
It appears from the foregoing that two simultaneous processes can occur when tumour cytosol is prepared in $HDK_{.15}$ in the presence of protease inhibitors and, subsequently, subjected to activation conditions (Scheme 1).

Figure 55. **Analysis of Transformed Oestrogen Receptor from Human Breast Cancer in Gradients Containing 0.15M KCl**

Human breast tumour was homogenized in $HDK._{15}$ as described in Section 2.2.3.2 (patient CB). Immediately on homogenization, DFP was added to a final concentration of 10mM. Cytosol (protein concentration 6mg/ml) was labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 1h at 4^{o}C. Free steroid was then removed with DCC (0.25% w/v, final concentration). One set of aliquots was then loaded onto 5-20% gradients and centrifuged at 20^{o}C, for 11h at 42,000 rev/min in a Beckman SW 50.1 rotor (C). Another set of aliquots was warmed to 30^{o}C for 30 min followed by cooling to 4^{o}C for 5 min. (B), while keeping a further set at 4^{o}C as control (A). 200ul aliquots were then loaded onto 5-20% gradients and centrifuged at 45,000 rev/min for 11h at 4^{o}C in a Beckman SW 50.1 rotor. The receptor concentration of the cytosol was 76fmoles/mg protein with recovery of specific counts in gradient of 100% in A, 85% in B, and 98% in C.


cpm $^3HE_2$
500
400
300
200
100
A.
4.6S 7.1S
60min 4°C
30min 4°C
Run temp. 4°C
B.
4.6S
7.1S
60min 4°C
30min 30°C
Run temp. 4°C
C.
4.6S 7.1S
60 min 4°C
Run temp. 20°C
Top
0 10 20 30 40
Fraction Number

Scheme 1 (1) (2)



Comprising 4S proteolyzed form	comprising 4S aggregating type	possibly 4S dimer	comprising 8S + higher molecular weight species

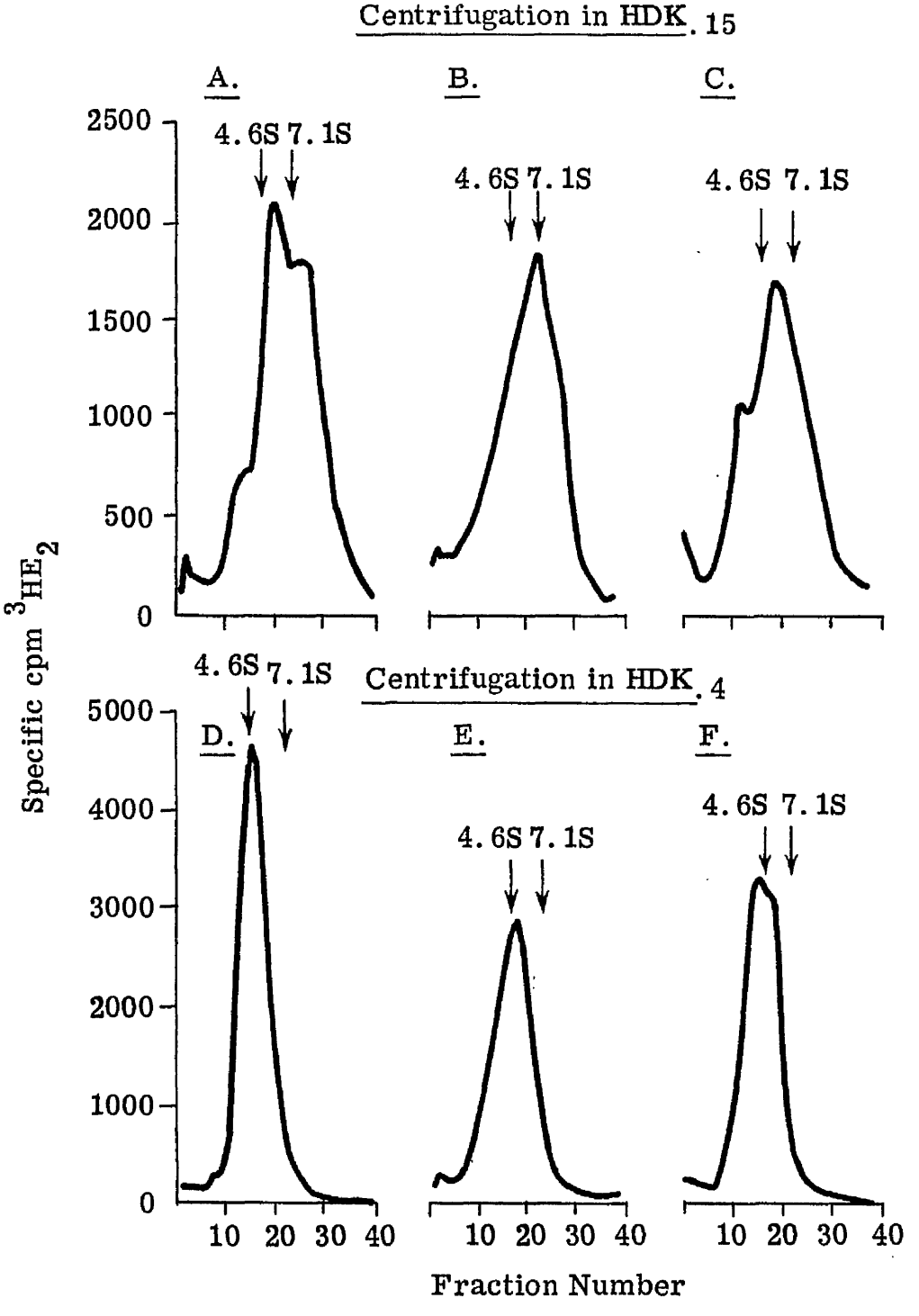
Depending on the protease content of the tumour cytosol, mechanism (1) and/or (2) shown in Scheme 1, is dominant.

In Figure 54A non-activated cytosol in 0.15M KCl at $4^{o}C$ showed a clear 8S shoulder, together with the suggestion of a 4S shoulder, in addition to the major 6S peak. However activation conditions led to the disappearance of the 8S shoulder (Figure 54B, similar to the disappearance of the 6S form in Figure 55B). After activation conditions, therefore, there is a clear increase in the amount of 4S but the 6S and 8S peaks are lost. A proportion of receptor is aggregated to the bottom of the tube and a large proportion of radioactivity appears to have been released from the receptor specially, as shown in Figure 54B, in the presence of 0.4M KCl gradients. It is possible that the 4S form of receptor, extracted initially in $HDK_{.15}$, is possibly aggregated to the 6S form and further aggregated to the higher molecular weight species or is proteolyzed to the 4S non-aggregating type (Scheme 1, Figure 54 and 55). It was therefore decided to see if aggregation could be accelerated by homogenizing tumours in low salt rather than in buffers containing 0.15M salt.

For the experiment illustrated in Figure 56, the tumour was homogenized in HD buffer instead of $HDK_{.15}$. DFP was then introduced. Figure 56A shows that again a 6S + 8S profile (with a shoulder at $\sim$4S) could be observed when centrifugation was carried out in 0.15M KCl at

Figure 56. **Effect of Salt Concentration and Temperature on the Sedimentation Profile of Oestrogen Receptor from Human Breast Cancer Cytosol**

Human breast tumour cytosol was homogenized in HD (patient SN) as described in Section 2.2.3.2. Immediately after homogenisation DFP was added to a final concentration of 10mM. Cytosol (protein concnetration 4.8mg/ml) was labelled with 5 x 10^{-9}M $^{3}HE_2$ ± 1000-fold excess DES for 1h at 4°C. Free steroid was then removed with DCC (0.25% w/v, final concentration). Two sets were then immediately loaded onto 5-20% gradients prepared in either HDK.$_{15}$ (B) or HDK.$_{4}$ (E) and centrifuged at 42,000 rev/min for 11h at 20°C in a Beckman SW 50.1 rotor. Two further sets were heat activated at 30°C for 20 minutes, cooled to 4°C for 5 minutes and then loaded onto 5-20% gradients prepared in either HDK.$_{15}$ (C) or HDK.$_{4}$ (F). Control aliquots at 4°C were also loaded onto 5-20% gradients prepared in either HDK.$_{15}$ (A) or HDK.$_{4}$ (D). These gradients (A, C, D and F) were then centrifuged at 50,000 rev/min for 11h at 4°C in a Beckman SW 50.1 rotor. Arrows indicate the position of ^{14}C-labelled marker proteins. The peak position between the sedimentation markers in A and C is at ~6S. The receptor concentration of the cytosol preparation as determined by taking aliquots after DCC treatment was 587fmoles/mg protein with recovery of specific counts in the gradient representing 96% in A, 85% in B, 88% in C, 103% in D, 97% in E and 100% in F.


Centrifugation in HDK. 15
A.
B.
C.
4.6S 7.1S
2500
2000
1500
1000
500
0
Specific cpm $^3HE_2$
Centrifugation in HDK. 4
D.
E.
F.
5000
4000
3000
2000
1000
0
10 20 30 40
Fraction Number

4°C. Now, in contrast to the previous results, centrifugation at 20°C led to a 7-8S peak (Figure 56B) suggesting that the 4S and 6S are present in the aggregated form. Surprisingly however, warming cytosol to 30°, 30' prior to centrifugation in 0.15M KCl containing gradients at 4°C, yielded a dominant 6S profile with a 4S shoulder (Figure 56C). This result contrasts with the previously observed aggregation plus conversion to the 4-4.6S form under such conditions (Figures 53-55). It therefore appears that, in the low salt conditions, heating this tumour cytosol to 30°, 30' prior to analysis in 0.15M KCl gradients at 4°C, led to the formation of 6S form. This suggests that either there was no aggregation to high molecular state or that the aggregation is reversible under these conditions. The same cytosol used in Figure 56A, B and C, when subjected to 0.4M KCl gradient following various conditions yielded only the 4.6S state (Figure 56D, E and F) although in Figure 56F there was a shoulder present at ∿5S and there was no loss of counts seen towards the top of the gradient. It is essential, therefore, to establish if the 6S form represents the active form, that is to say if the 6S form observed in 0.15M KCl gradients represents the same type as the two subunit structures of progesterone receptor. The formation of 6S at 4°C for the oestrogen receptor (as indeed is the case for progestoner receptor) may take place as a result of removal of low or macromolecular weight inhibitors during centrifugation.

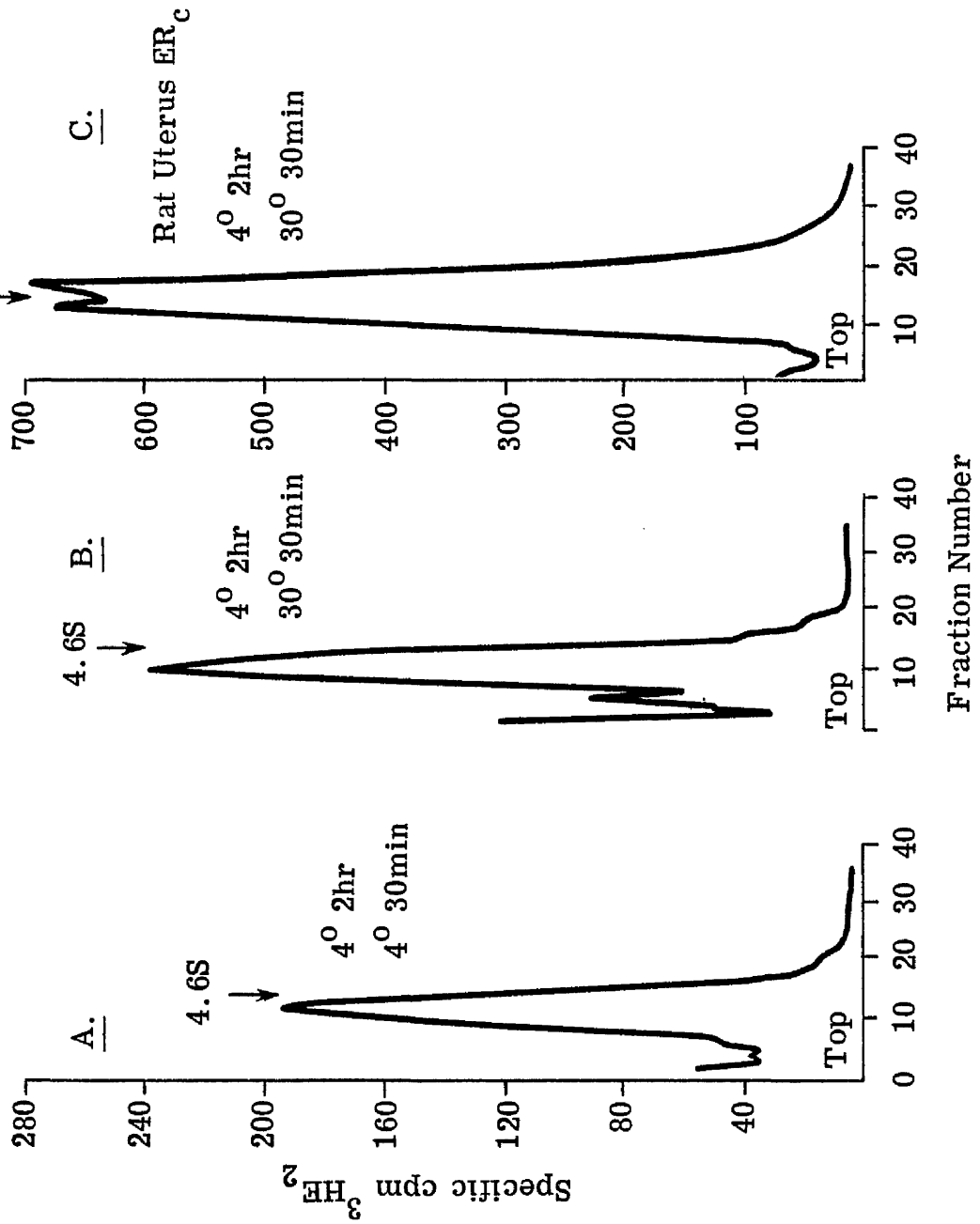
3.2.3.3 The 4S⟶5S Transition of the Soluble Oestrogen Receptor from the Human Endometrial Tissue

3.2.3.3.1 Effect of Warming to 30°C for 30 min. prior to Analysis

Warming human endometrial cytosol to 30°C for 30 min followed by analyzing the cytosol in 0.4M KCl gradients (Figure 57A and B), led to

Figure 57. **Transformation of Human Endometrial and Stored Immature Rat Uterine Cytosol Oestrogen Receptor**

Human endometrial cytosol was prepared in HD as described in Section 2.2.3.2 (protein concentration was 3mg/ml). Aliquots were labelled for 2h at $4^{o}C$, with 5 x $10^{-9}M$ $\pm$ 1000-fold excess DES. An aliquot was then kept at $4^{o}C$ for an additional 30 min (A) while a second aliquot was warmed to $30^{o}C$ for 30 min (B). Warmed cytosol was then cooled to $4^{o}C$ for 5 min and unbound counts from both sets were stripped with DCC (Section 2.2.3.2) prior to SDGA. Stored rat uterine cytosol was also prepared in HD (protein concentration 2.6mg/ml), labelled, heat activated and DCC stripped in a similar manner (C). Sedimentation profile was then observed on 10-30% linear gradients prepared in $HDK._{4}$. Centrifugation was for 17h at 50,000 rev/min in a Beckman SW 50.1 rotor at $4^{o}C$. Arrow indicates the position of ^{14}C-labelled marker protein. Receptor concentration in fmoles/mg protein for human endometrial cytosol was 98 in A with 100% recovery in gradient and 137 in B with 80% recovery in gradient. Receptor concentration for rat uteri (C) was 601 fmoles/mg protein with 93% recovery in gradient.


A.
4.6S
4° 2hr
4° 30min
B.
4.6S
4° 2hr
30° 30min
C.
4.6S
Rat Uterus ER$_c$
4° 2hr
30° 30min
Specific cpm $^3HE_2$
Top
Fraction Number

an increase in the total binding capacity. This is in contrast to the results presented under the same conditions for breast tumour tissue cytosol (Figure 48.1 + 48.2). However, there was no change in the sedimentation value from 4S⟶5S, confirming the situiation with the human breast tumour cytosol and in contrast to the results obtained with immature rat uterine cytosol included in Figure 57 (C). In contrast to the ∼100% 5S conversion obtained from fresh immature rat uteri, only ∼50% of sucrose/glycerol stored uteri could be transformed to the 5S form. The sucrose/glycerol storage procedure is known to affect the stability of rat uteri ER_c (Figure 21). However, both fresh and stored endometrial ER_c showed no such transition. In some cases of human endometrial cytosol, the receptor, after activation, sedimented as a single peak at ∼ 3S, implying a different proteolytic content of different cytosols. Similar findings are reported by Notides et al. (1976) for human myometrial tissue.

Figure 58 shows the effect of temperature of activation on the sedimentation profile of human endometrial cytosol, under 0.4M KCl and 4°C centrifugation conditions. The purpose of this experiment was to find out if the 30°, 30' heating procedure was longer than needed to activate or transform (4S⟶ 5S) the receptor and might even be promoting a 5S⟶ 4S degradation. A reduced temperature of 20°C was, therefore, used with no difference in sedimentation value (Figure 58). A similar temperature-dependent rise in total binding capacity (as seen in Figure 57) was obtained.

3.2.3.3.2 Effect of Elevated Centrifugation Temperature, Inclusion of Protease Inhibitors and Salt Concentration

Figure 59 shows that only in the presence of a protease inhibitor (Leupeptin) can the 6S be maintained during elevated temperature centrifugation run. Inclusion of DFP also showed a similar effect. As

Figure 58. **Effect of Temperature on the Sedimentation Profile of Human Endometrial Cytosol Oestrogen Receptor**

Human endometrial cytosol was prepared as described in Section 2.2.3.2 (except that protein concentration obtained was only 2mg/ml). Aliquots were labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 2h at $4^{o}C$. An aliquot was kept at $4^{o}C$ (A) while another aliquot was warmed at $20^{o}C$ for 30 min. Another aliquot was warmed at $30^{o}C$ for 30 min. Warmed aliquots were cooled to $4^{o}C$ for 5 min. DCC stripped (Section 2.2.3.2) and analyzed on 10-30% w/w linear sucrose density gradients containing 0.4M KCl. Centrifugation was for 18.5h at 50,000 rev/min in a Beckman SW 50.1 rotor at $4^{o}C$. Arrow indicates the position of ^{14}C-labelled marker protein. Receptor concentration in fmoles/mg protein obtained was 104 in A, 117 in B and 151 in C. Recovery of receptor was near 100% in all cases.

Effect of temp. on the transformation of human endometrium ER_c.
centrifugation temp. 4°C. SDG containing 0.4M Kcl

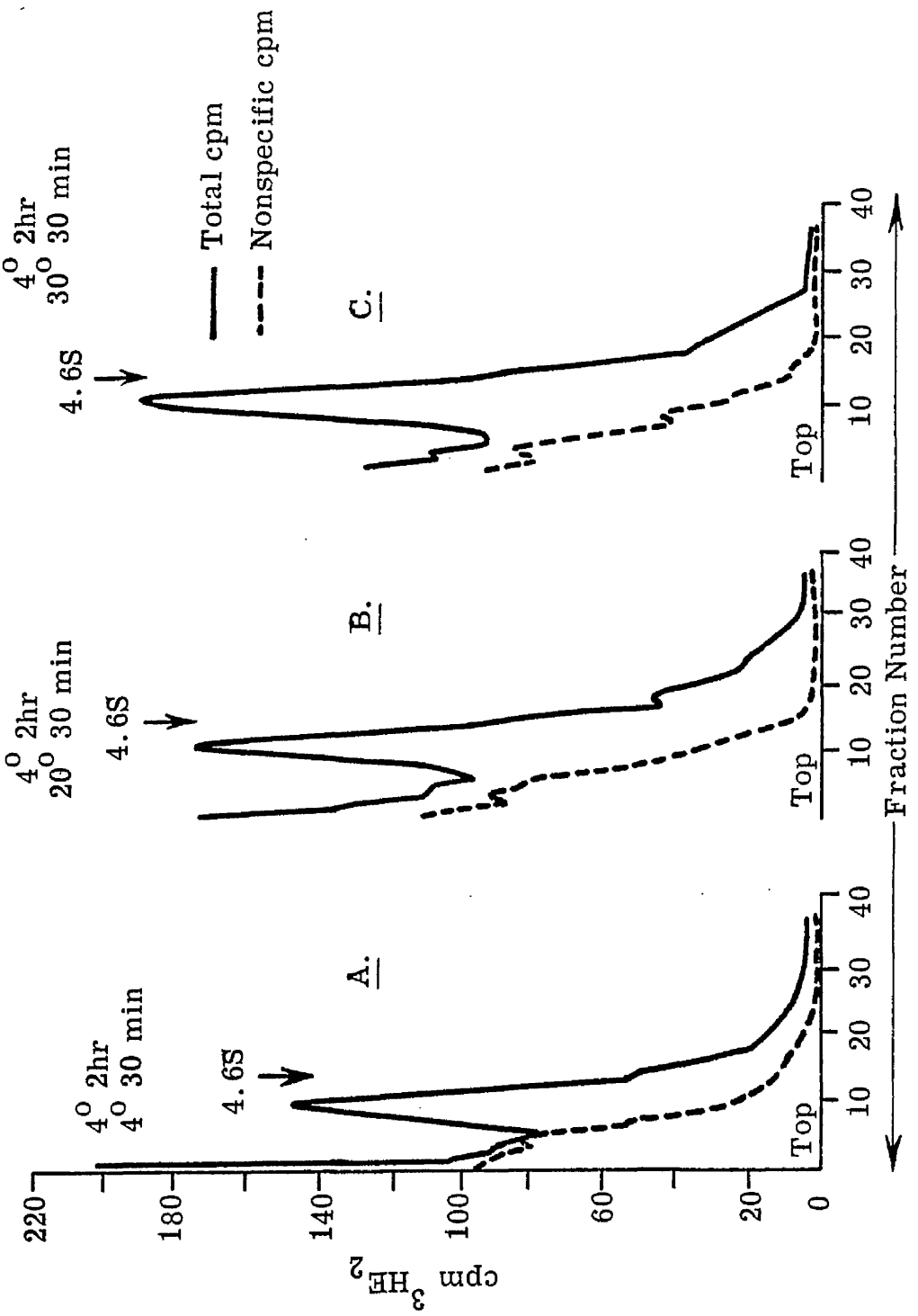
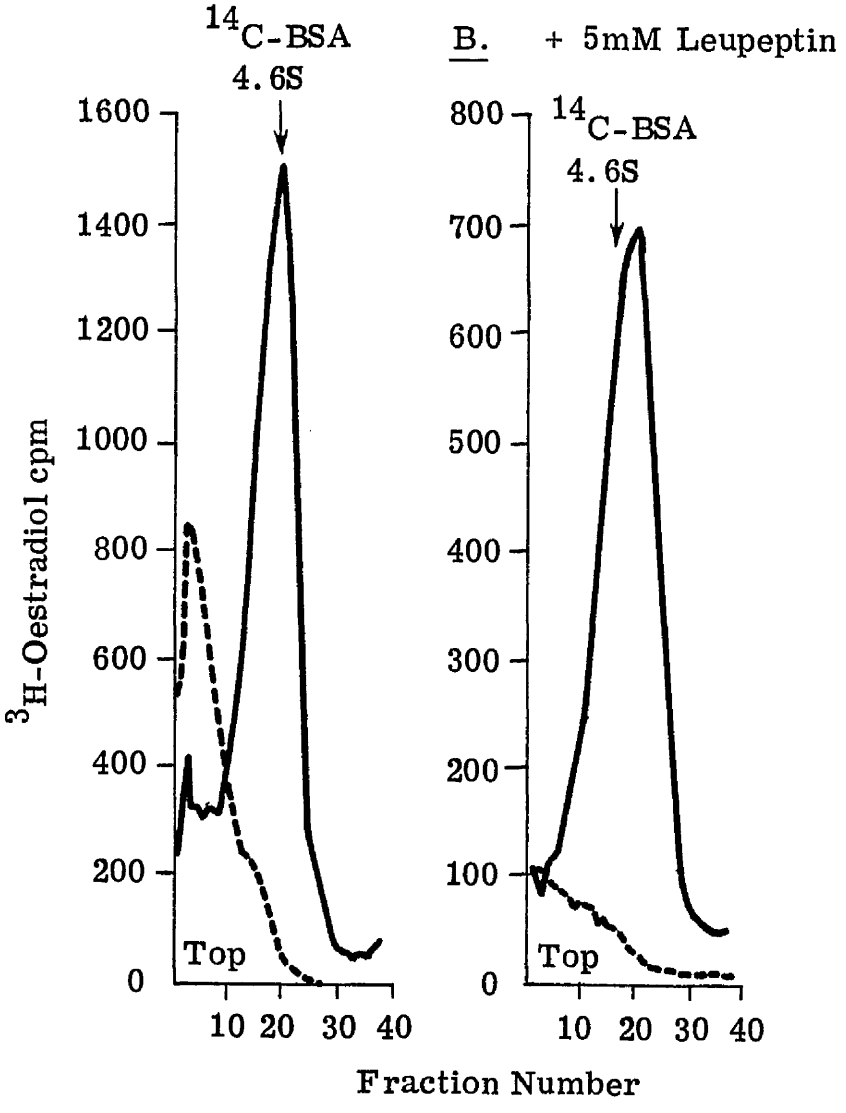


Figure 59. **Effect of Protease Inhibitor Leupeptin on the Sedimentation Profile of Oestrogen Receptor from Human Endometrial Cytosol**

Two different endometrial samples were used. In (A) human endometrium was homogenized in $TDK._{15}$ as described in Section 2.2.2.6. Cytosol was prepared (Section 2.2.2.1) and the protein concentration obtained was 6.8mg/ml. It was then labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 1h at 4°C. Free steroid was removed with DCC (0.25% w/v, final concentration) and 200ul aliquots loaded onto 5-20% linear sucrose density gradients prepared in $TDK._{15}$. Centrifugation was for 8h at 50,000 rev/min in a Beckman SW 50.1 rotor at 20°C. Arrow indicates the position of ^{14}C-BSA (4.6S). Receptor concentration was 209fmoles/mg protein and recovery of specific counts in the gradient was 84%. Receptor peak is at 4.8S.

In (B), endometrial sample was homogenized in $HDK._{15}$ as described above. Cytosol preparation (protein concentration 6.2mg/ml) was labelled with 2×10^{-9}M $^{3}HE_2$ in the absence (——) or presence (....) of 2×10^{-6}M DES for 1h at 4°C. Free steroid was removed and aliquots loaded onto gradients prepared in $HDK._{15}$ as described above. Centrifugation was for 10h at 45,000 rev/min at 20°C in Beckman SW 50.1 rotor. Arrow indicates the position of ^{14}C-labelled marker protein. Receptor concentration was 137fmoles/mg protein and recovery of specific counts in the gradient was 77%. The position of receptor peak is at $\sim$ 6S (5.8S).


A. - Protease Inhibitor
^{14}C-BSA
4.6S
B. + 5mM Leupeptin
^{14}C-BSA
4.6S
^{3}H-Oestradiol cpm
1600
1400
1200
1000
800
600
400
200
0
800
700
600
500
400
300
200
100
0
Top
Top
10 20 30 40
10 20 30 40
Fraction Number

for the breast tissue cytosol, these protease inhibitors protect the aggregation property of the receptor.

Figure 60 demonstrates the effect of salt concentration on the sedimentation profile of human endometrial ER_c where 0.4M KCl is once again shown to inhibit the formation of the 6S peak even though DFP is present in the cytosol. This result is very similar to the one already presented for human breast tissue ER_c (Figure 51) and implies ionic interactions in the 6S formation.

Leupeptin concentration below 1mM were found to be ineffective and studies, therefore, were routinely conducted at 5mM. Optimum DFP concentration was not determined and the 10mM concentration used by other workers (Daxenbichler et al., 1980) was adopted. These inhibitors caused reduction in the binding capacity of the cytosol, as also observed by Lukola et al. (1980). Leupeptin if present during homogenization caused frothing, an observation in agreement with Sherman et al. (1980), with an adverse effect on receptor binding capacity. Its not known if inclusion of DFP during homogenization would have made any difference. The inhibitors were added immediately after homogenization.

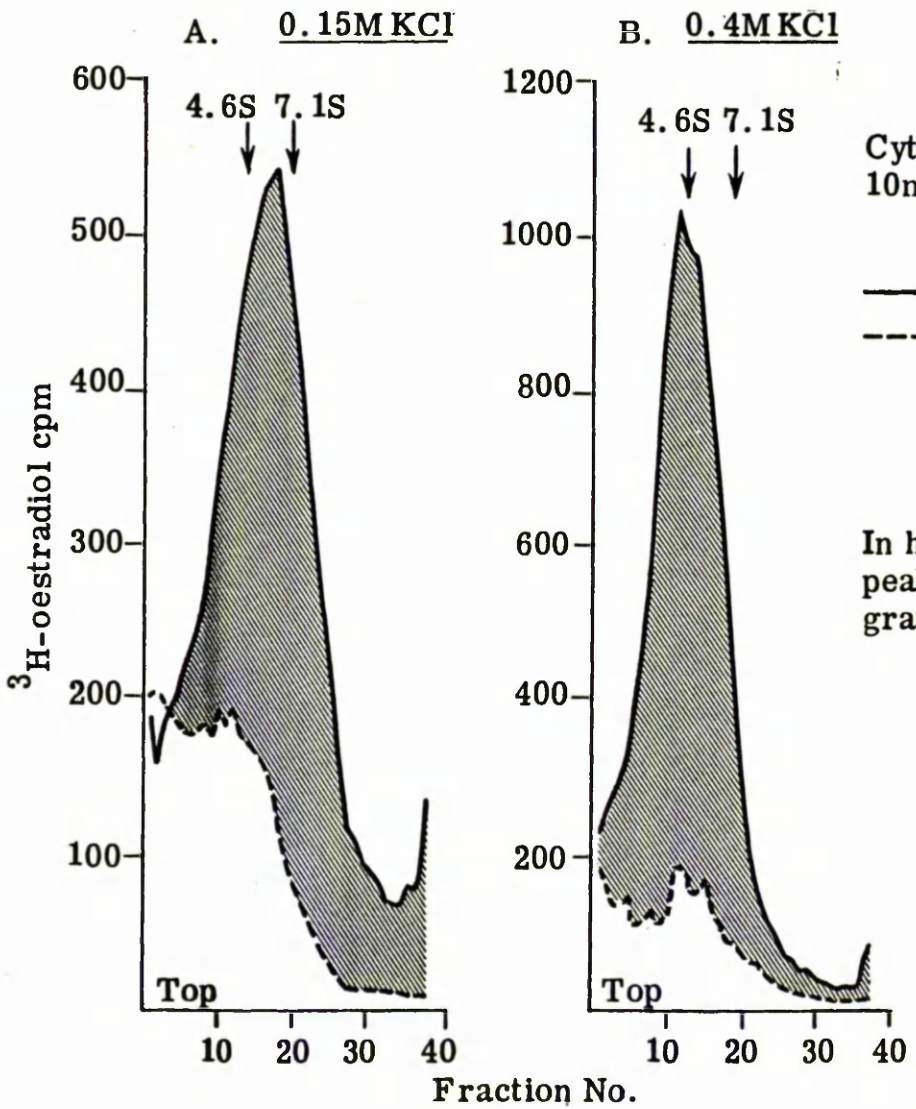
3.2.3.3.3 **Effect of Receptor Concentration**

It was observed that at low receptor concentration, 157 - 485 fmoles/ml, Leupeptin or DFP failed to protect the receptor aggregation site and the receptor sedimented at 4.0 - 4.8S. It is possible, however, that for other reasons the receptor may be initially in the 4S state. The 4.0 - 4.8S value observed with low receptor concentration is similar to that observed with high receptor concentration in the absence of protease inhibitors (Figure 59A - receptor concentration 1418 fmoles/ml). However lack of aggregation could also be due to (a) receptor concentration being below a certain critical level or (b) absence of other aggregating molecules.

Figure 60. **Effect of Salt Concentration on the Sedimentation Profile of Oestrogen Receptor from Human Endometrial Cytosol**

Human endometrial cytosol was prepared in $HDK._{15}$ as described in Section 2.2.3.2. Immediately after glass/glass homogenization the homogenate was made 10mM with respect to DFP. Cytosol (protein concentration 6.2mg/ml) obtained was incubated with 5×10^{-9}M $^{3}HE_{2}$ in the absence (——) or presence (....) of 5×10^{-6}M DES for 1h at 4^{o}C. After removal of free steroid with DCC (0.25% w/v, final concentration), 200ul aliquots were loaded onto 5-20% sucrose density gradients prepared in either $HDK._{15}$ (A) or $HDK._{4}$ (B). The gradient was then centrifuged at 42,000 rev/min for 9.5h in Beckman SW 50.1 rotor at 20^{o}C. The receptor concentration was 121fmoles/mg protein and specific percentage recovery in the gradient was 62% in A and 94% in B.

Effect of Salt Concentration on the Sedimentation Profile of Oestrogen Receptor from Human Endometrium



Cytosol prepared in the presence of 10mM Diisopropylfluorophosphate

—— 5×10^{-9}M ^{3}H-oestradiol

--- 5×10^{-9}M ^{3}H-oestradiol + 5×10^{-6}M Diethylstilboestrol

Centrifugation Temp. 20°C.

In high salt gradient the sedimentation peak is around 4.6S and in 0.15M KCl gradient the peak is at about 6S.

Further results are required to correlate the presence of 8S in low salt with the amount of 6S formed, and with DNA binding. The two determinations performed at 753 fmoles/ml (12.4 mg/ml protein concentration) and 852 fmoles/ml (6.2 mg/ml protein concentration) resulted in sharp 6S peak. The appearance of the 6S form, therefore, seems to be less dependent on high receptor content, in contrast to that observed with human breast tissue (Table 15) and may be related to the total concentration of ER_c being present in the 8S form in low salt conditions in the human endometrial cytosol.

3.2.3.3.4 **Effect of Sodium Molybdate**

Sodium molybdate is known to stabilize steroid receptors (Section 1.1.5.3.2) but it also inhibits transformation of the rat ER_c (Shyamala and Leonard, 1980). It was, therefore, decided to test this agent in human endometrial cytosol, in the transformation studies, in case the mode of action of sodium molybdate is different in different target tissues or under different experimental conditions. Sodium molybdate may protect the activated oestrogen receptor (6S) in a manner anaolgous to pyridoxal-5-phosphate, which activates glucocorticoid receptor but inhibits its DNA binding properties (Schmidt and Litwack, 1982).

Figure 61.1 and 61.2 show the effect of inclusion of sodium molybdate prior to centrifugation at 20°C. No 6S could be demonstrated under these conditions, presumably because of the inhibition of transformation. If sodium molybdate prevented receptor disaggregation, a prerequisite to transformation, then an 8S peak or higher aggregates should have been seen. Unless the original cytosol contained only the 4S non-aggregating receptor, sodium molybdate did not prevent proteolysis

Figure 61.1. **Effect of Sodium Molybdate on the Sedimentation Profile of Oestrogen Receptor from Human Endometrial Cytosol**

Human endometrial cytosol was homogenized in HED as described in Section 2.2.2.6. The homogenate was immediately divided into two portions and one was made 10mM with respect to sodium molybdate. Cytosol was then prepared (Section 2.2.2.6; protein concentration 5.0mg/ml) and labelled with 5×10^{-9}M $^{3}HE_2$ ± 1000-fold excess DES for 1h at 4°C. After removal of free steroid with DCC (0.5% w/v, final concentration), 200ul aliquots were loaded onto 5-20% linear sucrose density gradients prepared in HDK.$_{15}$. Centrifugation was at 20°C for 7.5h at 50,000 rev/min in a Beckman SW 50.1 rotor. Arrows indicate the position of ^{14}C-labelled marker proteins. The receptor concentration obtained was 128fmoles/mg protein with 56% recovery in the gradient for sodium molybdate free cytosol and 164fmoles/mg protein with 73% recovery in gradient for sodium molybdate containing cytosol.

Effect of sodium molybdate on the sedimentation profile of human endometrium ER_c. Centrifugation temp. 20°C. SDG containing 0.15M Kcl. Profiles obtained from same cytosol preparation

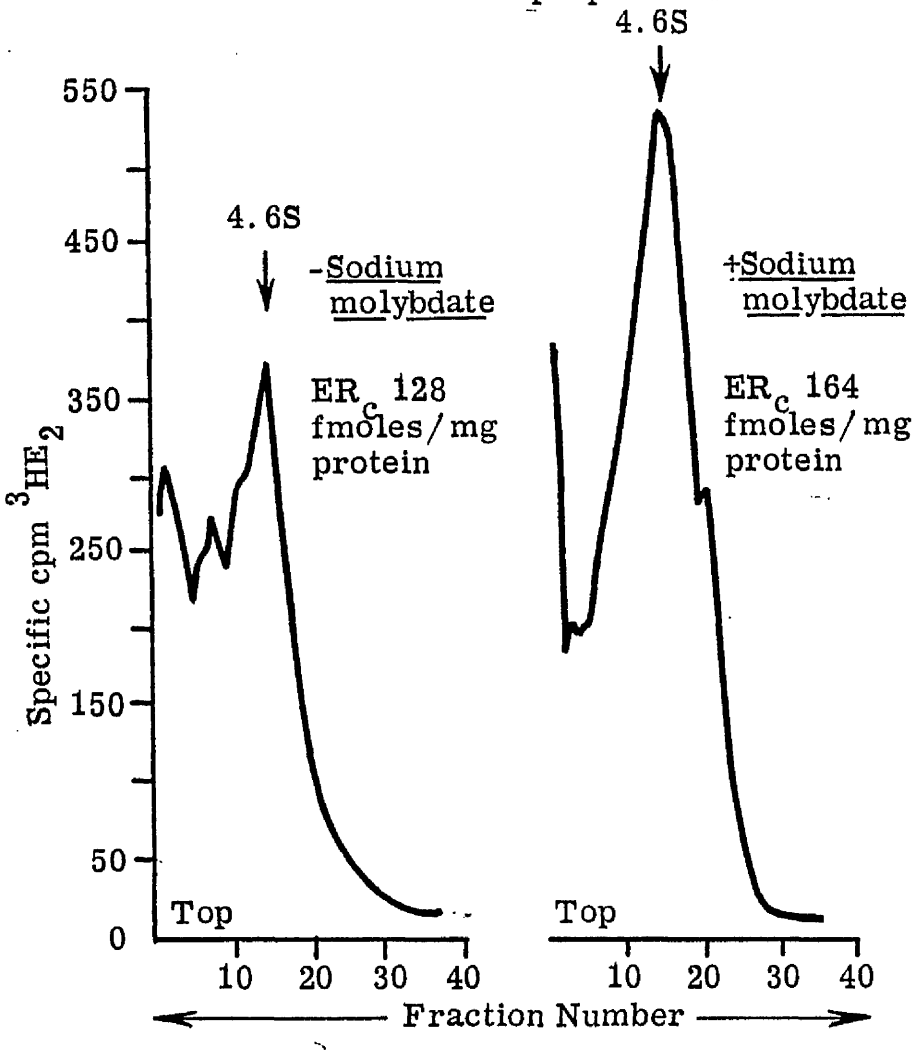
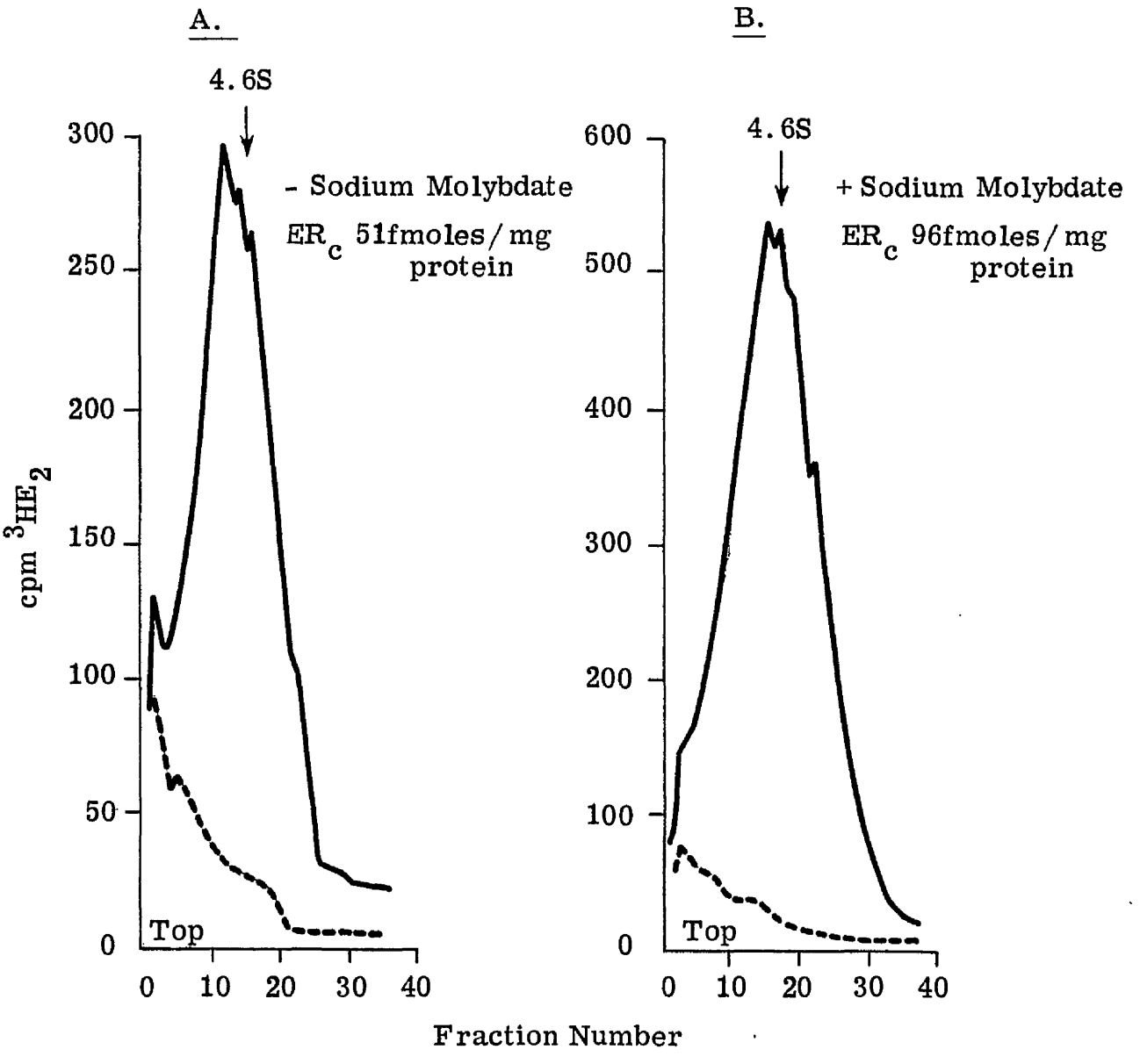


Figure 61.2. **Effect of Sodium Molybdate on the Sedimentation Profile of Soluble Oestrogen Receptor from Different Sections of Human Endometrium**

Two different portions of the same endometrial sample were homogenized in $HDK._{15}$ as described in Section 2.2.2.6 in the absence (A) or presence (B) of 20mM sodium molybdate. Cytosol was prepared as described in Section 2.2.2.6. (protein concentration 5.9mg/ml in the absence and 6.2mg/ml in the presence of sodium molybdate). Aliquots were labelled with 5×10^{-9}M $^{3}HE_2$ in the absence (——) or presence of 5×10^{-6}M DES (....). After removal of free steroid with DCC (0.25% w/v, final concentration), 200ul aliquots were loaded onto 5-20% sucrose density gradients prepared in $HDK._{15}$. Centrifugation was at 50,000 rev/min for 8h at 20°C in a Beckman SW 50.1 rotor. Arrow indicates the position of ^{14}C-labelled marker protein. The receptor concentration in A was 51fmoles/mg protein with percentage recovery of 80% and the receptor concentration in B was 96fmoles/mg protein with percentage recovery of 80%.


A.
4.6S
- Sodium Molybdate
ER$_c$ 51fmoles/mg protein
B.
4.6S
+ Sodium Molybdate
ER$_c$ 96fmoles/mg protein
cpm $^3HE_2$
Top
Top
Fraction Number

or dissociation of 8S form at 20°C. However, sodium molybdate does protect or even increase the binding capacity of the cytosol preparations (Figure 61.1 and 61.2). It is also possible that the 4.6S receptor in the Figures 61.1 and 61.2 has its aggregation site preserved but that sodium molybdate specifically inhibits aggregation. Sodium molybdate may also interact with other components required for transformation. A further consideration for inclusion of sodium molybdate was the possibility that it may interact with certain proteases or phosphatases, separating them on the gradient and permitting receptor to form the 6S complex (assuming that all necessary components for 6S formation co-sediment with the receptor).

3.2.3.3.5 **Effect of DCC Treatment**

It was found that DCC treatment of the labelled cytosol did not alter the formation of 6S complex when the cytosol, in the presence of DFP, was analyzed at 20°C in 0.15M KCl gradients. That is to say that DCC was not removing any factors required for the formation of 6S complex (data not shown).

In conclusion, human endometrial cytosol shows similar sensitivity to protease inhibitors for the formation of 6S complex as does human breast tumour cytosol ER_c. These proteases do not seem to be sensitive to sodium molybdate since elevated centrifugation runs yielded a 4S peak rather than higher S values and/or aggregation of receptor.

4. DISCUSSION

Despite several years of research into the cellular biochemistry of steroid receptors, the molecular mechanisms involved in steroid hormone action remain unclear. As yet, it is not even known as to exactly where in the cell the soluble empty receptor resides (Leake, 1976; Sheridan et al., 1979). It has, nevertheless, been shown that the genomic interaction of filled receptor leads to the differentiation of the target cells (Clark and Peck, 1979). It is the latter concept which has proved useful in selecting human breast cancer patients for endocrine therapy (Jensen, 1981). Sex steroid hormone receptor determinations have acquired an established position in the selection of such patients (there are also indications for the usefulness of this approach in the management of patients with other endocrine related diseases (Leake, 1981b)). However, several studies measure only the soluble receptor with the inherent assumption of measuring total functional receptor. Such methodology has resulted in the selection of receptor positive patients only 50% of whom respond to endocrine therapy (Hawkins et al., 1980). To circumvent the problem of 'false positive' patients, Wittliff and Savlov (1975) proposed analysis of the molecular forms of soluble oestrogen receptor (ER_c) on sucrose density gradient (SDG). They have proposed that it is the 8S ER_c complex which indicates functional receptor and that only those patients exhibiting this form of receptor in the tumour biopsy should be considered for endocrine manipulation. This proposal has been criticized (Freedman and Hawkins, 1980) and several investigators have been unable to demonstrate any relationship between the molecular form of receptor and response (Westerberg et al., 1978; Dao and Nemoto, 1980). The general consensus is that the 4S and 8S forms to not serve as individual independent prognostic parameters.

The work presented in this thesis was directed in part towards elucidating some of the reasons why such contradictory results are to be found in the literature. Some of the findings observed in cancerous tissue were related to those which are found in normal tissue, particularly the immature rat uterus, since this tissue is the best characterized with respect to oestrogen-receptor interaction (Figure 5). This work is presented in Section 4.1 below.

Another concept put forward by Wittliff et al. (1976) is that the reason why 4S containing tumours do not respond to hormone therapy is because they lack a second subunit. The second subunit combines with the 4S form to give the 6S complex which is claimed to be the active form of the receptor in vivo. This proposal was based on the fact that in physiological ionic strength only the 8S containing tumours yield a 6S complex. However Wittliff et al. (1978) have failed to detect such a form after heat activation. It is suggested in Section 4.2. that whereas Wittliff's view may be correct, the way it is presented in the literature is misleading. In vitro 4S can originate as a result of proteolysis, and several factors influence 8S→ 4S proteolysis (Section 4.1). Therefore, in vitro the 4S receptor may lose its ability to bind to the other subunit. The proteolytic activity of individual tumours (reflected in 8S→ 4S) may, however, be an indicator of tumour growth potential. It is shown in Section 4.2. that the 6S complex can be determined at higher temperatures but only in the presence of protease inhibitors and in 0.15M KCl gradients. The complex can also be formed at $4^{o}C$ in the absence of EDTA.

4.1. Clinical and Biochemical Aspects of Oestrogen Receptor Molecular Forms

In normal non-lactating breast, oestrogen receptor containing cells are present in very small numbers. Hormone sensitive cells increase

rapidly during pregnancy and lactation (Muldoon, 1978; Mohla et al., 1981). Malignant transformation of breast epithelial cells may initially involve one single cell or a number of cells. During growth of the tumour oestrogen receptor synthesis may be activated or abolished, and this may be related to the degree of differentiation of the tumour (Millis, 1980; Fisher et al., 1981). If activation of receptor synthesis occurs then the tumours will be hormone responsive and this forms the basis of hormone treatment (Section 1.2.5.1). On the other hand if the cells close down receptor synthesis then the tumours become hormone independent and are not likely to be hormone responsive. An intermediate situation may exist (Nenci et al., 1976).

Oestrogen receptor has gained much support as a prognostic index as well as in prediction of tumour response (Hawkins et al., 1980; Leake, 1981b). The subject of 'false positives' has been of great concern over the past few years. Several markers of hormone dependence have therefore been suggested but these have met with mixed success (Section 1.2.3.3). One such marker for hormone sensitivity has been the presence of the 8S molecular form of oestrogen receptor in human breast cancer (Wittliff and Savlov, 1975). Data from Wittliff's group shows a very consistant response rate to endocrine therapy of $\sim$ 75% if the tumour biopsy contained the 8S form. Patients with only the 4S form in their tumours do not show such a favourable response (Wittliff, 1980). Several other investigators found no significant relationship between receptor molecular form and response (Dao and Nemoto, 1980; Gapinski and Donegan, 1980). If they had used Wittliff's criteria of selecting patients then several potential responders would have been classified as non-responders to hormone therapy. It remains intriguing, however, as to why the invest-

gators have found such differences in the molecular form of receptor in relation to patient response.

4.1.1 Factors affecting Receptor Molecular Form

The data of Freedman and Hawkins (1980) provides evidence that most tumours initially possess the 8S form of ER_c. In rat mammary carcinoma no relationship could be detected between hormone dependence or independence of tumour and molecular form, both types exhibiting predominantly the 8S form (Vignon and Rochefort, 1978; Freedman and Hawkins, 1980). The method of storage affects the receptor molecular form as 8S is degraded faster than 4S receptor (Namkung et al., 1979). Measurement of ER_c content may also depend on the procedure used (Braunsberg, 1975; Poulsen et al., 1981). The results presented in this thesis substantiate the view that all tumours may initially possess the 8S cytoplasmic form. However, different tumours have favourable potential for converting the 8S form into the 4S form. This most probably is related to a specific protease(s), the concentration of which may vary from tumour to tumour or even within a single tumour. This protease(s) seems to alter only the aggregation property of the 8S form, not its oestradiol binding capacity. Different storage and assay procedures employed in the various laboratories, could therefore, lead to the variable distribution of 4S and 8S forms reported and reflect the different extents of 'exposure' to this protease(s). Jensen et al. (1975) could not demonstrate tumours containing only the 4S form in any receptor positive tumour. Further only a few cases of 4S containing tumours were reported by McGuire et al. (1975b). In keeping with this observation Freedman and Hawkins (1980) found only one receptor positive case (1/19) to possess only the 4S form. In contrast, a significant proportion of receptor positive tumours were found to contain only

the 4S form of receptor in the studies reported by Wittliff (1980) and Dao and Nemoto (1980).

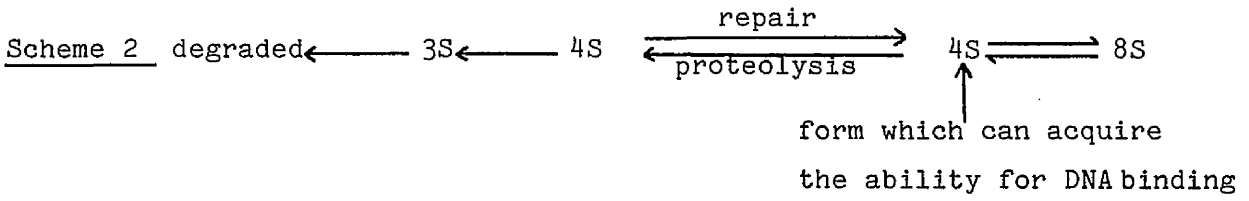
The results presented in Figures 11-29 and Tables 2-6 indicate that several conditions can affect the 4S : 8S distribution on the sucrose density gradient (SDG). The results presented in Figure 11 were obtained from a tumour with predominantly the 8S form of ER_c. After an incubation period of up to 150 min. with $^3HE_2$, there was no change in the sedimentation profile. It is not known whether further incubation of the same cytosol would have resulted in any change in the profile. However the fact that the receptor profile can change with time is clearly demonstrated in Figures 12.1 and 12.2. As shown in both these Figures, when the cytosol was labelled for 1h with $^3HE_2$ the profile obtained was of the 4S + 8S type (Figures 12.1A and 12.2A). When the same cytosol was incubated for 24h with $^3HE_2$ followed by DCC treatment and analysis, there was a decrease in the 8S peak height with an increased peak height in the 4S area. This demonstrates that most probably proteolysis of the 8S form of ER_c is taking place (Figures 12.1B and 12.2B). In addition, if the same cytosol, showing 4S + 8S profile after short periods of incubation, was incubated for 23h without $^3HE_2$ then labelled for 1h with $^3HE_2$ and subsequently treated with DCC, the receptor concentration obtained was found to be less than the control (1h incubation with steroid). There was also a complete loss of the 8S form and decrease in the 4S peak (Figures 12.1C and 12.2C) suggesting that empty receptor is more labile. This leads to either loss or alteration of the steroid binding site of the receptor. It is possible that the Type II sites (Panko *et al.*, 1981) represent Type I sites which have been enzymatically altered *in vivo* or *in vitro* (reflected in a high K_d value). Alternatively, the detection of Type II sites may not represent an *in vitro* artifact but the result of a physiologically meaningful process: Type II

cytoplasmic sites representing the degraded excess Type I sites or the newly synthetized receptor, and Type II nuclear sites, the degraded nuclear functional receptor - since nuclear Type II sites are correlated with response (Clark and Markaverich, 1981).

The two peaks noted in Figure 12.2 in the 4S area are also reported by others (Kute et al., 1978). This most probably represents receptors proteolyzed to different extents. In most experiments resolution of such peaks may be masked by their relative concentrations. Studies of Sherman et al. (1976) have led to the identification of mero-receptor ($\sim$3S), the lowest molecular weight form which can bind oestradiol. However, the physiological significance of such a form is not known. Another interesting consideration to emerge from the above results was that long term incubation with steroid results in 8S$\rightarrow$4S conversion with no loss of total binding capacity. In the absence of ligand, however, a similar conversion is followed by (or associated with) a further modification of the oestrogen binding site. Therefore an initial mild proteolysis of 8S$\rightarrow$4S (assuming 8S formation is independent of the presence of ligand) renders the receptor more prone to proteolytic digestion. Notides et al. (1973), however, report an opposite view. Studying human myometrial tissue they found a greater selective degradation of occupied receptor. Others also report that the steroid receptors are stabilized with ligand occupation, in agreement with our results (Peck et al., 1973b). Surprisingly though Kute et al. (1978) have reported they could not observe an 8S$\rightarrow$4S conversion with time. It is not clear, however, how many tumours were analyzed in their study. Tumours tend to vary in their proteolytic activity (Sherman et al., 1980). Time of incubation, therefore, seems to be an important parameter in the final profile obtained (Freedman and Hawkins, 1980; Tilzer et al., 1981).

The studies of Freedman and Hawkins (1980) and Gaubert et al. (1982) make it unlikely that if the 8S → 4S is physiologically meaningful, it takes place in the cytosol. Nevertheless, a nuclear location of such an event is a possibility. Lysosomes have been shown to be translocated into the nucleus as a result of oestrogen stimulation (Szego, 1975). It is found that limited tryptic digestion of receptor destroys the DNA binding site (Andre and Rochefort, 1973). However, Sala-Trepat and Vallet-Strouve (1974) show that Ca^{2+} stabilized receptor (which fails to bind to DNA, see Andre and Rochefort, 1973) can bind to chromatin. Dickerman and Kumar (1982) have indicated that a separate histone binding site may be present on the receptor. It is possible, therefore, that some of the receptor in the nucleus may lose its DNA binding site but remain bound to the chromatin with an intact oestradiol binding site and provide a 'buffering capacity' for the DNA bound oestradiol-receptor complex. This mechanism may be important at physiological temperature. In the same context, it may be of interest if the 8S → 4S converted receptor can be converted back to 8S. Muldoon (1980) has suggested that not all translocated receptor is degraded, indicating that a receptor molecule can be further used after triggering an initial event. The above studies with the human tissue imply the following sequence of events in a very simplistic view.

Scheme 2



The 4S form which may undergo mild proteolysis may become more prone to ultimate degradation. Microsome bound receptor or other membrane bound receptors (Muldoon, 1980; Parikh et al., 1980) may represent either newly synthesized receptor or receptor undergoing repair. Murayama et al. (1980b)

have suggested that a possible role of the 8S aggregate is to prevent proteolysis of receptor in vivo. It is also of interest that the progesterone receptor shows seasonal variation in functionality (Boyd and Spelsberg 1979; Boyd-Leinen et al., (1982). This may even indicate the variation of such a protease which could regulate receptor in vivo. Others have indicated that proteolysis of progesterone receptor occurs(Dougherty and Toft, 1980; Vedeckis et al.,1980)

In addition, Figure 13 shows that Ca^{2+} promotes the 8S $\longrightarrow$ 4S conversion resulting in a sharp symmetrical 4S peak. This sharp 4S peak was also characteristic of tumours which showed heavy aggregates at the bottom of the tube with disturbed 8S area, suggesting that at least some 4S molecules have lost the ability to non-specifically aggregate. Schneider and Dao (1977) observed a similar Ca^{2+} promoted effect which they could inhibit with PMSF. Others have found PMSF very ineffective in human tissue (Lukola and Punnonen, 1982). We therefore used DFP to suppress this enzymatic activity effectively (Figure 13C). The use of DFP (and Leupeptin) led to a lower value of total binding capacity being measured. This is in agreement with various authors (Notides et al., 1976; Lovgren et al., 1979). However it is difficult to state how such an effect is brought about. Some form of change in receptor structure is plausible. An effect of tetracine, for example, on the molecular form of oestrogen receptor is documented (Kim et al., 1982). Competition for the steroid binding site by protease inhibitors is also indicated (Baker et al.,1978). An alternative explanation is possible. Recently it has been demonstrated that mild trypsinization of receptor preparations from human breast cancer lead to an increase in the total binding capacity (Pettersson et al., 1982). It is therefore possible that, whereas in DFP-free cytosol this process is continually proceeding, in the DFP-containing cytosol this process will be

inhibited. The same may apply for the estimation of higher binding capacity obtained on overnight incubations (Figures 12.1 and 12.2). Both trypsinization and Ca^{2+} promoted 4S receptor formation have been shown to result in loss of the DNA binding ability of the receptor (Andre and Rochefort, 1973) although one report suggests that Ca^{2+} promoted transformation leads to enhanced binding to nuclei (Sala-Trepat and Vallet-Strouve, 1974). Interestingly Ca^{2+} promoted proteases from different species show different properties with respect to their sensitivity to DFP. Whereas the data presented in Figure 13 and by Rochefort & Bauleiu (1971) clearly indicate that Ca^{2+} promoted protease activity in the immature rat uterus cannot be inhibited with DFP, the data presented in Figure 13C and Figure 51 show that DFP does protect the human receptor. Notides et al. (1976) and Daxenbichler et al. (1980) have also found DFP very effective in human tissues.

Such data indicates that different proteases are present in different tissues within the same or different species. It is possible that the protease responsible for 8S → 4S conversion may be the same but that Ca^{2+} may be promoting other enzymatic processes which may hydrolyze not only DFP but also other proteins associated with the 8S form. Such an effect will render DFP ineffective. Recently, for example it has been shown that the 8S form of the glucocorticoid receptor is converted to the 4S form by RNAases (Tymoczko and Phillips, 1983) which presumably is independent of any proteolytic effect. Generally in the human breast tissue cytosol, incubation with DFP and heating to $30^{o}C$ for 30' for activation purposes led to receptor aggregation (Section 3.2.3.2.7). However, in some other tissues DFP had no effect. A report mentioning two different proteases with differing effects on receptor structure has just been published (Gregory and Notides, 1982). Ca^{2+} activated proteases have also been

described for progesterone receptor in chick oviduct (Vedeckis et al., 1980).

Figure 14 shows the effect of incubation of tumour cytosol with varying ligand concentration in the range 10^{-8} - 10^{-9}M. This tumour cytosol showed predominantly the 4S type of receptor. With increasing concentration of radioactive ligand there was an increase in the total receptor concentration measured during the 1h incubation at 4°C, although in going from 5 x 10^{-9} to 10^{-8}M this increase was relatively much lower than over the range 10^{-9} to 5 x 10^{-9}M. There was no increase in the proportion of 8S suggesting that the lack of 8S form is not due to different kinetic properties of the two forms. Similar results are presented by Wittliff et al. (1976) who found no difference in the kinetics of the 4S and 8S forms. It must be pointed out, however, that in the mouse mammary gland 4S and 8S forms did show variation in their ligand binding properties (Muldoon, 1979). Nevertheless, this only affects the very early part of association kinetics (first 10 min) and the 4S and 8S forms were from two different physiological states of the mouse. One essential feature demonstrated in Figure 14 is the 8S shoulder present. This is reproducible at all these ligand concentrations tested. This strongly suggests a possible 8S to 4S conversion, which therefore must only be affecting the receptor aggregation properties (or the availability of other proteins) and not the steroid binding site.

Figure 15 shows the effect of temperature on the interconversion of the receptor molecular forms. Two different effects were observed. In Figure 15, on keeping tumour cytosol at 20°C for 1h, there was an increase in the 4S region of binding with some aggregation of the receptor towards the bottom of the tube. There was also an increase in total binding capacity on warming. The increase in the 4S receptor concentration could

be a result of either unfilled 4S receptor being present or the conversion of 8S into the 4S form with some receptor forming heavy aggregates. That temperature promotes 8S⟶4S conversion was suggested by some other experiments (data not presented) where heated cytosol showed receptor present exclusively in the 4S form (without any change in receptor concentrations), in contrast to control cytosol kept at $4^{o}C$. The formation of heavy aggregates in these experiments were low suggesting that protease mediated 8S⟶4S conversion was dominant. Thus, the extent of aggregation (8S⟶Aggregates) and of proteolysis (8S⟶4S) is a balance which depends on the amount of proteolytic content of the particular tumour. These results also indicate that the proteolyzed 4S form is unable to aggregate but is relatively stable at raised temperatures. Kute et al. (1978) have presented similar results. Michel et al. (1981) have found that warming the cytosol obtained from various mammary carcinomas reduces the receptor ability to bind to Heparin-Sepharose, most probably indicating loss of receptor aggregating ability.

Figure 16 represents the experiment conducted to determine the optimum concnetration of DCC required for removal of free radioactive steroid without affecting the total binding. This tumour showed a profile which cannot be quantitated on SDG as the 4S peak extends with the aggregating material towards the bottom of the tube. Such tumours were classified as DCC^{+}, SDG^{+} and are further mentioned in Table 3.1. It is not clear as to what leads to such a profile. It probably represents loss of the 8S form, either as a direct result of receptor conversion from 8S⟶4S or dissociation of steroid from the 8S form. It is possible that such a profile could be a result of delay from the time of mastectomy to storage and/or assay. Nevertheless, the results presented in Figure 16 demonstrate that, with increasing concentrations of DCC, there was an increased removal of non-specifically bound counts without any significant effect on the specifically

bound radioactivity. The final DCC concentration of 0.5% (w/v) was chosen. Further increases in DCC concentration were avoided since this leads to both absorption of protein and stripping of receptor-bound steroid (Peck and Clark, 1977; Poulsen, 1981). It should be mentioned here that in the analysis of Peck and Clark (1977) the protein concentration was very low (1-2 uteri/ml) whereas in the present analysis protein concentration generally varied from 3-10mg/ml and therefore protein concentration will tend to negate the DCC adsorption effect. Figure 17A and 17B further show that when using the same final concentration of DCC, the non-specific components can vary from tumour to tumour and is independent of the protein concentration range tested (see also Poulsen, 1981). The blood proteins, binding the oestrogens (mainly SHBG) can affect the receptor measurement, but not to any great extent (Hahnel & Twaddle, 1979). SHBG has now been suggested to play an important role in carcinogenesis. In post-menopausal women, low SHBG binding capacity is regarded by most (Murayama et al., 1980e; Moore et al., 1982) to indicate a greater chance of developing breast and endometrial carcinoma. However, the prognostic significance of SHBG is controversial (Mason et al., 1981; Murayama and Asano, 1981). Nevertheless, the results presented in Figure 17A and B cannot be taken to indicate a difference in the SHBG level of various patients. This is because the nature of the tumour will dictate the extent of SHBG found in the cytosol of a particular section. It is further shown in Figure 17A and B that cytosol prepared from a relatively homogeneous tissue (immature rat uterus) does not show such a variation in non-specific counts as that in human breast tumour cytosol preparations.

4.1.1.1 Storage in Sucrose/glycerol

During the course of the present investigation it was shown that an alternative to liquid nitrogen can be used for storage of human

breast tumours. This involves storing tissue in sucrose/glycerol buffer (Section 2.2.1.1, Figures 18-20 and Table 2). Liquid nitrogen, the currently favoured form of storage for breast tumours has been found by many investigators not to be entirely satisfactory (Wittliff and Savlov, 1975; Leake et al., 1979). Three weeks from the time of storage, the receptor degrades although the 4S form seems to be relatively more stable than the 8S form (Namkung et al., 1979). Similar results were found in lyophilized breast tumour tissue (Janes et al., 1982). In the sucrose/glycerol buffer two different processes were observed - either there was retention of the 8S form for a very long time (Figure 18) or 8S was gradually degraded to the 4S form, apparently without loss of total binding capacity (Figure 20). The storage data in sucrose/glycerol shown in Figure 18 was obtained from the analysis of an axillary node tumour mass. There was no loss of the 8S form for up to 96 days. Even after 96 days receptor could still be detected using Scatchard analysis (Section 2.2.2.3, data not shown) with $K_d \sim 10^{-10}$M. However on the gradient the molecular form was lost. This apparently results from a certain extent of aggregation induced by storage (as also shown by Rochefort and Baulieu (1971) for stored cytosol) but predominantly from some form of alteration in the molecular structure of the receptor. The receptor molecules (perhaps, on separation from certain other proteins) appear very unstable on gradients. The same tumour when analyzed on the 517th day (Figure 19A) shows that once again there was a smear of counts across the gradient with some aggregation (<100% recovery of counts), when the cytosol was analyzed on a low salt gradient. The same cytosol when applied to a HDK.$_4$ gradient (Figure 19B) allowed the detection of a 4S peak with some loss of radioactivity towards the top. This most probably represents the dissociated receptor form from about the 4S region to the bottom of the tube (and including aggregated receptor) which is now

concentrated in the 4S area. One, however, cannot ignore the possibility that for some unknown reason, KCl may in fact stabilize the aged receptor. Again, on the 517th day the receptor concentration was detectable with the DCC method.

It is shown in Figure 20 that in contrast to the previously mentioned retention of the 8S form for a long period in sucrose/glycerol buffer, some tumours show an 8S → 4S conversion after very short periods of storage. Depending on the time of analysis these tumours could be classified into 8S or 8S + 4S types. Due to the unavailability of enough tumour material, a fact also recognized by Sherman et al. (1980), it was not possible to determine a complete conversion of 8S into the 4S form. However, the very few tumours presented in Table 3.1 in the 4S alone class probably arose as a result of such an interconversion since some of these showed a second section in the 4S + 8S class. This result clearly highlights the importance of measuring tumour molecular form as soon after mastectomy as possible if molecular form is to be used as a discriminant. This applies to every type of storage system used. Results presented in Tables 5 and 6 further substantiate that in different tumours, varying concentrations of 4S and 8S could be found, probably reflecting different concentrations of protease(s) being present.

It therefore seems that storage of human breast tissue in sucrose/glycerol buffer either preserves the 8S form or results in the loss of this molecular form into the 4S form. This does not affect the steroid binding site for a considerable duration of storage (Figure 20 and Table 2), whereas long term storage may result in loss of molecular form and/or receptor concentration. This may be significant since even short-term storage in liquid nitrogen generally leads to a loss of

receptor concentration compared to control (Namkung et al., 1979; also seen by Toppila et al. (1982) for gynaecological tissues). In the present investigation, tumours showing high receptor values were studied for molecular forms. Nevertheless Scatchard analysis conducted by Miss Marion McMenamin in our laboratory showed that in tumours with very low receptor concentrations (<20 fmole/mg protein), both cytosol and nuclear receptors can be preserved for up to at least 12 weeks.

The sucrose/glycerol storage results of human breast tissue suggest that in cases where storage in liquid nitrogen is delayed with deleterious effect on receptor, the surgeon can section the biopsy and store it in sucrose/glycerol buffer at -20°C prior to shipment to the biochemical laboratory. Hasson et al. (1981) have stressed the importance of avoiding such a delay. They found receptor levels in specimens from mastectomy to be significantly lower than the initial frozen section specimens. In Hasson's study, however, no consideration was given to intratumoural variation which could occur (Section 3.1.3). Our results clearly show that sucrose/glycerol can be employed for such a purpose and may also be used for the analysis of molecular forms but that the latter, if required, must be determined rapidly (within 7 days). Sucrose/glycerol buffer (or other storage methods) should be used only if the fresh sample cannot be analyzed. An efficient coordination between the biochemical laboratory and surgical theatre staff is required for the analysis of fresh tumours. However, this is not always practical. In addition the number of samples that can be processed and the time factor involved in sucrose density gradient procedures are drawbacks of SDGA. The recent application of high pressure liquid chromatography should prove useful (Pavlik et al., 1982). It is possible that, keeping the time factor constant, the various molecular forms may be indicative of

some form of tumour characteristic, such as possible extent of protease present and its association with hormone response and/or metastasis. It is interesting that plasminogen activator and receptor content have been found to be correlative by Sutherland (1980); plasminogen activator may also be involved in 8S→4S conversion, as noted by Sherman et al. (1980). Taking various factors into account, it has been shown that sucrose/glycerol buffer is a better method of storage than liquid nitrogen. The 8S→4S conversion observed by various investigators therefore seems to be the result of methodology involved and does not represent an in vivo state although such a process may still take place in vivo.

For reasons unknown, sucrose/glycerol buffer does not preserve the immature rat uterine ER_c (Figure 21). If this is a result of leaching out of the receptor into the medium such leaching could not be determined, probably as a result of the very low concentration of protein in the medium for which the DCC assay is unsuitable (Poulsen, 1981). The indication however, was that the receptor was degraded since storage for 1 day resulted in the appearance of a previously non-existant 4S peak followed by a drastic reduction in both the 4S and 8S forms. The protease (or phosphatase or other enzymatic activity) involved in receptor degradation seems to be present in immature rat uterus but is compartmentalized in some mammer. This degradation of receptor may involve a different cellular metabolism of receptor other than the 8S→4S conversion seen in human breast tumours, indicating perhaps different protease(s) in different target cells. Gregory and Notides (1982) have recently demonstrated two different types of proteases with different effects on receptor structure. This protease, whether present in nucleus or cytoplasm, may get artifactually activated during storage or during homogenization. Lysozomal membranes may be sensitive to storage and

nuclear membrane may similarly be prone to rupture as a result of post storage thawing. Chromatin associated serine protease activity has been demonstrated in the rat liver (Tsurugi and Ogata, 1982) but this does not appear to be the protease encountered here since DFP, a serine protease inhibitor was found to be of limited effectiveness in the immature rat uterine cytosol (Figure 50). The latter, however, was not stored but fresh cytosol. Plasminogen activators have been found in immature rat uteri (Peltz et al., 1983) but these were indicated to be inhibited by DFP. The situation therefore remains unclear.

4.1.1.2 Effect of Ionic Strength and Sodium Molybdate

The results presented in Figure 22 were directed towards studying the effect of ionic strength and sodium molybdate on the sedimentation profile of ER_c from human breast cancer. The low salt (HED) profile of the predominant 8S form (Figure 22A) was changed to the predominant 4S form when the same cytosol was loaded onto $HEDK._{15}$ gradients. A similar effect is observed if the salt concentration is increased to 0.4M. This suggests that the 8S complex is sustained by relatively weak ionic associations between protein molecules and that probably in vivo the receptor exists in the 4S state (Stancel et al., 1973a). However since it is not possible to determine the intracellular protein concentration, the actual in vivo state of the receptor will perhaps remain unresolved for a considerable period of time and for this reason, the 8S complex so formed in low salt conditions cannot be disregarded. The 8S complex could arise from either self-association of receptor or as a result of aggregation with other soluble proteins. The exact role of such an association is not known but Murayama et al. (1980c) have shown that the 8S complex arises as a result of specific proteins present in

the soluble fraction which associate with the 4.5S complex (native form). Similarly the molecular constituents of the 8S progesterone receptor complex are also composed of specific associations of proteins with the 4S complex (Murayama et al., 1980d). The formation of the 8S complex is thought to prevent the receptor from undergoing proteolysis in vivo (Murayama et al., 1980b). However as shown in Figure 12.1 & 12.2, the 8S complex is liable to undergo degradation to the 4S form in vitro. This most probably arises prior to centrifugation analysis when an 8S $\rightleftharpoons$ 4S interconversion will make the 4S available for proteolysis. As has been suggested previously, this degradation may take place as a result of artifactual release of certain nuclear proteolytic enzymes on cell disruption (assuming the process of 8S $\longrightarrow$ 4S is physiologically significant), therefore the soluble 8S complex may still serve as an indicator of the extent of this protease(s).

The formation of the 8S complex (or higher aggregates) may be an in vitro indication of the receptor's ability to form such complexes in vivo. This may be a way of preserving the 8S complex in the cytosol since large molecular weight compounds will not diffuse across the nuclear membrane (Gorski and Gannon, 1976). There have been reports of specifically associated inhibitors of activation with the receptor - perhaps the 8S complex is a reflection of such an association. Steroids may then allow the receptor to dissociate into the 4S form (in vivo), the form which now either possesses or acquires the ability to bind to DNA. The ability of receptor to undergo aggregation may serve another important function in the nucleus. The possibility of a positive co-operative effect of receptor on certain genomic sites for induction of message has been suggested by Yamamoto and Alberts (1976). Perhaps, then, such aggregation of receptor reflects an in vivo requirement for an association amongst

receptors at a regulatory site (multimeric complexes). The report of Govindan et al. (1982) have suggested a similar proposition for the glucocorticoid receptor.

Several workers have recently focused attention on the compound sodium molybdate which promises to be an important tool in understanding the receptor molecular forms and the process of activation (reviewed in Grody et al., 1982). It has been claimed recently that, in the presence of sodium molybdate the receptor sediments faster than 8S (Sherman et al., 1980). However, it is shown in Figure 22C that the inclusion of sodium molybdate in the present work did not result in any significant increase in sedimentation value (cf Figure 22A). This is in agreement with the data of Mauck et al. (1982) and Moncharmont et al. (1982), who studied the effect of molybdate in calf uteri, and the data of Krozowski and Murphy (1981) who studied human myometrial and MCF-7 human breast cancer cell receptor.

The proposed mechanism of molybdate action include (i) inhibition of phosphatase (Sando et al., 1979 a,b), (ii) inhibition of RNAase and protease (Chong and Lippman, 1981-82), (iii) direct interaction with receptor or associated proteins (Noma et al., 1980), (iv) intercalating Ca^{2+} ions (Ratajczak et al., 1981), (v) possible interaction with sulphydryl groups (Kalimi and Banerjee, 1981). It is easy to see that if more than one of these mechanisms is involved, then variation in experimental techniques may account for disagreements in the literature. Although sodium molybdate has been shown to be useful in both qualitative and quantitative assessment of receptor in human breast cancer (Anderson et al., 1980), as yet no data is available which shows that the overall rate of receptor positivity and response has been increased. In any case the difference in receptor

concentration shown in Figure 22, in the presence or absence of molybdate cannot be ascribed solely to molybdate since two different sections of the tumours were analyzed (Section 3.1.3).

Figure 22 demonstrates another important but scantily studied feature. The results are those of a human male breast cancer patient. Male breast carcinomas are very frequently found to be receptor positive and this may relate to early presentation at the surgery, while the tumour is still relatively more differentiated. Both the higher incidence of male breast tumours being receptor positive and possible application of hormone treatment to them is recognized (Gupta et al., 1980; Friedman et al., 1981; Kraybill et al., 1981; Pegoraro et al., 1982). Another feature presented in Figure 22 is the consistence of ER_c concentration when expressed per unit DNA which may imply that male breast tumour is a more concentrated and homogeneous mass of tumour cells than is obtained with female breast carcinoma.

4.1.1.3 **Protease Inhibitors**

Figure 23.1 and 23.2 represent the effect of two protease inhibitors, PMSF and Trasylol, on receptor sedimentation profile. Although Schneider and Dao (1977) used PMSF for inhibiting the effect of a protease(s) in human breast tumours, others have found it ineffective in human tissue (Lukola and Punnonen, 1982). It may be important however, that Schneider and Dao's study involved only low temperature, salt dependent effect on proteolysis of receptors whereas most other investigators have used higher temperatures (e.g. for heat induced transformation). Increased temperature may activate certain other proteolytic enzymes or enzymes responsible for degradation of PMSF itself. In Figure 23.1, however, it is shown that, in the absence of

any heat induced effects, PMSF was unable to protect the appearance of some 4S form of receptor. There may be other reasons for the presence of a minor 4S peak and these include dissociation during centrifugation and proteolysis of 8S during storage. Since the PMSF effect was studied in only two tumours, which were both 8S dominant (low level of protease(s)) the effectiveness of PMSF at low temperatures cannot be deduced from the present work.

Attempts to stabilize receptor molecular form with Trasylol were unsuccessful (Figure 23.2). The 8S form was particularly affected and this probably involved both loss of receptor into the 4S form and aggregation. It is not possible to comment on Trasylol promoted aggregation but the 8S $\longrightarrow$ 4S conversion could be attributed to the introduction of ionic strength into the cytosol. Trasylol, which is used mainly for injections into humans to suppress certain proteolytic events in the blood, is provided in isotonic medium. It is therefore possible that the 4S area seen in Figure 23.2, in the presence of Trasylol, represents salt-induced 8S dissociation. This is stressed since two different sections of the tumour were analyzed and the 4S : 8S distribution may simply be a result of intra-tumoural variation. Trasylol therefore does not prevent the appearance of 4S receptor although the formation of 4S possibly may have occurred endogenously during storage or at the re-warming process. If the tumours are stored prior to analysis then the efficacy of any protease inhibitor on the molecular form should not be judged on separate sections. Birnbaumer et al. (1983) have also cautioned against the assumption that molecular forms obtained in the presence of Trasylol and PMSF represent the non-degraded form of receptor. These authors were studying chick oviduct progesterone receptors. This is not to infer that PMSF should not be used at all. For instance, Wilson and

French (1979) have found it very useful for the analysis of androgen receptor.

4.1.1.4 **Buffers**

It has been documented that buffer systems can affect the receptor conformation on SDGA (Gaubert et al., 1982). On this basis it could be argued that the very small number of predominantly 4S containing tumours detected in this present series was a result of the use of Hepes (Table 3.1). Figure 24, nevertheless, shows that results obtained with Hepes were comparable to those obtained in Tris and Hepes was therefore not the cause of present results. Gaubert et al. (1982) have found that, at least in the mouse mammary system phosphate buffer prevents any 8S $\longrightarrow$ 4S conversion. Hepes was used in the present investigation since its PK_a value is close to the pH at which the experiments were conducted. It is not known if this in any way influenced the separation of molecular forms on the gradient. For example, the separation of receptor from other proteins during centrifugation may lead to certain receptor sites being exposed to different chemical environments in various buffers, which may influence the final configuration (see Mauck et al., 1982). Another point demonstrated in Figure 24 is that storage in Hepes (sucrose/glycerol buffer) does not result in any alteration of sedimentation profile whether analyzed on Hepes or Tris gradients.

4.1.1.5 **Protein Concentration**

The protein concentration of tissue cytosol has been shown to alter receptor sedimentation profiles (Stancel et al., 1973a, b). However, in the protein concentration range used in the present results, 3-10mg/ml,

no significant variation in the sedimentation profile was obtained (Figure 25). The receptor was found to sediment at 7-8S at the tested protein concentration range of 2.4-11mg/ml. This most probably indicates that the 4S + 8S profile seen in other tumours is independent of protein concentration and is related to other factors. Protease(s) effects seem to be the major candidate but the effect of the protease itself should be carefully interpreted. It may not always be the case that the 8S⟶4S conversion requires the proteolysis of the 8S form. Recently it has been found that there are other protein factors which may be required for the conversion of 4S⟶8S (Murayama et al., 1980a,b,c; Colvard and Wilson, 1981) and, therefore, if these factors are destroyed or are present in limiting amounts, the 4S : 8S distribution will be affected. In the same context RNA has been implicated as one such factor in determining the 8S form in the glucocorticoid receptor system (Tymoczko and Phillips, 1983). If this also applies in the case of the oestrogen receptor then extraction of RNAase in the soluble fraction may influence the 4S : 8S distribution. Sherman et al. (1980) used a protein concentration range 3-12mg/ml for their analysis and report similar results. In addition they have pointed out that at least 40fmole/mg of receptor should be used for SDGA for correct determination of the hydrodynamic properties of receptor.

4.1.2 **Distribution of Various Molecular Forms**

The distribution of various molecular forms of oestrogen receptor in human breast tumours fell predominantly into the 8S and 8S + 4S class with variation in the concentration of each in the latter (Figures 26B, C, D and Table 3.1 and 3.2). A few tumours showed the predominant 4S profile, as shown in Figure 28.1A. A subclass was found

to represent what has been designated as DCC^+, $SDG^{\pm}$ (Table 3.1 and Figure 16). These showed quantifiable receptor by DCC analysis but not by SDGA. This situation may arise as a result of proteolysis on the gradient or during the storage procedure or as a result of destruction prior to storage. Some other authors have also had to use the DCC^+, $SDG^{\pm}$ term in cytosols where 8S could not be demonstrated clearly (Iino et al., 1980). It must be mentioned that in accordance with other published results (Geier et al., 1979; Freedman and Hawkins, 1980) very few tumours showed the predominant 4S profile. Even amongst the tumours which showed predominant 4S form in one section, some showed a second section in the 4S + 8S class, although this was rare. These two sections were not necessarily studied on the same day but within 60 days of storage, the criteria set for the present work. The storage data (Table 2) had already indicated that there are some tumours in which this time period does not alter the sedimentation profile and therefore this variation obtained between two sections may represent,(i) a greater concentration of protease in one section, possibly linked to the position of the section within the tumour (Section 3.1.3), (ii) experimental variation. It becomes increasingly important that investigators should set unified criteria not only for storage but also for the duration of storage. With the same reasoning, the vast majority of tumours falling into the 4S + 8S type (Table 3.1) could be the result of such an interconversion taking place during storage and/or the post storage warming (to $4^{\circ}C$) period. This similar problem may be inherent in the liquid nitrogen method of storage (Jensen et al., 1975; Dao and Nemoto, 1980) and lyophilization of tissue (Janes et al., 1982). These methods usually do not take into account the relative positioning of the section within the tumour. It is therefore possible that tumour powder used for analysis

may have acquired a large quantity of protease from within the central section and this will therefore influence the sedimentation profile. The influence of protease in the distribution of variable 4S : 8S was also indicated by Tilzer et al. (1981).

During the present investigation the receptor rich tumours (DCC assay) were generally selected for gradient analysis. This decision was made for reasons which include (i) a minimum value of receptor concentration is required for hydrodynamic analysis (Sherman et al., 1980), (ii) if an intratumoural variation of receptor molecular form was to be found then this should be clearly demonstrated and (iii) since the availability of tumour sample is limited and further sections were sometimes required for further biochemical analysis, a demonstration of a clear 8S receptor was felt necessary (based on literature survey). This selection of tumours for analysis is probably reflected in the very high number of 8S containing tumours detected (Table 3.2). Nevertheless, others have shown that receptor rich tumours can be predominantly the 4S type (Wittliff et al., 1976; Dao and Nemoto, 1980) and similarly that low receptor content tumours can be predominantly 8S (Freedman and Hawkins, 1980).

In no case in the present results (Table 3.1 and 3.2) was the receptor present exclusively as 4S, as shown for example in the Figure 2 of the paper by Wittliff and Savlov (1975). There always seemed to be either a shoulder present in the 7-8S area or some form of aggregated material in this region. This probably also provides the clue that 8S$\rightarrow$4S conversion must have occurred. It, therefore, seems likely that a complete conversion of 8S$\rightarrow$4S is most probably what Wittliff's group was observing. As previously noted the 8S$\rightarrow$4S conversion is a consequence of the

handling of tumours e.g. method and extent of homogenization, time and method of storage, time and method of thawing of stored tissue, extent of time lapse from biopsy to storage or assay. All these factors may contribute towards the final 4S : 8S ratio obtained. In this context, it is not surprising that several authors have concluded that inclusion of the 4S value, irrespective of whether or not 8S is present, can increase the predictive value of ER_c in relation to response (Dao and Nemoto, 1980; Gapinski and Donegan, 1980) in keeping with the observation that tumours showing higher receptor content are likely to show a greater chance of response. It therefore is difficult to resolve why Wittliff's result showed such a consistent and close relationship between molecular status and response. It is possible, but unlikely, that a sub group of patients, probably of an advanced stage may be referred to the surgeons from whom Wittliff's group obtain samples. It is also possible that, in fact, Wittliff's group may be employing some form of very strict criteria with respect to storage etc. which is giving them such reproducible results and this in fact may be reflecting some form of predictive nature of the proteolytic content of the tumours. It may be possible to reproduce these results in other laboratories but identical conditions will be required for such an achievement. The need for inter-laboratory quality control is an absolute requirement for such assays and the need for this has recently been recognized (King, 1980; Zava et al., 1982), although present work in this context is being directed towards measuring receptor content, not the molecular form. It may be argued that SDGA will not increase the 70% or so response rate obtained by simple DCC assay of both soluble and nuclear oestrogen receptor (Leake et al., 1981).

4.1.2.1 **Intratumoural Variation**

Results have already been presented in the literature

recognizing intratumoural variation with respect to receptor concentration and in extreme cases such variation may lead to diagnosis of one section being receptor positive and another receptor negative (Poulsen, 1981; Izuo et al., 1982). In general, it was found that the tumour preserves its receptor status in different sections, whether the sections were taken randomly or selectively (Figure 27 and Tables, 5, 6 and 7), however, the relative concentration of the molecular forms can vary. As far as is known no study has been reported where intratumoural variation with respect to molecular form has been analyzed. Kiang and Kennedy (1977) have studied simultaneous and sequential biopsies in the same patient on SDG and report that these are consistant in their qualities and quantities. However, recent work has shown that not all receptor positive primary tumours retain receptors in the secondaries (Leake et al., 1981; Harland et al., 1983).

In the present work 4 tumours were found (4/74) which showed one section in the 4S class and a second section in the 4S+8S class (Table 3.1). It is therefore concluded that in extreme cases, situations can arise where a false estimation of receptor molecular status could be detected (discussed below).

With respect to receptor concentration, it was found that the central sections showed lower soluble and nuclear receptor concentration than the peripheral sections (Table 4). This was, however, not always the case. Sometimes the peripheral sections showed lower receptor values than the central sections. These results are generally in agreement with others with respect to variation in the soluble receptor (Hawkins et al., 1977; Masters et al., 1978; Tilley et al., 1978; Poulsen, 1981; Izuo et al., 1982). However, with respect to the

nuclear receptors these results are at difference with those presented by Silfversward et al. (1980). It is not possible to comment as to why such a difference has been noticed. Recent studies with human endometrial carcinoma (Castagnetta et al., 1983) would tend to favour the results shown in the present work. The loss of both soluble and nuclear receptor towards the central area of the tumour is probably a consequence of that section representing the oldest part of the tumour with altered blood supply (Folkman and Cotran, 1976) and resulting necrosis, not visible to the naked eye.

Table 5 also presents results from tumour sections studied selectively (Figure 9) except that here the sedimentation pattern was also judged. With respect to receptor concentration, the results were no different to those already discussed for Table 4. The molecular status of the tumour was generally also preserved whether the sections were studied selectively (Table 5) or randomly (Table 6). However, where both the 4S and 8S forms were detected, these were found to vary among different sections of the same tumour. Figure 27 illustrates one such example. The protein/DNA ratio indicated in the associated table (shown in Figure 27) suggests that the low concentration of receptor in the periphery could be the result of a lower cellular content of the peripheral section, as indicated by the low DNA value. DNA, however, cannot be taken as an absolute index of tumour cell number since there may be non-receptor containing normal or cancerous cells present. Histochemical analysis (Nenci, 1981) has revealed that such situations prevail in human breast carcinomas. In addition leukocyte infiltration of some tumours could give a false estimation of receptor value when expressed per unit DNA, as has been indicated by Silfversward et al. (1980). Importance of measuring tumour cell content has been stressed (Hawkins

et al., 1977; Van Netten et al., 1982) although some investigators have failed to relate receptor to tumour content (Wittliff et al., 1976). Castagnetta et al. (1983) have reported that a more meaningful comparison between human endometrial carcinoma sections can be made if receptor concentration per unit DNA are compared. Human endometrial carcinomas may be relatively more homogenous than breast tumours since Romano et al. (1979) have reported that wide variations with respect not only to DNA content but also receptor expressed per unit DNA can be obtained in human breast tumours. In addition, whereas the human endometrial carcinoma intratumoural study has revealed that there exists an 'all or none' phenomenon with respect to receptor concentration in different sections, this process is much more gradual in human breast cancer. Nevertheless, from the very small number of tumours analyzed (Table 5 and 6) it is indicated that DNA may provide a better index of representing receptor concentration than that expressed per unit protein (e.g. tumour number 1 in Table 5). The maximum variation obtained in receptor concentration with respect to protein concentration was 13.7 fold whereas the maximum variation on DNA basis was 4.5 fold. Axillary nodes analyzed, however, showed an opposite effect, in that the intratumoural variation increased when receptor concentrations were expressed per unit DNA (tumour number 2 in Table 6 and tumour sample represented in Table 7). However, since lymph nodes are more prone to leukocyte infiltration, DNA may only be of value for primary tumours. Auer et al. (1980) have shown that tumour cells can contain an aneuploid condition, but that such a situation is usually associated with receptor negative tumour cells. The foregoing conclusions are purely preliminary since the number of tumours analyzed was small. A detailed analysis is needed. What has been clearly shown, is that tumour ER_c 4S : 8S ratio can vary between different sections of the same tumour. In addition to other possible reasons for such a situation, one reason must include the

variation of protease content in different regions of the tumour. It cannot be said at present, however, whether this variation is a reflection of variability of cellularity or variations within individual cells.

Figure 28.1 and 28.2 show two examples where the tumour can be interpreted and classified into two different categories, depending on which section was analyzed. Routine analysis may be limited by the amount of material available, a difficulty recognized by others (Sherman et al., 1980). In Figure 28.1, one tumour section shows a predominant 4S profile whereas an 8S peak can also be clearly demonstrated in a different section. This is an example of extreme variation noted in 4/74 tumours. Figure 28.2 is an example of a case where such variation was found in the 4S form. The tumour shown in Figure 28.2 can be classified as either a 4S + 8S type or as a predominant 8S type. In the present work, for uniformity both sections were classified as a 4S + 8S type (Table 3.1) although section B should really have been classified as a predominant 8S type. Nevertheless, the quantitation of 4S receptor in such profiles is subject to considerable error because of the nature of the 4S peak ($\sim$26%). However, with respect to criteria for relating tumour profile with response, both sections indicate the patient as a potential responder. It is also important to point out that in Figure 28.2B, a proportion of receptor was present as heavy aggregate. If this represents only the 8S form then the proportion of 4S receptor was overestimated and this section should have been classified as 8S type.

It has previously been indicated that certain tumours may possess factors which can lead to receptor aggregation at the bottom of the centrifuge tube. Receptor aggregation has been observed in other systems (Rochefort and Baulieu, 1971) and seems to represent a general

property of receptor. Polysaccharides may be involved (Nishizawa et al., 1981; Kim et al., 1982). However, self-association of receptors and association with other proteins cannot be ignored (Stancel et al., 1973a; Murayama et al., 1980a,b,c). This aggregation property cannot be ascribed solely to in vitro artifacts. An in vivo function for aggregation is possible or may relate to cell cytoskeletal structure (Mueller et al., 1978; Schliwa et al., 1981; Barrack and Coffey, 1982). Aggregation was quite prominant in some of the human breast tumour cytosols analyzed in low salt gradients (Tables 5 and 6). Such aggregation can lead to a false estimate of the 4S : 8S ratio if only one form is able to aggregate. This is not the case in Figure 28.1, where there was complete recovery of specific counts on the gradient and therefore no aggregation. In one case studied (data not shown), 1h incubation at $4^{o}C$ showed a high amount of receptor aggregation. However, in the same cytosol when incubated for 24h at $4^{o}C$, aggregation was not observed. Instead, a considerable rise in 4S concentration occurred. One explanation of such a situation is that only 8S aggregates and all 8S is degraded to 4S during the 24h incubation.

4.1.2.2 **Protease Mediated 8S ⟶ 4S Conversion**

The varying distribution of 4S and 8S forms within the same tumour prompted an investigation to determine if 8S⟶4S conversion may take place between homogenization and gradient analysis. Immature rat uteri are known to contain only the 8S form. Immature rat uterine cytosol was mixed with human breast tumour cytosol. The result (Figure 27) shows that 8S ⟶4S conversion does occur on mixing these cytosols, even at $4^{o}C$. The tumour used for this experiment gave the 4S + 8S profile. Receptor concentration of the tumour cytosol was low to avoid interference with the resolution of the rat receptor.

Sato et al. (1981a) have also performed similar experiments and have shown that such 8S⟶4S conversions could be accelerated by temperature and lead to loss of DNA binding by receptor. In a similar context, some preliminary results (data not shown) indicated that immature rat uterine cytosol, after mixing with breast tumour cytosol, failed to give a 4S⟶5S transformation. This same proteolytic process may be involved in Ca^{2+} promoted effects (Figure 13; see also Schneider and Dao, 1977). It is concluded from these data that a protease(s) activity is present in human tissue which also recognizes immature rat uterine receptor.

Analysis of mature rat uterine cytosol showed the presence of two molecular forms (Figure 30) similar to those found in certain human breast tumour cytosols. The rat receptor molecular form seems to fluctuate to a certain extent depending on the stage of the oestrous cycle. The 4S : 8S ratio seems to be highest at oestrous when the uterus has just passed the period of maximum exposure to oestradiol. The ratio seems to be lowest at metoestrous, the time of lowest oestradiol stimulation. Oestrogen induced trypsin like activity in the mature rat uterus has been reported by some authors (Katz et al., 1976; Kneifel et al., 1982). Hakim (1980) has also shown a variety of oestrogen-induced proteases in human tissue. In human breast tumours a similar effect is observed since increased cleavage of human breast tumour ER_c is correlated with plasminogen activator (Sherman et al., 1980). Fluctuation of progesterone receptor with the oestrous cycle in guinea pig uterus has been reported (Milgrom et al., 1972). Mammary gland and other tissues have all been shown to contain various molecular forms of receptor (Section 1.2.2.2.1, Milgrom et al., 1973c) and seem to depend on the hormonal background of the animal. Gaubert et al. (1982) have shown that this 8S⟶4S conversion in cytosol can be prevented by homogenizing the tissue in either

phosphate buffers or buffers containing sodium molybdate. Such a specific mechanism in which the receptor steroid binding site is not affected suggests a true in vivo role for this protease. It may be of significance that such a process (8S⟶4S) seems to be common to other steroid receptors (Hazato and Murayama, 1981; Prins and Lee, 1982). Investigators have also located large concentrations of such a protease in nuclei, affecting both oestrogen (Murayama & Fukai, 1981) and progesterone receptor (Vedeckis et al; 1980). Such a protease might serve a nuclear function either regulating the amount of receptor which can bind to correct DNA and/or acceptor sites/initiating receptor degradation. Fractionation may release this protease into the soluble fraction. Serine proteases are known to be present in chromatin (Carter and Chae , 1976; Tsurugi and Ogata, 1982).

It is suggested that 5S may represent the form which has a higher affinity for oestradiol (Weichman and Notides, 1977) and this may be linked to the duration of stay of receptor on the chromatin. The presence of a high concentration of protease may explain why some 8S containing tumours fail to respond to endocrine therapy. The protease(s) may not allow sufficient time for the receptor to bind to and activate DNA. Therefore the tumour is non-responsive. The salt extraction of nuclear receptor may not allow the detection of heterogeneity in nuclear receptor since present investigations all use high salt gradients (4S cytoplasmic receptors may comprise of two forms, see Section 4.2.2). However, Andre et al. (1978) have used micrococcal nuclease to digest nuclear receptor and have found two forms on low salt gradients, the 4S and the 6S form. It is even possible that the 8S⟶4S conversion may, in fact, be the signal for a rapid degradation of the receptor. A conformational change in receptor protein may be the signal required

for such degradation, as is found for other proteins (Wheatley, 1982). Such an 8S⟶4S conversion really represents a 4S aggregating type⟶4S non-aggregating type conversion. The 4S aggregating type (8S) may also be released by other mechanisms, such as dephosphorylation (Auricchio et al., 1982) and this possibly contributes towards the recycled receptor. At least some of the recycled receptor is able to undergo a second round of translocation (Muldoon, 1980). It is possible that some of the proteolysed receptor may undergo repair in the cytoplasm. Detection of microsomal bound receptor may represent both newly synthesized and receptor undergoing repair (Muldoon, 1980; Parikh et al. 1980). It should be pointed out that the effect of dephosphorylation is measured as loss of oestradiol from nuclei and may not indicate loss of receptor itself.

A possible model of steroid hormone action is proposed (Section 4.3).

4.2. Activation/Transformation Study

The terminology used in steroid receptor research, with regard to activation and molecular transitions has led to much confusion! The two terms, activation and transformation (4S→5S) of oestrogen receptor, have been used interchangebly to describe the state of receptor which has acquired an increased affinity for DNA or chromatin. True transformation, nevertheless, has only been observed for oestrogen receptors. The recent use of the term activation to describe the form of receptor which, after phosphorylation (Sando et al., 1979a,b) acquires the ability to bind hormone, has further confused the issue. Unless specified, the latter concept should be disregarded with reference to the present discussion.

Activation and transformation of oestrogen receptor may not be independent events. Nevertheless whether activation proceeds, occurs concomitantly with or follows transformation is unclear. Activation may be taken to mean (a) the dissociation of receptor from inhibitors and modulators, to give the active form (active implying the availability of receptor for DNA binding, but prior to acquisition of DNA binding ability), (b) a simple conformation change (no 4S→5S required) common to all steroid receptors and (c) association of another subunit(s) to the 4S subunit to yield the 5S receptor which now represents the DNA-binding form. Support for concept (c) comes from the fact that nuclear oestrogen receptor shows properties similar to the 5S form that can be generated in the cytosol (Notides, 1978). If, however, the second subunit(s) (Thampan and Clark, 1981) were to be a nuclear protein then concepts (a) and (b) are possible. Data is consistent with the existence of inhibitory factors mentioned in (a) but there remains no proof in vivo.

The study of Bailly et al. (1980) has suggested that receptor

activation (to the DNA binding form) is a first order process, and consequently independent of a second subunit. The same study has further suggested that transformation is a second order process which involves binding of another subunit. Their methodology however, is open to criticism. In their method, rapid dilution of high salt cytosol for DNA binding purposes can, itself, induce a 4S⟶5S change (Notides, 1978). Further, separation of low receptor concentrations on overnight sucrose density gradients may promote 5S⟶4S transition giving a false indication that 4S represents the active form. In the absence of any detailed knowledge of 4S⟶5S equilibrium the correlation of test tube data with that of SDGA should be interpreted very carefully.

It may be important that it is only after heat activation that receptor triggers RNA polymerase activity (Mohla et al., 1972). This heat activation is associated with a 4S⟶5S change for the oestrogen receptor (Figure 46). The 5S complex may represent a functional unit required to form a tight complex with DNA and essential for the triggering off of a signal. The formation of multiple 5S complexes (aggregation) is also a possibility. The fact that such a 5S complex is not observed for other steroid receptors does not argue against the formation of such a complex. The 5S human receptor can be shown to form under physiological conditions (Notides et al., 1976). The glucocorticoid receptor recently visualized electronmicroscopically (Govindan et al., 1982) seems to comprise a dimer on the DNA. Therefore the possibility that there is an equilibrium between a higher molecular weight (active?) form and the conventional 4S form for other steroid receptors seems likely but such an equilibrium is strongly in favour of the dissociated form. Proteolysis during extraction and the fact that the second subunit may be a nuclear protein for receptors other than oestrogen, may also obscure transformation studies.

At present, the only known form of regulating receptor-DNA interaction seems to be the regulation of receptor concentration itself (Clark and Peck, 1979). The process of receptor activation may normally prevent excessive gene activation. In recent years this idea has been reinforced by studies of DNA-binding of oestrogen-receptor and anti-oestrogen-receptor complex (Rochefort and Borgna, 1981; Katzenellenbogen et al., 1981; Evans et al., 1982).

4.2.1. **The DNA-cellulose binding assay**

To further assess the role of the DNA-binding ability of receptor as one mechanism of control in normal cells, the DNA binding assay was developed. Yamamoto and Alberts (1974) have reported that the binding of receptor to double stranded DNA is not a sequence specific effect, yet the observation (Simons et al., 1976) that the activated-receptor complex binds strongly to DNA-cellulose has proved very useful as a measure of the active receptor. Using the DNA-cellulose competitive binding assay (Kallos and Hollander, 1978), Mulvihill et al. (1982) have demonstrated the presence of specific DNA binding sites for hormonally responsive genes (for progesterone receptor). Use of DNA-cellulose has also been made by Pfahl (1982) to demonstrate specific binding of the glucocorticoid-receptor complex to the mouse mammary tumour proviral promoter region. The use of purified nuclei to study oestrogen receptor-chromatin interaction has proved of little success, especially with the mammary gland (Park and Wittliff, 1977). DNA-cellulose has proved a very useful and convenient tool for studying activated oestrogen-receptor complex (Park and Wittliff, 1977; Sato et al., 1981a; Katzenellenbogen et al., 1981). It has also proved useful for analysis of oestrogen-receptor and drug receptor interaction in other systems (Evans et al., 1982).

4.2.1.1 **Immature Rat Uterine Soluble Oestrogen Receptor**

It was shown that DNA-cellulose does not retain any significant free steroid (Table 8). This was found to be the case whether steroid was provided in buffer or in receptor depleted cytosol. This result is in agreement with that of Pfahl (1982). In the presence of receptor, however, radioactive steroid binds to DNA-cellulose (Table 9.1 and 9.2). The bound counts can be extracted in part with salt (Table 9.2). The DCC assay used (Park and Wittliff, 1977) most probably underestimates the salt extracted receptor bound counts but heat-dependent activation can still be demonstrated (Table 9.1). After several washings of the DNA-cellulose bound receptor and subsequent salt extraction receptor is relatively pure and most probably adsorbs to the DCC (Poulsen, 1981; Powell et al., 1981). The extraction buffers contain 0.4 - 0.6M salt which itself, has been shown to effect the DCC assay (Peck and Clark, 1977).

Underestimation of salt-extracted activated receptor by DCC analysis was also indicated by the results shown in Figures 31 and 32. If the salt extracted receptor, without any previous DCC stripping, was loaded onto low salt gradients the receptor aggregated to the bottom of the tube leaving some free steroid trailing towards the top of the gradient. In high salt, however, a 5S peak can be detected but the trailing effect of free steroid was persistent. The receptor most probably looses steroid during centrifugation due to low protein concentration, an effect also seen by Bailley et al. (1980) and Nishizawa et al. (1981). However, if the receptor is extracted from DNA-cellulose in buffers containing protein and then loaded onto gradients containing protein up to 70% of the steroid could be shown to be still macromolecular bound (Figure 32). This sedimented as a sharp 5.5S peak particularly in BSA containing gradients

(Figure 32B). It is believed that the rest of the counts may also be macromolecular bound but that these bind to the sides of the cellulose-nitrate tube during centrifugation. This binding appears to be very tight since even alcohol extraction of the tube was unable to yield 100% recovery of counts. This use of proteins was also made by Yamamoto (1974), where better results were found in the presence, than in the absence of proteins. However, as shown in Figure 32C, the choice of protein should be made carefully since in the presence of human-γ-globulin the process of receptor extraction was inefficient. Thus, both DCC and SDG (in the absence of exogenous proteins) may underestimate the total concentration of DNA-cellulose bound activated receptor. Finally, the activated complex sediments as a 5S species.

When using DNA-cellulose, the use of competitor was found unnecessary (Table 9.2), and both Figures 31 and 32 confirm that no non-specific component could be detected on the gradients. DNA-cellulose therefore selectively retains the oestrogen-receptor complex and confirms that it is a DNA binding protein. Other DNA binding proteins are known including the lactose repressor, which binds to specific sites on DNA other than their specific sites in the gene-control regions (Bourgeois and Pfahl, 1976). It is not known if the nucleotide sequence of these sites is different or the same. Similarly certain stretches on calf thymus DNA (DNA-cellulose) may preferentially retain the oestrogen-receptor complex. One great advantage of the DNA-cellulose assay is that it omits the need for receptor purification, a process which may remove certain components required for specific selection of DNA binding site (Mulvihill *et al.*, 1982).

Temperature dependence of immature rat uterine ER_c - DNA interaction

is shown in Figure 33. It was found that 30°C for 30 min. provided maximum binding to DNA (80-90% of total input; also see Tables 10 and 11). Significant ($\sim$ 30%) binding was also found in non-activated cytosol. This indicates that a proportion of receptor is activated even at 4°C possibly as a result of 0.15M salt present in the incubation mixture, salt may be dissociating inhibitors (Section 1.1.5.3.2). Traish et al. (1979) have found that in intact uterine cells the formation of the 5S complex (activated receptor) is a temperature dependent event but can take place at 4°C over a longer time interval (22h). Gschwendt (1980) used a chromatographic procedure for his DNA-cellulose assay and reported 80% binding of activated oestrogen-receptor complex from chick oviduct. These results suggest that on heat activation oestrogen receptor either acquires another subunit(s) with a DNA binding site on it or else that the receptor itself unfolds to expose a DNA binding site(s).

The 80-90% DNA binding ability of immature rat uterine oestrogen-receptor complex is in disagreement with the results presented by Bailly et al. (1980) and for glucocorticoid receptor by Le Fevre et al. (1979). Whereas the differences reported by Le Fevre et al. can be ascribed to different systems being studied, the differences obtained with Bailly et al. are more difficult to explain. These possibilities can be put forward - (a) in our experiments, fresh cytosol was always used whereas Bailly et al. used uteri stored for up to 4 weeks in liquid nitrogen which must have destroyed some of the DNA binding potential of receptors (also see Figure 57C which was obtained from uteri stored in sucrose/glycerol for just 1 day - there was a loss of 5S forming potential in these cytosols), (b) Bailly et al. used EDTA in their buffers, an agent which is known to inhibit activation (Sala-Trepat and Vallet-Strouve, 1974; Sato et al., 1978a), (c) the experiments conducted by

Bailly et al. involved up to 8-fold dilution of cytosol, a process which would be unfavourable for a bimolecular reaction. It should also be noted that the failure to show a temperature dependent activation step for uterine cytosol by Sato et al. (1981a), may also be due to previous storage.

Although a direct comparison of DNA-cellulose and nuclei is inappropriate, it is interesting that in vivo translocation also allows 90% of the receptor to move to the nuclear fraction (Williams and Gorski, 1972). The 5S complex formed in vitro therefore may possess the same properties of binding to DNA as in vivo. However the other subunit present in the 5S complex (Thampan and Clark, 1981) may perhaps direct the receptor to correct acceptor sites (Leake, 1976).

In the present study it was observed that $\sim$ 70% of the total counts bound to DNA-cellulose were salt extractable. This involved one salt extraction at $4^{o}C$ for 1h with buffer containing 0.6M KCl (Figure 33, Table 10 and Table 11). This procedure is similar to that used by others (Clark and Peck, 1976; Katzenellenbogen et al., 1980) for whole nuclei. An interesting feature to emerge from the present data was that a constant proportion of total bound receptor was extractable from the DNA irrespective of the use of activated or non-activated cytosol for the binding incubation. This implied that a fixed proportion of activated receptor binds to higher affinity sites on the DNA and is insensitive to a single salt extraction, that is, there must be an equilibrium between tight-binding and loose-binding sites on the DNA. The possibility of two different types of 5S receptor has been ruled out (Juliano and Stancel, 1976) although Ruh et al. (1981) point out that heterogeneity within receptor may occur. Dickerman and Kumar (1982)

have shown that receptor is capable of distinguishing between various deoxynucleotides. The deoxyguanosine in particular shows much greater affinity for the receptor and there is a possibility, therefore, that a stretch of deoxyguanosine may serve as a salt resistant site.

The physiological significance of differential extraction of receptor from nuclei is currently a matter of debate (Clark and Peck, 1979; Barrack and Coffey, 1982; Ikeda et al., 1982). Nevertheless it is interesting that, when using whole nuclei, the proportion of receptor that is salt extractable (Katzenellenbogen et al., 1980) is similar to that from DNA-cellulose (present study). Ruh and Baundendistel (1977) have proposed that antioestrogen-receptor complexes only bind to salt extractable sites. Barrack et al. (1983) have found a difference in the proportion of salt extractable androgen receptor from normal and cancerous human prostate. Perhaps DNA-cellulose could shed further insight into the specificity of this reaction. Some studies involving antioestrogen-receptor complex binding to DNA-cellulose are already in progress (Evans et al., 1982). Whereas Katzenellenbogen et al. (1981) have found fewer binding sites on DNA for the antioestrogen-receptor complex, Evans et al. (1982) report that the number of binding sites are the same but that the anti-oestrogen-receptor complex has a lower affinity for the DNA. The latter may be related only to salt extractable sites.

Maximum binding of activated receptor to DNA occurred after a constant time of approximately 1h with 100ug DNA (Figures 34 and 35). Interestingly, however, when the effect of contact time of DNA with a fixed receptor concentration was studied (Figure 34) the alcohol extractable sites were filled very rapidly but the lower affinity site(s) (salt extractable sites) showed a time dependent occupation.

The saturation obtained must be a consequence of receptor limitation. The above also rules out the possibility that access of $HDK._6$ buffer to the DNA-bound receptor is the limiting factor in the salt extraction efficiency. At shorter contact times of receptor with DNA-cellulose the binding equilibrium between salt extractable and salt resistant sites must be in favour of the salt resistant form since proportionately there were more salt resistant counts at shorter contact times. Spelsberg (1976) has shown the existence of multiple affinity acceptor sites on chromatin. It appears from recent experiments that chromatin associated proteins are involved in determining the specificity of binding whereas binding to naked DNA is non-specific (Ruh and Spelsberg, 1983). In keeping with the above, Figure 35 shows that whereas 100ug DNA provided the optimum concentration for measuring total activated receptor in the system, the alcohol extractable counts increased with increasing DNA concentration.

Figure 36 shows that using a fixed amount of DNA (100ug), increasing receptor concentration reveals non-saturable binding in the range tested (confirming the data of Ruh and Spelsberg, 1983). This confirms that in the previous experiments DNA binding sites for the receptor were not limiting. The protein concentration in the entire system was kept constant by adding a heated ($37^{o}C$ for 2h in the absence of steroid) aliquot of the same cytosol to the required volume. It was noted, surprisingly, that the heated cytosol was still able to bind about 50% of the specific counts represented by the intact cytosol. However, these counts did not interfere with the assay for the reasons explained in Section 3.2.1.2.6. In addition a recent paper by Leach et al. (1982) has suggested the presence of a heat stable factor in the glucocorticoid receptor system which acts similarly to sodium molybdate. This factor

both stabilizes the unbound receptor and prevents its DNA binding. It should be remembered that fresh cytosol was used in the present investigation which may be significant with respect to the above result. When sodium molybdate was introduced into the cytosol either just prior to activation or from the time of incubation with radioactive steroid, the receptor activation, as judged by the DNA-cellulose assay was inhibited (Figure 37). The possible modes of action of this compound were discussed in Section 1.1.5.3.2 and Section 4.1.1.2. Interestingly, it was found that when homogenization was performed in the presence of sodium molybdate the activation seen at $4^{o}C$ was completely abolished (Figure 38). Nevertheless, heat activation always provided $\sim$ 20% receptor activation irrespective of the stage at which sodium molybdate was introduced (Figures 37 and 38). This most probably indicates that molybdate only slows down the rate of activation. A similar observation was also made by Mauck et al. (1982) for calf uterine oestrogen receptor. During the course of the present investigation it was noted that EDTA also provided a similar result (data not shown). It has been suggested that molybdate may chelate endogenous metal ions (cf. EDTA) which may be required for activation (Ratajczak et al., 1981). There is also some evidence that receptors may be metalloproteins (Lohmar and Toft, 1975; Shyamala, 1975; Schmidt et al., 1981), and therefore a direct interaction of molybdate or EDTA with receptors themselves cannot be excluded. It has been reported by (Cong and Lippman, 1981-82) that ATP-promoted oestrogen receptor activation in MCF-7 cells is also only partially inhibited with sodium molybdate. If the effect of molybdate or EDTA is on the rate of activation then an extended time course should promote further activation. For EDTA indeed this has been found to be the case (Hyder and Myatt, unpublished observation) but only after overnight incubation at $4^{o}C$. Although a similar effect at higher temperature

is expected, there is also the process of deactivation which follows receptor activation (Buchi and Ville, 1976; also found for glucocorticoid receptor by Aranyi, 1983). It therefore appears that EDTA and molybdate may play a common role in inhibiting receptor activation. Bailly et al. (1980) in their attempt to distinguish between activation and transformation used EDTA in their buffers, hence that data should be interpreted with great care. EDTA has been known to inhibit not only activation but also transformation (Jensen and DeSombre, 1972; Sato et al., 1978a).

It was noted (Figure 37 and 38) that whereas in the absence of molybdate the difference between salt extractable and alcohol extractable counts was approximately 4-5 fold, this difference in the presence of molybdate was only 2 fold. Molybdate seems to have a greater effect on the DNA-binding of salt extractable counts. The significance of this observation is not clear.

4.2.1.2. Human Breast Tumour Soluble Oestrogen Receptor

In contrast to the DNA binding results of immature rat uterine receptor, the level of DNA binding by human breast tumour cytosol receptor was far less and more variable ($\leq$30%) (Figures 39 and 40). Due to lack of sample availability not many samples could be analyzed, but the results presented are generally in agreement with those of others (Park and Wittliff, 1977; Sato et al., 1981a). Figure 39 confirms that temperature dependent activation of oestrogen receptor takes place in human breast tumour cytosol but this is far less dramatic than that observed for rat uterine receptor (Figure 33). Sato et al. (1981a) found that whereas some tumour cytosol preparations showed temperature dependent activation others lost the DNA binding ability on heating. In another report (Sato

et al., 1981b) it is mentioned that in oestrogen-independent mouse Leydig cell tumour, heating cytosol for activation destroys the DNA binding ability whereas dialysis at low temperature promoted DNA binding. Proteolysis of receptor is indicated. The use of DFP in the present investigation (Figure 40) suggests that serine protease(s) in tumour cytosols destroy the DNA binding site of receptor. This either results from proteolysis of the DNA binding domain of receptor itself or of another component which during the activation conditions binds to the receptor. This result is consistent with those reported for other human tissues (Notides et al., 1976; Lukola and Punnonen, 1982). Human tissues in general possess protease(s) which are absent or not active in immature rat uterine tissue.

DFP was found to suppress the total oestrogen binding capacity of tumour cytosol. The possible reasons for this are presented in Section 4.1.1. In human tissue DNA binding studies it was found necessary to compute the DNA-binding obtained in the presence of competitor. In some cases this was significant because of the very low percentage of receptor binding to DNA-cellulose (see also Park and Wittliff, 1977; Sato et al., 1981a and Table 12).

It is difficult to explain why after taking the necessary precautions (inclusion of 0.15M KCl and DFP) the total receptor binding to DNA does not exceed $\sim$30% of the input concentration (30% was the maximum binding obtained using DNA-cellulose). It is possible that there is a large amount of receptor activation inhibitor or that there is absence of a factor(s) promoting activation. There is also a suggestion that in the human uterus, post-menopausal receptor is non-functional (Strathy et al., 1982) with respect to DNA binding. Nevertheless, as shown

in the next section, a very large proportion of human breast tumour receptor is capable of binding to soluble DNA (especially the 8S form) and therefore the problem does not seem to be lack of factor(s) required for DNA binding. The problem seems to be associated with the heating step. In vitro transformation studies (Section 3.2.3.2.7) revealed that this process leads to receptor aggregation (especially in the presence of DFP) or receptor proteolysis from 8S⟶4S (sometimes even in the presence of DFP). Aggregation may therefore mask the DNA binding site and proteolysis may lead to the loss of DNA binding ability. Limited heat dependent activation may result from both receptor present in the active form and some active receptor dissociating from the aggregated material during the incubation with DNA-cellulose.

It is interesting that Lukola et al. (1980) and Charreau and Baldi (1977) found that a greater nuclear uptake can be observed if the acceptor is present during the warming step. This would suggest that the active receptor, in the absence of an acceptor, becomes rapidly deactivated (see Aranyi, 1983) either in the aggregated form or as a proteolyzed form. In the present investigation and in those of others (Park and Wittliff, 1977; Notides et al., 1976; Sato et al., 1981a), the receptor was incubated with DNA-cellulose after heat activation. These results strongly suggest that it may not be entirely correct to conclude from in vitro experiments that there is an excess of inhibitor(s) or lack of activation factor(s) when DNA or chromatin binding could not be observed or was limited (Haselbacher and Eisenfeld, 1976; Fox, 1977; Shen et al., 1979; Strathy et al., 1982). Thus loss of DNA binding may result from an artifactual loss of activating factor(s), acquisition of inhibitory factor(s) or alteration in receptor structure. The aggregation and proteolysis problem may also be responsible for the apparent lack of

temperature dependent activation in mature rat uterine cytosol (Myatt et al., 1982a) and in the variable level of receptor binding to oligo-deoxythymidine observed by Thrower et al. (1976) who were using mature rat uteri irrespective of oestrous stage (see also Figure 30).

Table 13 indicates that the DNA-cellulose bound human receptor is more difficult to extract with salt than the activated immature rat uterine soluble receptor. In fact, the situation is reversed, that is, there are about 70% alcohol extractable counts compared to approximately 30% in the immature rat uterine DNA-bound receptor. Once again, a similar situation is observed with intact nuclei prepared from other human tissues (Katzenellenbogen et al., 1980). It has been found by Kasid et al. (1982) that in the MCF-7 human breast cancer cells, the processed receptor is inaccessible to salt extraction. This observation lends further support to the view presented by Love et al. (in press) that by salt extracting, most investigators would underestimate nuclear receptor in human breast tumours. However there is one possibility which we cannot ignore in explaining the differential salt extractability of DNA-cellulose bound human oestrogen receptor. It is possible that most of the DNA-bound receptors are loosely attached and are released during the washing procedure, giving a false distribution.

4.2.2 Interaction of Cytoplasmic Oestrogen Receptor - Soluble Calf Thymus DNA

Low salt SDGA, used by Park and Wittliff (1980), involving ER_c interaction with soluble calf thymus DNA showed that a vast majority of soluble receptor from human breast tumour (especially 8S) was capable of interacting with DNA (cf results in previous section). However,

setting up of this assay required a control experiment. Immature rat uterine soluble receptor was used (Figure 41). It was shown to sediment primarily at 8S (Figure 41A) in the absence of DNA. On adding DNA to the cytosol, this peak was shifted to the bottom of the tube in conjunction with the DNA (also see Majumdar and Frankel, 1978). Since immature rat uterine cytosol generally contained a much lower protein concentration compared with human breast tumour cytosol, it was decided to introduce a large amount of non-specific protein into the assay system to determine its effect on DNA binding (Figure 41C). Cytosol was made 10mg/ml with respect to BSA and then, after incubation with $^{3}HE_{2}$, DNA was added. The receptor was still able to bind to DNA and sediment to the bottom of the tube. There was however, a small 4.6S peak of $^{3}HE_{2}$, co-sedimenting with the ^{14}C-BSA marker suggesting that some $^{3}HE_{2}$ has been non-specifically associated with the BSA. The possibility however, is not discounted that there may be other proteins in the tumour cytosol which may block DNA binding ability, by either directly interacting with the receptor or by interacting with the DNA. The latter seems unlikely since such a vast excess of DNA was used (1mg/ml). It is seen in Figure 41 that not all receptor binds to the DNA and there is a minor peak left behind on the gradient. Possible explanations must include (a) insufficient time for equilibration between DNA and receptor, this may in turn depend on the equilibrium between the quasi-stable states of the receptor (Kim et al., 1982), (b) lack of other factors required for activation, (c) dissociation of receptor during centrifugation and (d) a possible equilibrium between activated and non-activated receptor.

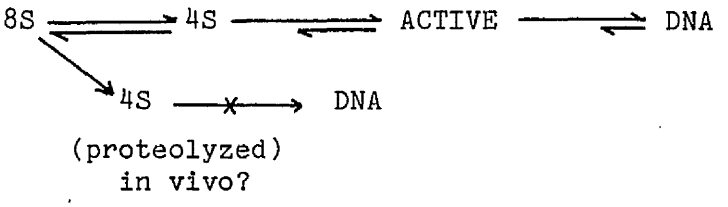
The same assay when repeated for human breast tumour cytosol, containing predominantly the 8S form, showed a similar result (Figure 42). However two tumours which were found to contain very distinct 4S and 8S

peaks, showed that only the 8S peak was displacable with DNA. An example is presented in Figure 43. This result is in agreement with Park and Wittliff (1980) who propose that low salt 4S receptors are inactive in DNA binding and this therefore supports their theory of unresponsiveness of 4S containing tumours. However, this is not always the case. Some tumours were found where a vast majority of the 4S peak also bound to the DNA (Figure 44). The data therefore clearly demonstrates a heterogeneity in the ability of 4S receptor to bind DNA.

It is suggested that the proteolyzed 4S receptor fails to aggregate to 8S and does not readily bind to DNA (Rochefort and Baulieu, 1971; Lukola and Punnonen, 1982). The 8S dissociated form represents a 4S form which can acquire the ability to bind to DNA. The proteolyzed 4S receptor may bind to DNA but with very low association kinetics (probably non-specifically). Situations are known where such weak associations do occur (Muldoon, 1981 ; Mataradze et al., 1982). There may be several reasons why an intact 8S dissociated receptor is found in the 4S region. Possibilities may include (a) relaxation of 8S structure as a result of mild proteolysis (b) absence or limitation of certain other components which are known to interact with intact 4S receptor to form the 8S form (Murayama et al., 1980b). These situations could arise as a result of storage or experimental handling. The possibility that intact 4S forms can exist has recently been demonstrated by Colvard and Wilson (1981) for the androgen receptor.

An interesting result was found in one case where there was only partial 4S and 8S binding to DNA. Whereas lack of 4S binding came as no surprise, the absence of 8S interaction was unusual. Perhaps such an aggregated receptor does exist in the cytoplasm of the cell

preventing translocation of receptor into the nucleus in the absence of ligand. Ligand most probably shifts the equilibrium towards the intact 4S complex which moves to the nucleus and binds other factors to become activated receptor (5S).



A similar equilibrium model was recently reported by Raaka and Samuels (1983) for the glucocorticoid receptor. It has also been noted by Tymoczko and Phillips (1983) that RNAase treatment of glucocorticoid receptor increases DNA binding ability and shifts the molecular form to 4S. Wrange and Gustafsson (1978) also found that partial proteolysis of the glucocorticoid receptor shifts the sedimentation profile towards the lower molecular form and increases the DNA binding ability of the glucocorticoid receptor. However further proteolysis led to destruction of the DNA binding site. For oestrogen receptor it is clear that molybdate prevents 8S⟶4S dissociation and simultaneously prevents transformation of the receptor to the 5S form (Muller et al., 1982). All this suggests that an 8S⟶4S dissociation is essential for activation of receptor. In Figure 45, the most probable reasons for the receptor to exist in the 8S complex were (i) a large concentration of inhibitor or (ii) a relatively high protein concentration of the cytosol compared to other analyses. A possible proteolysis of the 8S receptor DNA binding site cannot be ignored, although this seems unlikely.

It is interesting that in contrast to the temperature dependent activation previously shown (Figures 33 and 39) no such requirement was necessary for the analysis just presented. This most probably indicates

that 4S⟶activated state is promoted by the presence of DNA (Yamamoto, 1974). It is also possible that such a vast excess of DNA (1mg/ml) may actually bind the receptor non-specifically. Some of these experiments were therefore repeated in the presence of 0.15M KCl (data not shown). Receptor was shown to bind to DNA but, under these conditions, it was not possible to conclude which molecular form bound as all receptor is initially 4S (e.g. see Figure 22).

These results indicate that the human breast cancer soluble receptor sedimenting in the 4S region of the gradient may comprise both proteolyzed receptor and dissociated 8S receptor. The latter remains in equilibrium with the 8S form.

4.2.3 Receptor Transformation

The 4S⟶5S transformation of oestrogen receptor has been suggested to represent a physiological event (Traish et al., 1979). Nevertheless, it has been suggested that such a process does not occur in human breast tumours (Fazekas and MacFarlane, 1980). Results presented in this thesis suggest that such a transformation may be demonstrated under defined conditions.

4.2.3.1 Immature Rat Uterine Receptor Transformation

In accordance with transformation results presented from intact uterine cells (Traish et al., 1979), Figure 46 shows the in vitro temperature and time dependent formation of the 5S peak. In addition Figure 46A (low temperature control) shows a shoulder at about 5S which could explain the small extent ($\sim$30%) of DNA-cellulose binding seen in the non-activated cytosol (Table 10). These results would suggest that

it is the 5S form which binds to DNA. This transformation step most probably involves binding of another subunit to the native 4S receptor. Nevertheless, it is debatable whether the 5S form represents a homodimer (Notides et al., 1981) or a heterodimer (Thampan and Clark, 1981). It is likely that the second subunit either provides the DNA binding site or allows some conformational change in the receptor molecule exposing the DNA binding site. This 5S complex obtained is indistinguishable from that obtained from salt extraction of intact nuclei (Notides, 1978) or DNA-cellulose (Figure 32B). In the present investigation it was found that the 5S form could be demonstrated in either 0.15M or 0.4M KCl gradients following heat activation of either low salt or 0.15M KCl containing uterine cytosol. However, heat activation in low salt buffer followed by low salt gradient analysis led to aggregation of the receptor to the bottom of the tube (data not shown).

It is further shown in Figure 47 that if the activation was followed by progressive dilution and sedimentation analysis, then there was a loss of counts towards the top of the gradient. This most probably results from the lack of protein environment which may help the receptor to retain the DNA-binding subunit. It could be suggested that such a process is one of the ways of terminating receptor occupancy of the acceptor site - the release of a second subunit from the receptor will lead to a relaxed K_d for E_2 (or vice versa) and subsequent loss of response. Some of the results presented by Bailly et al. (1980) involved separation of receptor on 0.4M KCl gradients, followed by an 8-fold dilution (to reduce salt concentration) and subsequent DNA-binding analysis. Those results, therefore, are expected to show only suboptimal binding (in fact only 30-40% DNA binding was observed in their case). Although not clear from Figure 47, dilution always led to a shift of the 5.5-5.6S

peak (Figure 47A) towards the 4.6S marker (Figure 47B) suggesting an effect of dilution on the 4S⟶5S equilibrium. However, as previously suggested, the 5S form may be still intact on dilution and releases E_2 only as a result of lack of protein environment. This could be checked by post-labelling the gradients. The possibility of a 'pulling out' effect of E_2 by the cellulose nitrate tube should also be considered and future experiments should be conducted with protein coated tubes.

4.2.3.2 Transformation of Human Breast Tumour and Normal Endometrial Cytosol Oestrogen Receptor

The simple view that loss of 4S⟶5S transformation in a ER_c^+/ER_n^- tumour may explain the defective receptor, was not readily upheld and these studies are relatively incomplete.

It was found that the 4S⟶5S transformation just described for immature rat uterine receptor (Section 4.2.3.1) cannot be demonstrated in either ER_c^+/ER_n^+ (Figures 48.1 and 48.2) or in ER_c^+/ER_n^- (data not shown) breast tumour cytosol. The absence of 4S⟶5S is not confined to cancerous tissue. Normal human endometrial samples also fail to show such a transition (Figures 57 and 58).

In breast tumour cytosol, there was generally a loss of receptor binding after activation conditions (30^o, 30') but the extent of this varied from experiment to experiment. In contrast human endometrial samples generally showed a rise in total specific binding after activation, implying exchange of endogenously bound oestradiol (or other metabolites).

Kute et al. (1978) and Fazekas and MacFarlane (1980) reported

a similar lack of 4S⟶5S transformation in human breast cancer cytosol. A similar result was found for lactating mammary gland receptor (Park and Wittliff, 1977). Nevertheless, Notides et al. (1976) had previously reported that human myometrial receptor, which under identical conditions fails to show 4S⟶5S change, does undergo transformation but only in the presence of DFP, lower ionic strength (0.15M compared to 0.4M KCl usually used) and elevated temperature during centrifugation. They suggested that the equilibrium between 4S⟶5S is different for the human oestrogen receptor than for the rat receptor.

The methodology of Notides et al. (1976) was therefore adopted for some of the present experiments with the exception that the ammonium sulphate precipitation step was omitted. This latter step was avoided because (1) other subunit(s) which may be involved in activation/transformation might be eliminated (2) some results have already provided evidence that ammonium sulphate precipitation may affect the acceptor recognizing ability of the receptor (Feit and Muldoon, 1983).

The effect of elevated centrifugation temperature (20°C) on 4S⟶5S change was studied, first without the inclusion of protease inhibitors. Human breast tumour cytosol receptor showed a slight increase in its sedimentation value from 4S to 4.6S (co-sedimenting with the BSA marker) (Figures 49A and B). However this shift was found to be very variable and seemed to depend to an extent on receptor concentration (Table 14). A small conformational change or association of certain factor(s) to the 4S receptor cannot be ignored but the 4.6 to 4.8S seemed to be the maximum value obtained under these conditions. The sedimentation profile of ^{14}C-labelled markers were not affected by ionic strnegth, an observation in agreement with others (Katzenellenbogen et al., 1980; Ledden et al., 1981). The immature rat uterine cytosol

receptor failed to sustain its 5S form under these conditions and there was either a loss of oestradiol, or the appearance of both 4.6S and aggregated forms(Figures 49C andD and Figure 50A). The 4.6 - 4.8S peak was also found in human endometrial cytosols (Figure 59A).

It is believed that the 4.6S - 4.8S receptor represents the proteolyzed receptor which failed to retain its aggregation property. There was a complete recovery of counts on the gradient. The presence of aggregation potential of receptor has been shown to be correlated with its DNA binding ability (Rochefort and Baulieu, 1971; Lukola et al., 1981). Another possibility which must be considered is that we may simply be observing the 4.6S as a result of loss of other subunit(s) required for aggregation either to the 5-6S state or to the bottom of the tube.

Using DFP, a potent serine protease inhibitor, receptor sedimenting in the 6S region can clearly be demonstrated (Figure 51A). In the absence of DFP this value fell to 4.6S. It is further shown (Figure 51A) that the 6S form undergoes aggregation either as a result of self-aggregation or aggregation with other molecules. This process, however, most probably arises during the earlier part of centrifugation. A similar result was found for human endometrial receptor using either DFP or leupeptin (Figure 59B and 60A). Bailly et al. (1980) have previously shown that immature rat uterine 5S form also undergoes further aggregation on heating, and a similar effect was observed here in Figure 50A and B. DFP however, failed to conserve the 5S form of rat receptor (Figure 50B). Leupeptin was found to preserve the 5S form in rat cytosol in a similar fashion to the 6S form in human breast tissue (Notides, personal communication). This is suggestive of a heterogeneity in enzymes responsible for receptor metabolism. Use of DFP

or leupeptin alone was found to be ineffective in some tumour and endometrial cytosols. Heterogeneity of proteases, affecting receptor probably occurs in both tissues of different species and within the same individual or even tissue (Hazato and Murayama, 1981; Gregory and Notides, 1982).

Mixing human breast tumour cytosol with immature rat uterine cytosol was found to abolish the aggregation potential of rat receptor (data not shown). This not only prevented 4S $\longrightarrow$ 5S change but also inhibited the DNA binding potential (also see Sato et al., 1981a, b).

It is interesting that experiments (conducted at 20^{o}C) which show loss of aggregation potential of receptor still leave the oestradiol binding site intact. In such cases the receptor is neither degraded nor the oestradiol dissociated. This most probably is a result of an early separation of proteolytic components (or protease required factors) on the gradients (Charreau and Baldi, 1977). The oestradiol-receptor interaction is most probably sustained by hydrophobic interactions since such interactions are known to increase with temperature (Notides et al., 1976).

The formation of 6S complex in human tissues was found to be sensitive to ionic interaction (Figures 51 & and 60). Increase in ionic strength from 0.15M to 0.4M KCl in gradients resulted in a single peak at around 4.6S. This was found to be the case irrespective of the presence of DFP. This suggests that the formation of 6S complex is a result of interaction of other subunit(s) with the 4S receptor through ionic bonds. The fact that immature rat uterine 5S receptor can be observed in 0.4M KCl gradients (at 4^{o}C) suggests that bonds other than ionic bonds are involved (Notides et al.,1976).

It was further observed that the formation of 6S complex in human breast tumour cytosol was dependent both on the concentration of receptor and its molecular form (Table 15 and Figure 52). The presence of both 4S and 8S low salt profile resulted in a 4S + 6S peak when centrifuged through 0.15M KCl gradients at 20°C. If the concentration of 4S and 6S were such that these could be resolved then a profile, as shown in Figure 52A, was seen. Otherwise a very broad peak extending from 4.6 to 6S was noticed (Table 15 and Figure 55C). The results from human endometrial cytosol showed a lesser degree of dependence on receptor concentration for the formation of 6S complex. This most probably is a result of the initial presence of only the 8S form in the cytosol, in the presence of DFP (results not shown) thus, it seems likely that the receptor which forms an 8S complex in low salt yields the 6S form in the presence of 0.15M KCl.

The possibility that 6S form may represent the active form of oestrogen receptor in human breast cancer tissue was initially suggested by Wittliff et al. (1976). However they were unable to demonstrate such a form after heat activation (Wittliff et al., 1978). The possibility that such a form does exist at elevated temperature is demonstrated in present results (Figure 51). It was later found that the 6S form can also be demonstrated at 4°C, as was reported by Wittliff et al. (1976) (Figure 54). The formation of 6S complex most probably results from dissociation of inhibitor(s) during the early part of centrifugation and possibly allowing receptor dimerization. This explains why DNA binding cannot be observed at 4°C in a test tube assay. The reason why Notides et al. (1976) may have failed to see the higher molecular form receptor in human myometrial tissue at 4°C could have been due to either (a) different reaction mechanisms in different

tissues or (b) due to their use of ammonium sulphate which would then alter the 4S⟶5S transition by altering the structure of receptor or removal of some other components which would promote formation of 5S receptor.

The results presented in Figure 51 - Figure 56 help to explain why investigators have failed to observe both the 6S form after heat activation and DNA binding, even in the presence of DFP. (The results Section 3.2.3.2.6 and 3.2.3.2.7 should be consulted for explanation of Figures 53-56). Most investigators failed to see 6S or DNA binding due to proteolysis since such work was generally done in the absence of protease inhibitors (Kute et al., 1978; Sato et al., 1981a). Proteolysis may also explain why in other systems a lower in vitro DNA binding value is recorded or different molecular forms observed (discussed in Section 4.2.1.2). In the presence of DFP some investigators still find a low DNA binding value (Lukola and Punnonen, 1982; present results e.g. Figure 39). This could be explained by some of the results observed on SDGA that although DFP prevented proteolysis, aggregation was still taking place (Figure 51). This aggregation may mask the DNA binding site. However, in some analysis, the DFP effect was overridden and both proteolysis and some aggregation occurred.

One tumour was found to show the 6S form after heat activation (Figure 56) in the presence of DFP and centrifugation at $4^{o}C$ in 0.15M KCl gradient. However, this tumour was homogenized in HD rather than the usual $HDK._{15}$. It remains to be answered whether this result is significant. Homogenization in KCl may extract or activate certain proteins which the low salt buffer may not. It was previously noted (Figure 22) that extraction of tumours with HED and subsequent analysis

on HEDK.$_{15}$ at 4°C yielded only a 4S peak. EDTA may have prevented the 4S→6S transition. (This result is in variance with those presented by Wittliff et al. (1976), who showed that low salt 8S containing tumours formed a 6S peak under these conditions - Wittliff et al. were however, using Tris in their assay which has been shown to accelerate activation (Notides, 1978)).

Before analyzing the significance of 6S form so detected, two further results which were obtained with human endometrium must be discussed. First it is seen in Figures 61.1 and 61.2 that sodium molybdate on its own failed to prevent the proteolysis of receptor to the 4S form. Sodium molybdate has also been suggested to function as a protease inhibitor (Chong and Lippman 1981-82). However, at 20°C centrifugation it fails to function in a similar manner to DFP. It was expected that sodium molybdate might combine with protease(s), separate it on gradient and then allow 4S→6S transition. Alternatively, once the 6S form is formed it may prevent degradation. However, this did not take place. Not all proteases are protected by sodium molybdate (Hazato and Murayama, 1981). Sodium molybdate has been found to be less effective with increasing ionic strength (Moncharmont et al., 1982). There is also the possibility that in these tissues receptor existed as 4S initially due to prior degradation, though endometrium was usually used fresh. Finally the effect of DCC was tested on receptor transformation (data not shown). It was found that analysis either before or after DCC treatment yielded the similar 6S peak. DCC therefore did not alter the 6S pattern seen in the absence of any DCC treatment suggesting that DCC was not absorbing any necessary additional proteins.

The results presented in the literature indicate that the human nuclear receptor sediments at 4S (Geier et al., 1979; Katzenellenbogen

et al., 1980) but these experiments involved 0.4 - 0.6M KCl extraction and gradient analysis. Results shown in Figure 51 would argue that either only one subunit is being extracted or that the transformed form extracted is dissociating during centrifugation in 0.4M KCl. Another possibility which must be considered is that KCl extraction may release certain proteases from the chromatin which could result in the proteolysis of the 6S form. Serine proteases are demonstrated on the rat liver chromatin (Tsurugi and Ogata, 1982).

A recent report by Linkie (1981-82) has shown that transformed receptor can be extracted from human endometrial nuclei. However, the procedure used by Linkie for extraction and assay, is not clear. It seems that Linkie used glycerol during analysis which is known to prevent aggregation of receptor to those components which sediment to the bottom of the tube. It may be significant that micrococcal nuclease digestion of chromatin-bound receptor showed a 6S complex (note no KCl was used) (Andre et al., 1978; Rochefort and Andre, 1978). Progesterone receptor only initiates transcription when present in the 6S state and glucocorticoid receptor shows a possible dimer on electronmicroscopical examination of DNA bound protein. Eckert and Katzenellenbogen (1982) have recently detected a higher molecular weight nuclear antioestrogen-receptor complex from MCF-7 breast cancer cell nuclei. The high molecular weight form is not seen with oestrogen-receptor complex. It may be that antioestrogen helps the receptor to retain the second subunit with greater affinity. This would allow a greater retention time of receptor on the chromatin, perhaps preventing recycling and other oestrogenic effects. The importance of 6S form should not be disregarded at present and further research must be directed to optimise conditions for its

detection. In vitro transcription work using isolated 4S, 6S and 8S forms would be instructive.

4.2.4. **CONCLUSIONS**

(a) Several factors were shown to influence the human tumour cytosol oestrogen receptor in low salt gradients. Some of these factors could be clinically significant and lead to misinterpretation of data. It is therefore essential to establish interlaboratory quality control with respect to molecular forms, if this criteria is to be used for clinical work. At present, however, the gradient method is more time consuming and uneconomical than the simple measurement of both soluble and nuclear receptor measurements in relation to determining patient response. The problem of the amount of sample available is increasing, probably as a result of a trend towards an earlier detection of disease. Gradient analysis requires at least 400-500mg tissue. However, the recent application of high pressure liquid chromatography may circumvent these problems. In any case if the measurement of molecular forms is to be pursued for clinical application a set criteria must be established.

(b) In contrast to the earlier reported results in the literature, it was shown that a human breast tumour 6S oestrogen receptor complex can exist at elevated temperatures (20°C) in 0.15M KCl gradients. This may be analogeous to the 5S form in that the human 6S complex may bind to the DNA. The presence of 6S complex was dependent on inclusion of DFP or leupeptin in the cytosol and on the presence of a predominant 8S complex in low salt. Previous reports have most probably measured the proteolyzed 8S complex as 4S on high salt gradients. The present studies have only suggested, and not proved that the 6S form may be the DNA binding form.

4.3. **A Proposed Model for Steroid Hormone Action**

With the wealth of contradictory data in the literature, it

is difficult to propose a single model for steroid hormone action, that will not be subject to criticism. Nevertheless, combining the results presented in the literature with those presented in this thesis, the following simplified model is presented. This may be valid for both the rat and human tissue.

In vivo, oestradiol enters the cell by passive diffusion and binds to its specific receptor. The empty receptor is most probably located in the localized areas of the soluble fraction, that is, is not freely available in the cytoplasm (Figure 62A). It may be present attached to membranes, cytoskeleton or as high molecular weight aggregates. This process would prevent not only proteolysis but also the movement of receptor onto the chromatin. The in vitro 8S formation may be a manifestation of receptor aggregation potential. Binding of ligand by the receptor would allow it to detach itself and be available throughout the free water content of the cell. This process would allow receptor activation to take place either in the cytoplasm or in the nucleus.

The process of activation in vitro (Figure 62B) of the soluble receptor (in the absence of proteases) would have to take into account the 8S⟶4S equilibrium. The 8S in itself most probably represents an inhibitory complex of the receptor. It will only be the available 4S receptor which is able to undergo activation to the 5S (or 6S) form. This is based on observations that processes which lead to disruption of 8S complex increase the rate of activation. Conversely, compounds which stabilize the 8S complex prevent activation and 4S⟶5S formation. In vitro, the release of proteases from tissues (abundant in human tissue) can modify the process of activation and the process would then depend on intact 4S receptor available (or other proteins required to bind to

Figure 62. **A proposed model for oestrogen receptor interaction in vivo (A) and the relationship between various molecular forms in vitro (B).**

* = The S values given are those accepted in the literature to represent different molecular species. These are not to be taken as absolute values.

E_2 = oestradiol

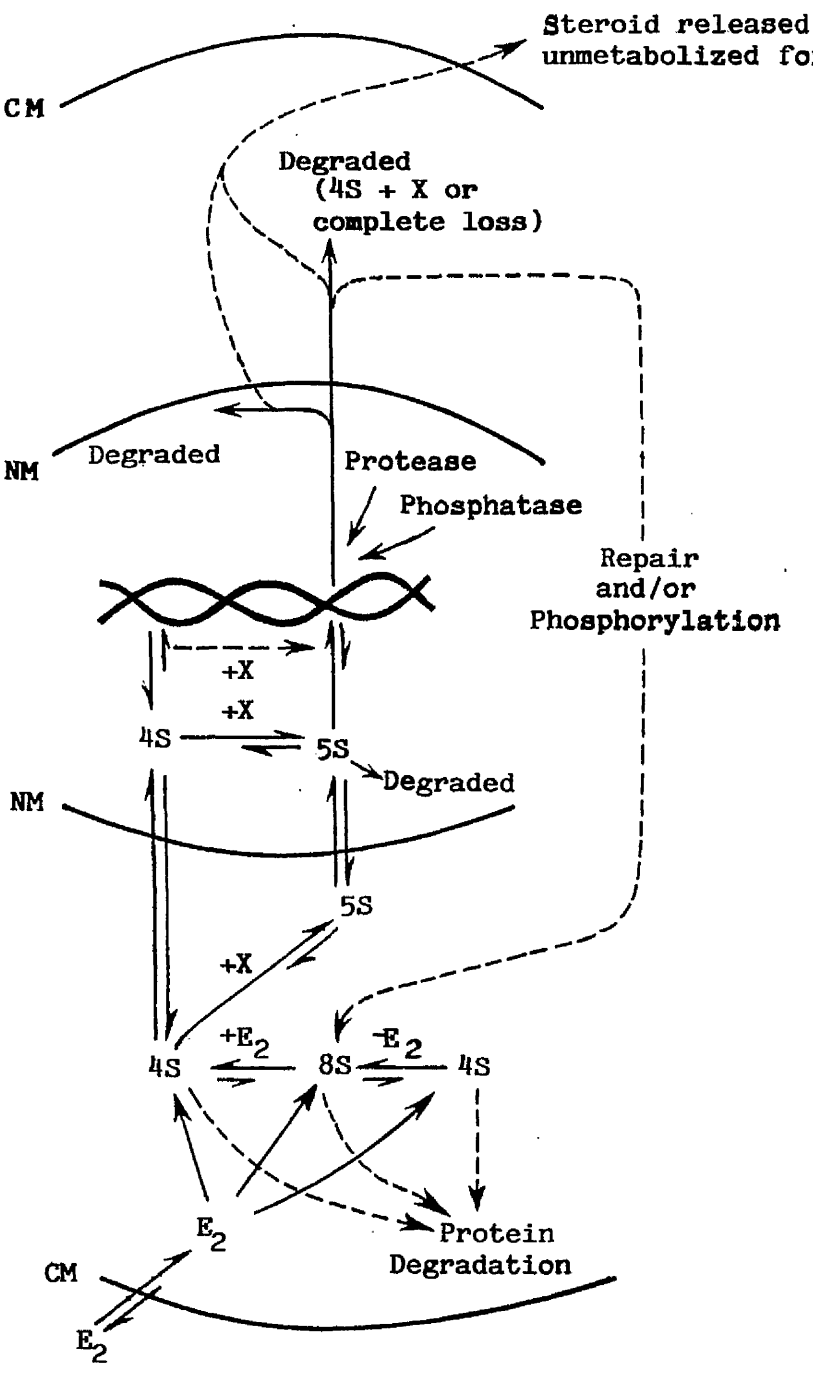
X = a second subunit(s) required for the binding of oestrogen receptor to DNA.

CM = Cell membrane

NM = Nuclear membrane

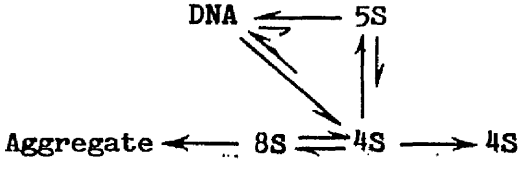
 = DNA

A. In vivo state*



B. In vitro state

Soluble receptor



the 4S receptor). At present therefore, it seems that most confusion in literature relating activation with transformation and/or DNA binding ability stems from artifactual proteolysis. The proteolysis of receptor *in vivo* may however hold physiological significance, as discussed below. Another parameter influencing receptor activation seems to be the great tendency of the intact receptor to aggregate. This may mask the DNA binding site of the receptor.

The *in vivo* binding of receptor to chromatin should be saturable, at least to the specific sites. The gene sequences to be transcribed may possess certain specific regions in or around the start of transcription region. Receptor may also be involved in binding to other structures in the nucleus (including the nuclear matrix).

It is more difficult to suggest how the receptor is released from the nucleus (Figure 62A). It is possible that several mechanisms are involved in this process depending on whether a receptor protein molecule is to be preserved or degraded. Dephosphorylation of receptor is indicated as one such mechanisms. However, a dephosphorylation mechanisms explains release of oestradiol from the nucleus, but the fate of receptor is still unclear. More interestingly, specific proteases are found in certain target tissue nuclei which have been shown to have a very strong K_m for the receptor. It will be interesting if these proteases are involved in not only regulating the activated receptor but also in their final degradation.

It is yet to be established what is the nuclear molecular form of oestrogen receptor in the human tissue. Salt releases the 5S form of

receptor from immature rat uterine nuclei but only the 4S form from nuclei obtained from human tissues. It is not possible to conclude from these results that 4S represents the nuclear form in human tissues since the interaction of 4S receptor with other components may be relatively weak. Some other procedures of isolation have indicated higher molecular form nuclear receptor. The problem therefore remains unresolved.

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