Oestrogen Receptors and the functionality of the Receptor Mediated Pathway in Human Breast Cancer

Brian Parmar BSc. (Hons)

Institute of Obstetrics and Gynaecology,
Hammersmith Hospital,
Du Cane Road,
London, W12 0HS.

Submitted for the degree of PhD in the Faculty of Medicine at the University of London,
June, 1985.

A B S T R A C T

The presence of steroid-receptors in human breast cancer may indicate their hormonal sensitivity. However, the presence of oestrogen receptors does not guarantee the functionality of the receptor mediated pathway. This has been assessed by the co-measurement of cytosol and nuclear oestrogen, and cytosol progesterone receptors. The distribution of oestrogen and progesterone receptors in primary and metastatic human breast cancers are evaluated in relation to the endocrine status of the patients.

Human breast cancer cytosol oestrogen receptor possesses an oligonucleotide binding domain distinct from the steroid binding site, which is assessed by its ability to bind to oligo(dT)-cellulose (activated receptor). A functional significance of the presence of activated oestrogen receptor is suggested by its association with the presence of cytosol progesterone receptor, as well as its association with a better response rate to endocrine therapy and a favourable prognosis in patients with breast cancer.

The higher incidence of activated oestrogen receptor and progesterone receptor in grade II tumours compared to grade III tumours suggests a correlation with the state of differentiation.

Furthermore, the relationship between the molecular form of the cytosol oestrogen receptor and its degree of activation has been evaluated to elucidate the mechanism of steroid hormone receptor interaction in human breast cancer. Breast tumour cytosol oestrogen receptors existed as a form with an apparent molecular weight of 300K alone or together with a form of apparent molecular weight 60K in low ionic strength buffer. The proportion of activated receptors was significantly higher in tumours containing both 300K and 60K forms of the receptor. Manipulations of cytosol which promoted oestrogen receptor activation were associated with with an increase in the 60K form with a corresponding decrease in the 300K species. The presence of sodium molybdate inhibited receptor activation and stabilised the receptor in the 300K form.

Activation of human breast cancer cytosol oestrogen receptor is apparently associated with the generation of the 60K receptor species, probably by disaggregation of the 300K form. A functional significance of the presence of the 60K receptor may be suggested by the small but significant increase in the cytosol progesterone receptor content of tumours containing both 300K and 60K oestrogen receptor.

TO

MY PARENTS

Acknowledgements

I am indebted to Dr. John White for his scientific acumen, friendship and encouragement during the course of the study. I wish to thank Professor M. G. Elder and Dr. Leslie Myatt for their support and direction. I am extremely grateful for the many scientific and other discussions with Tim Scane, Julian Guest, Matthew Rose, Dave Glance, Katherine Phillipson and Peter Moore. Finally, the moral support of Rudra Desai has been tremendous.

List of Abbreviations

CER	Cytosol oestrogen receptor
CPR	Cytosol progesterone receptor
NER4	'Unoccupied' nuclear oestrogen receptor
NER30	'Total' nuclear oestrogen receptor
DES	Diethylstilboestrol
EDTA	Ethylenediaminetetra-acetic acid
PED	10mM phosphate/1.5mM EDTA/1mM DTT/pH 7.4
PEDG	10mM phosphate/1.5mM EDTA/1mM DTT/30% glycerol/pH 7.4
DCC	Dextran coated charcoal
DNA	Deoxyribonucleic acid
BSA	Bovine serum albumin
dT	Deoxythmidylate
dA	Deoxyadenylate
dC	Deoxycytosine
dG	Deoxyguanosine
dI	Deoxyinosine
DT	oligo(dT)-cellulose binding of cytosol oestrogen receptors (i.e. activated receptor)

PREFACE

Reasons for this Study

Interaction of the cytoplasmic oestrogen receptor with nuclear components is neccessary to regulate gene expression and evoke a complete cellular response (Clark & Peck, 1979). The presence of cytosol oestrogen receptors alone in tumour tissue therefore does not guarantee the functionality of the receptor pathway of oestrogen action or of cellular responsivity.

The increase in ability of the cytoplasmic steroid-hormone receptor complexes to interact with nuclear components is defined as 'activation'. Activation can be assessed *in vitro* by the ability of the cytoplasmic steroid-hormone receptor complexes to bind to artificial nuclear matrices such as DNA- or oligo(dT)- cellulose (Yamamoto & Alberts, 1975; Thrower et al., 1976; Park & Wittliff, 1977; White et al., 1981; Myatt et al., 1982a). The objectives of this study were to:

(1): investigate the ability of human breast tumour cytosol oestrogen receptor to bind to oligo(dT)-cellulose and to define the factors that regulate this process,

(2): compare the ability of the cytoplasmic oestrogen receptor to bind to oligo(dT)-cellulose (i.e. the activated oestrogen receptor) with the presence of nuclear oestrogen and cytoplasmic progesterone receptors (both indices of a functional oestrogen receptor pathway) to evaluate whether the presence of the activated oestrogen receptor is a determinant of the functionality of the oestrogen receptor pathway,and

(3): analyse the molecular forms of the human breast tumour cytoplasmic oestrogen receptor in relation to its ability to bind to oligo(dT)-cellulose in order to determine the mechanisms underlying receptor activation.

C O N T E N T S

FIGURES

TABLES

CHAPTER ONE

INTRODUCTION

A: Cancer Biology

Definition

Cancer is a term often used synonymously with the terms neoplasm or tumour to signify malignant growth. A slightly modified version of the definition proposed by Ewing (1940) is that 'a neoplasm is a disturbance of growth characterised primarily by an excessive proliferation of cells without apparent relation to the physiological demands of the organ involved'. However, a variety of neoplasms arise from all types of human tissues and they show marked variations in biological behaviour. It is therefore difficult to formulate a simple definition of 'cancer' that is appropriate for all neoplasms.

Willis' (1960) definition of "a neoplasm as an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of normal tissues and persists in the same manner after cessation of stimuli that evoked the change", helps distinguish neoplasms from other malformations such as inflammatory and hyperplastic lesions.

Classification and Nomenclature

Neoplasms are classically divided into two main groups based on the biological behaviour of the tumours. Benign neoplasms have limited growth potential, remain localised and encapsulated, are highly differentiated and do not metastasise. A benign tumour of surface epithelium is usually referred to as a papilloma, whereas one of glandular epithelium is an adenoma. In connective tissue, benign

tumours are named after their cells of origin, for example, fibroma and lipoma are benign tumours arising in fibrous tissue and adipose tissue respectively. In some benign tumours, both epithelial and connective tissue elements are involved in the neoplastic proliferation, for example fibroadenoma of the breast.

In contrast, malignant neoplasms proliferate rapidly, are poorly differentiated and spread throughout the body so that if they are not treated early, they invariably cause death. The basic characteristic of malignant neoplasia is an abnormality in cells that is caused by reduced control over growth and function. This may lead to serious adverse effects on the patient as a result of invasive growth and metastases. The cellular proliferation in malignant tumours is not completely autonomous. In addition to the dependence of the tumour on the host for its blood supply (Brem & Folkman, 1975), its growth may be affected by hormones (Muhlbock, 1972), drugs (Mortimer & Lipsett, 1979) and the patient's immunological mechanisms.

Malignant tumours are described by the terms carcinoma (epithelial in origin) or sarcoma (connective tissue in origin), and usually qualified by a prefix or an adjective describing the specific tissue or site of origin; an adenocarcinoma of the breast is a malignant neoplasm of glandular epithelium and a fibrosarcoma is a cancer arising in the mesodermal fibrous tissue. If a tumour is metastatic from another tissue, it is described, for example, as an adenocarcinoma of the breast metastatic to liver.

The histological classification of tumours defines the growth patterns and ability of the tumours to metastasise and hence determine prognosis. The histological appearance of neoplastic proliferation may follow progressive changes from a normal to a cancerous state (figure

1). However, these changes are not applicable to all sites and although in-situ carcinoma is found in the uterine cervix, prostate, breast, bronchus and alimentary tract, objective interpretation is difficult.

Staging

Staging is a measure of the extent of disease at the time of presentation. It may be either based on clinical examination together with biochemical, radiographic and immunologic investigations, or pathologically - based on histologically proven disease in biopsy specimens. Accurate surgical and pathological information is essential for detailed classification of the disease and in deciding treatment and predicting prognosis. A standardised classification system for describing the extent of tumour progression has been devised by two major agencies - the Union Internationale Contre le Cancer, UICC (1978), and the American Joint Committee for Cancer Staging, AJCSS (1979) using the tumour, node, metastases (TNM) system.

In this clasification, the T categories define the size of the tumour, N describes the extent of involvement of regional lymph nodes and M describes the presence or absence of distant metastases. The TNM system is used for all cancers, although the exact criteria used vary with each organ site. The classification provides a standard reference for comparing the efficacy of different treatment regimens. The TNM system applied to breast cancer is modified to include stage groupings (UICC, 1978; AJCCS, 1979) and does have some therapeutic and prognostic importance.

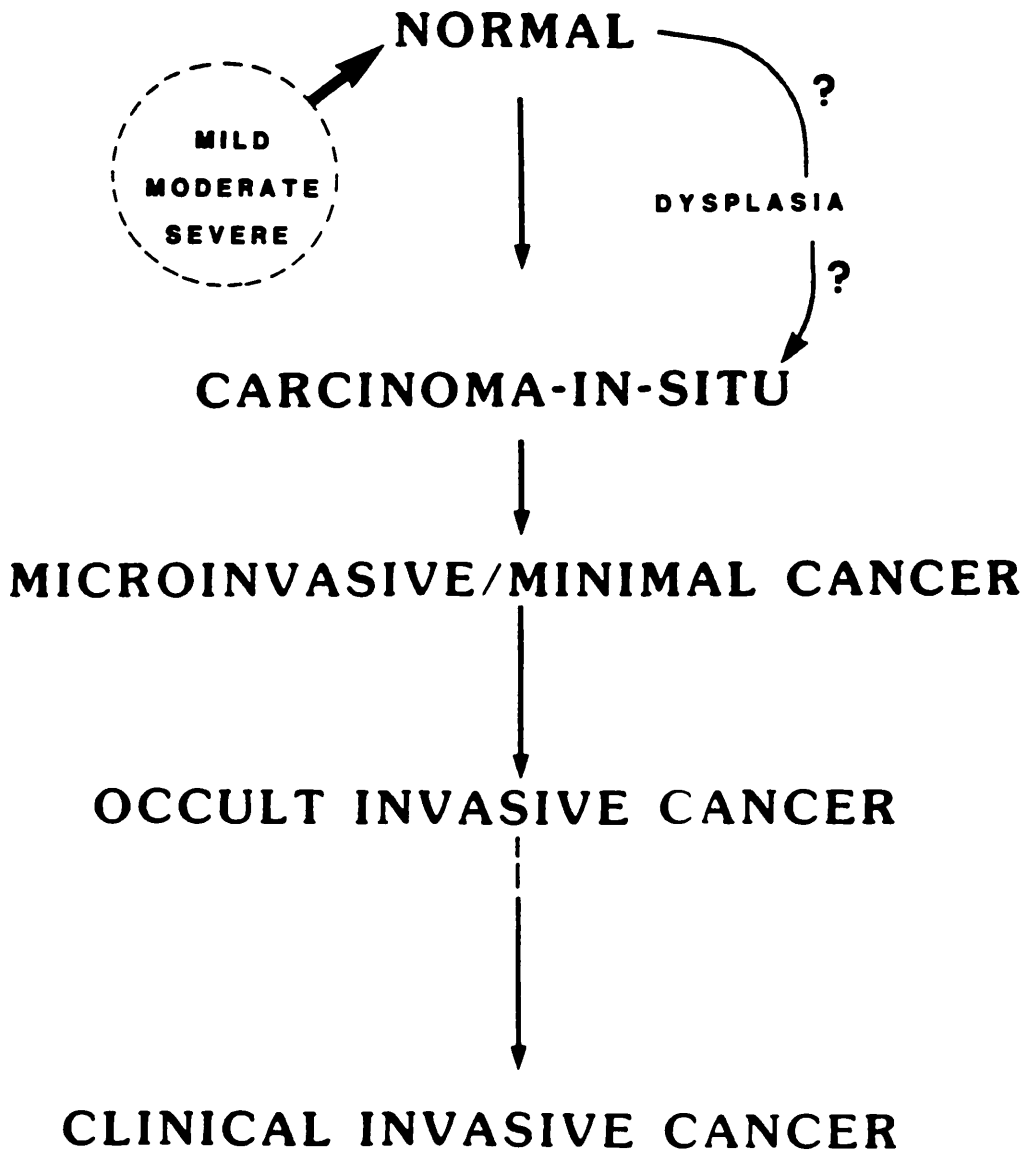


Fig. 1. Pathway of histological progression from a normal to invasive cancer.

Cellular Proliferation in Cancer

Malignant neoplasms develop from cells of normal tissues of the body that are capable of dividing or cells that have acquired this ability. The normal process of cellular differentiation leads to a fully differentiated state that is incapable of dividing again. Under some circumstances that are not fully understood, cells are transformed by interaction with carcinogens into a cell type that is capable of continued proliferation which does not reach the normal state of complete differentiation. Terminally differentiated cells may achieve this as a result of dedifferentiation (Uriel, 1976). This process is called malignant transformation. The genes controlling cell proliferation are somehow permanently switched 'on' when they should be 'off', and the genes controlling differentiation are either expressed partially or not expressed at all. Sachs (1980) proposed that an alteration from inducible to constitutive expression of genes in specific pathways required for growth results in malignant transformation. These transformed cells no longer require the physiological inducer for growth (i.e., they become autonomous) and thus growth is uncoupled from differentiation. Constitutive expression of other specific pathways, such as glucose utilisation and/or foetal proteins, then causes blocks in differentiation and results in further development of malignancy.

Carcinogenesis

The model of carcinogenesis has evolved from the early concept of a single mutational event, as suggested by Boveri in 1914, and has progressed through a 'two-stage' initiation and promotion model (Berenblum, 1941; Friedwald & Rous, 1944) to a multi-stage process

(Wolman & Horland, 1975) (Fig:2). The latter models provide a reasonable explanation of the long latency phenomenon of carcinogenesis. The initiation and promotion stages of carcinogenesis have been demonstrated in the mouse skin (Berenblum & Shubik, 1947), the mammary gland, thyroid and urinary bladder and in cell-culture systems (Miller, 1978).

Initiators and Promoters

An initiating agent is a chemical, physical or biological agent capable of directly and irreversibly altering the native molecular structure of DNA in the cell. The initiation phase requires only a brief exposure to a potent initiating agent (oncogenic viruses, radiation or chemical carcinogens)(figure 2) and it must be heritable so that the initiated cell can propagate the malignant alteration to its progeny (Pitot, 1981).

A promoting agent is one that alters the expression of genetic information of the cell. Examples of such agents include hormones and drugs which in themselves do not alter the molecular structure of the genome but affect its expression (figure 2). These agents may affect the hormonal milieu by stimulation or inhibition of secretion, altered rates and routes of metabolism, and changes in receptor mechanisms (Mortimer & Lipsett, 1979). The promotion phase is a gradual process and requires a prolonged exposure to the promoting agent (Boutwell, 1974; Berenblum, 1978). This phase occupies the greater part of the latent period of carcinogenesis, and is partially reversible (figure 2).

Tumour promotion can be divided into at least two phases (figure 2). First, the cell proliferation phase which propagates the initiated damage and leads to the appearance of a mass of malignant cells. This

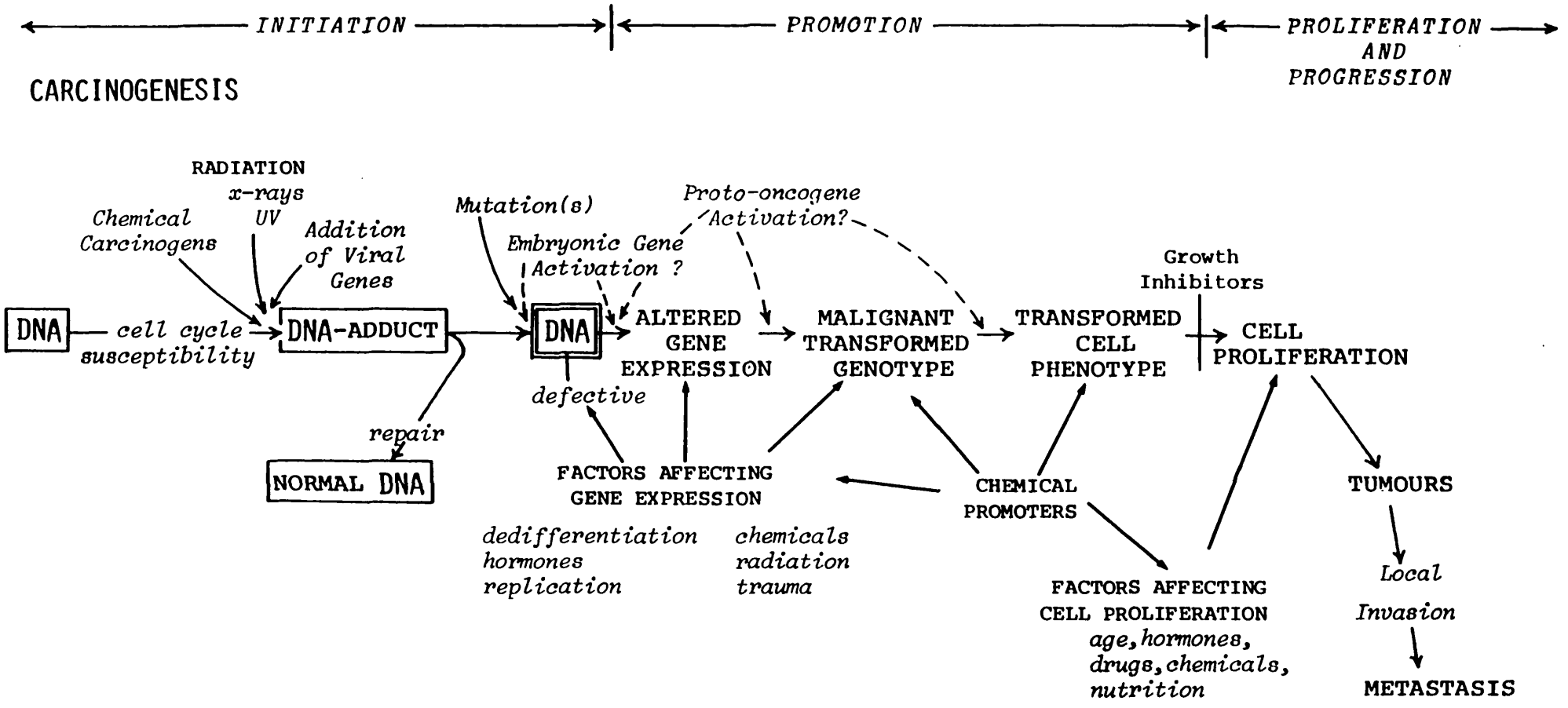


Fig. 2:Schematic representation depicting the interrelationships in Stages of Carcinogenesis.

phase is partially reversible. Most promoting agents are mitogens for the tissue in which they promote tumour growth, for example, oestrogens in the mammary gland and endometrium. The second phase is tumour progression. This phase requires continued proliferation of altered cells during which loss of growth control (Sachs, 1980) and an escape from host defence mechanisms becomes the predominant characteristic. This allows growth to progress to a clinically detectable tumour.

Theories of Carcinogenesis

Numerous hypotheses have been advanced to explain the molecular basis of carcinogenesis. All the various mechanisms postulated involve an alteration in gene expression either by a direct alteration of DNA, or by activation and repression of normal genes without alteration of DNA. Malignant changes can be initiated by somatic mutational events (Boveri, 1914; Kauffman, 1971), expression of viral oncogenes (Bittner, 1936; Gross, 1978; Temin, 1976; Huebner & Todaro, 1969), specific chromosomal changes (Rowley, 1977; Klein, 1979; van der Riet-Fox et al., 1980; Sandberg, 1973), gene derepression (Neuwald et al., 1980; Rosen et al., 1980) or a combination of these events.

Two general hypotheses of carcinogenesis can be developed from these numerous ideas. One is that malignant transformation occurs as a result of changes in genetic structure related to the somatic mutation concept, which advocates biochemical lesions in the structure of DNA as the major single cause of neoplastic transformation (Boveri, 1914; Burnet, 1978; Straus, 1981). This can involve addition of new genetic material (such as viral genes), deletion of genes, changing them by point mutations or chromosomal changes. The second hypothesis is based on the epigenetic theory or developmental changes and involves

alteration in genetic expression or derepression of embryonic genes without a direct alteration of DNA (Busch, 1976; Rubin, 1980; Temin, 1971). A more recent hypothesis postulates that all normal cells contain homologues of viral oncogenes which can be activated by physical, biological or chemical carcinogens resulting in neoplastic transformation

Gene Alteration Hypothesis

A major line of evidence for the occurrence of somatic mutations in neoplasia is the appearance of chromosomal abnormalities in many tumours (Mulvihill et al., 1977). Studies by Fialkow (1972) revealed that tumours arising in females heterozygous at the X-linked glucose-6-phosphate dehydrogenase (G6PDH) locus express only one allele, whereas normal tissues in the same female are mosaic for the alleles. Somatic mutations also play an important role in some childhood cancers (Knudson, 1973, Knudson et al., 1976). A chromosomal aberration occurs in chronic myeloid leukemia in which the Philadelphia chromosome results from a deleted chromosome 22 translocated to another chromosome, usually number 9 (Rowley, 1973; Mark, 1977; Festa, 1979). Specific chromosomal changes have also been described with high frequency in meningiomas (Mark, 1974), Burkitt's lymphoma (Zech et al.,1976) and acute leukemias (Rowley & Potter, 1976). These changes are only seen in neoplastic cells.

Integration of oncogenic viruses with the host cell genome can also induce cancer (Varmus et al., 1973; Doerfler, 1968; Huebner & Todaro, 1969; Klein, 1972). Of the two general classes of oncogenic viruses, the DNA viruses become directly incorporated into the host cell genome (Black, 1968), and the RNA viruses become incorporated after

formation of a DNA provirus by reverse transcription (Temin, 1974). In these cases, carcinogenesis occurs as a result of added viral oncogene as opposed to the mutationally altered genome. The viral aetiology of cancer has been demonstrated in mouse mammary carcinoma (Bittner, 1936), leukemias (Gross, 1974) and a variety of other neoplasias in animals (Tooze, 1973). However, the role of viruses has been demonstrated in only a few human cancers (Heath et al., 1975); cancer of the uterine cervix (Rawls et al., 1969), Burkitt's lymphoma (Burkitt, 1963), nasopharyngeal cancer in the Chinese (Kaschka-Dierich et al., 1976; Simons et al., 1976) and Hodgkin's disease in early life (Correa & O'Connor, 1971).

Gene Activation/Repression Hypothesis

This hypothesis is based on the epigenetic model in which cancers may develop as a result of activation (and repression or derepression) of specific genes rather than by actual alteration of DNA itself. Foetal genes in the adult organism are normally repressed, but there is evidence that these genes may be activated in neoplasia (Bush, 1976), and the products of foetal gene expression may interact with the genome to accelerate growth and invasiveness. The most convincing evidence for the epigenetic basis of neoplasia comes principally from studies demonstrating that teratoma cells innoculated into normal blastulae may exhibit complete reversion to the normal phenotype on the development of the organism (Mintz & Illmensee, 1975; Illmensee & Mintz, 1976). These experiments suggest that, in teratomas at least, it is unlikely that irreversible genetic alterations or mutations have occurred, and the cancerous state is the result of activation and repression of genes by enviromental factors.

Oncogene Hypothesis

It was proposed in 1969 by Huebner & Todaro that transforming genes (onc genes) of retroviruses pre-exist in normal vertebrate cells as latent cancer genes that may be transduced by retroviruses without onc genes or activated by carcinogens. These cellular homologues of viral oncogenes are termed proto-oncogenes, or cellular-oncogenes (c-onc genes). There is evidence for such c-onc genes corresponding to several viral oncogenes (Bishop et al., 1980; Oskarsson et al., 1980; Parada et al., 1982; Bishop, 1983). In 1981, Pall proposed that the initial event in carcinogenesis is a mutation, brought about by radiation or chemical carcinogens, that produces a single tandem-duplication of a c-onc gene; this would result in a doubling of the gene product, but is insufficient to transform the cell. The altered cells are also proposed to undergo infrequent unequal sister-chromatid crossing-over, generating a chromatid with three tandem c-onc genes and one with only one. Amplification of the c-onc gene may occur in this way after many subsequent rounds of DNA replication and cell division; additional unequal sister-chromatid exchanges may occur generating still higher duplications until the minimal amount of gene product is synthesised to transform the cell. This model provides a resonable explanation for the long latency of carcinogenesis because a considerable amount of time would elapse before the appearance of the transformed cell, since each unequal crossing-over would be relatively rare and each must occur in a different cell cycle. It is in agreement with the 'two-stage' mechanism of carcinogenesis (Berenblum, 1941; Friedwald & Rous, 1944). Amplification and increased expression of c-myc (a cellular homologue of the avian leukemia virus) sequences have been observed in human premyelocytic leukemia (Dalla-Favera, 1982; Collins & Groudine, 1982),

a colon cancer cell line (Alitalo et al., 1983) and a mouse adenocortical cell line (Schwab et al., 1983).

An alternative hypothesis has been proposed by Klein (1981) involving translocations of c-onc genes from their normal chromosomal site to another site. As a result of this movement the expression of the c-onc gene might be changed since it would be adjacent to different genes which may be expressed at a higher level, that is, the expression of the c-onc gene at this new site is regulated differently. This would result in over-expression which could then lead to neoplastic transformation. Evidence for Klein's hypothesis has come from studies on Burkitt's lymphoma by Della-Favera et al., (1982). They demonstrated that the c-myc gene normally located on chromosome 8 is moved to the immunoglobin heavy chain locus on chromosome 14. The consequence of this movement appears to be that the c-myc gene in Burkitt's lymphomas is expressed in higher amounts than in normal cells (Erikson et al., 1983). Similar observations have been made involving translocations of the c-abl gene (a cellular homologue of the murine Ableson leukemia virus) in chronic myeloid leukemia (de Klein et al., 1982) and the c-mos gene (a cellular homologue of the Moloney murine sarcoma virus) in acute myeloid leukemia (Neel et al., 1982).

It has not been formally demonstrated that movement of c-onc genes by chromosome translocations is a causative event in neoplastic transformation but it seems unlikely that so many tumour-specific chromosome translocations would involve these genes by chance.

The general models described are probably not separate or mutually exclusive, but rather one or more of the above mechanisms may be operating in concert in the genesis of the many diverse types of malignancies.

Progresion Of Neoplasia

The transition of a transformed cell from a very low degree of malignancy to a very rapidly growing, virulent neoplasm is termed progression of neoplasia. The mechanism of tumour progression is not established. It may involve selection of cell types that have acquired the ability for rapid proliferation and a faster metabolic rate. These cells become the predominant cell type, and in many instances this involves a change in karyotype (Mark, 1969). Recently, Goldenberg et al.,(1974) suggested that tumour progression may result, at least in part, from the fusion of neoplastic cells with normal cells. This would produce marked changes in karyotype, a characteristic of neoplastic progression, and may also explain how neoplastic cells could escape immunologic surveillance in the host. Kraemar et al., (1974) showed that neoplastic cells had extremely rapid growth rates and a high degree of aneuploidy. They suggested that the neoplastic cells had lost the ability to maintain a stable karyotype and become aneuploid. Neither of these theories explain tumour progression except that it appears that aneuploidy may be essential for rapid proliferation.

Metastasis

Anatomically, a number of pathways are available for metastasis. Metastatic cells can enter the vascular system, by invasion of the vessel wall, and be passively carried by the bloodstream or remain at

the site of vessel invasion where they proliferate and continue to shed emboli into the circulation (Fidler, 1978). Another common route is intravasation of the microvasculature or the lymphatic system draining the area of the tumour (figure 4), and some tumours may spread directly to the peritoneal cavity. Blood vessels within the primary tumour may also offer malignant cells a port of entry into the circulation.

The process of metastasis involves a series of sequential steps in which malignant cells are released from the primary tumour and disseminate to distant sites where they proliferate to form new tumours. It begins with the local invasion of the surrounding tissue by cells from the primary tumour (Trinkaus, 1976). This may be facilitated by tissue-digestive enzymes such as lysosomal hydrolases, collagenases and proteinases such as plasminogen activator and thiol proteinases (Roblin et al., 1975; Weiss & Poste, 1976; Strauli & Weiss, 1977; Kleinerman & Liotta, 1977; Poste, 1977; Mullins & Rohrlich, 1983). Surgical manipulations may also inadvertently assist the spread of tumour emboli (Salsbury, 1975).

After entry into the circulation, some degree of non-specific trapping of tumour cells occurs simply as a result of mechanical factors. However, these factors alone are not sufficient to explain the sites of tumour cell arrest and metastasis formation in animal and humans. In humans, certain cancers have a predisposition to metastasise to particular organs (Fidler, 1978; Regato, 1977; Tarin, 1976). Carcinoma of the breast and lung metastasise to adrenal glands (Viadana et al., 1973) and carcinoma of the prostate frequently metastasises to the bone. Experimental studies using radiolabelled tumour cells injected into the circulation have shown that circulating cells arrest in a wide variety of organs but metastases only occur in certain organs

(Fidler, 1978). Similar observations have been made by Nicholson (1978) using highly malignant murine neoplasms.

It has been suggested that once in the circulation, tumour cells may be transported singly or in clumps attached to blood platelets, red blood cells, leukocytes etc. (Warren & Vales, 1972). There is some evidence that tumour cells adhere to platelets and stimulate platelet aggregation or enhance arrest in the microvasculature (Yogeeswaran & Salk, 1981). This would facilitate the adhesion of tumour cells to the subendothelial matrix via platelet-bridges protecting the attached tumour cells from shear forces in the circulatory system and enhancing tumour cell survival due to the release of platelet derived growth factors. Platelet-tumour cell interactions may also protect against destruction by host macrophages by physical shielding of tumour cells within the emboli and increase the ability of tumour-platelet emboli to arrest in the vessel (Honn et al., 1983). The interactions between the vessel wall and tumour cells and platelets may be inhibited by exogenous prostacyclin, prostaglandin I_2 (PGI_2) (Fantone et al., 1982). Nafazotrom, which has the ability to stimulate endogenous production of PGI_2 and prevent its breakdown, appears to be an effective antimetastatic agent (Honn, 1982).

Following implantation in the vessel wall, the tumour cells exit (extravasate) from the vessel and invade the surrounding tissue possibly by mechanisms similar to those responsible for the initial invasion. The malignant cells proliferate to form small colonies called micro-metastases around the blood vessels. Further growth to form macro-metastases of clinical importance requires growth of new blood vessels (angiogenesis) to provide nutrition and support proliferation (Folkman & Greenspan, 1975; Gullino, 1978).

B: Hormones in Cancer

Hormones play an important role in the aetiology and pathogenesis of cancer in a variety of animal and human neoplasms (Beatson, 1896; Lathrop & Loeb, 1913; Lacassagne, 1948; Moon et al., 1950; Huggins et al., 1959; Segaloff & Maxfield, 1971; Muhlbock, 1972). Their role is more evident in experimental carcinomas than in human cancers (Bulbrook, 1966; Clifton & Sridharan, 1975; Carter, 1980). Hormones regulate growth and function of several human cancers; namely, cancers of the breast (Muhlbock, 1972), prostate (Huggins, 1967) and endometrium (Sex Hormones, IARC Monographs, 1979). However, the role of hormones in human carcinogenesis *per se* is yet to be conclusively demonstrated (Sex Hormones, IARC Monographs, 1979; Siiteri et al., 1980).

Hormones are important physiological factors in controlling cell division and proliferation; in this way, they play a major role in homeostatic cellular mechanisms (Takizawa et al., 1970; Lupulescu et al., 1978; Lupulescu, 1980). In normal target tissues, growth is controlled endocrinologically, however a prolonged phase of abnormally rapid growth may occur in the presence of a long-term endocrine imbalance. It has been suggested that a sustained high level of cellular proliferation is important in the promotion and progression of carcinogenesis (Furth, 1975). High proliferative activity may increase the susceptibility of the hormone-sensitive tissue(s) to carcinogenic event(s) (Clifton, 1961), or the hormones may act as promoters of the carcinogenic process (Lipsett, 1979). Berenblum (1978) has suggested that hormones may influence the process of carcinogenesis at any of the stages including initiation, promotion and progression (i.e. hormone dependent tumours) of the disease.

At the cellular level, steroid hormones enter their target cells and bind to specific proteins, termed `receptors', in the cytosol. The hormone-receptor complex then translocates to the nucleus where it modulates transcription (O'Malley & Means, 1974). The oestrogen-receptor complex in the nucleus associates with the operator loci of specific oestrogen operons that contain functional genes (cistrons) resulting in increased transcriptional activity (Sajdel & Jacob, 1971; Tsai et al., 1975). Thus, oestrogen actions generally reflect gene derepression (O'Malley et al., 1972; O'Malley & Means, 1974). Therefore, during long-term hormonal imbalance there may be excessive gene derepression which would result in abnormally rapid growth, and this may facilitate activation or derepression of other genes including the putative c-onc genes giving rise to neoplasia; probably oestrogens act in this way to promote and maintain the carinogenic process.

C: The Breast

Structure

The breast is a modified sebaceous gland which consists of 15-20 lobes, each containing many lobules interconnected by alveolar tissue, blood vessels and ducts (figure 3). When fully developed, the lobules consist of clusters of rounded alveoli (or acini) which open into the smallest branches of the milk-collecting ducts. These come together to form larger lactiferous ducts, each draining a lobe of the gland. The lactiferous ducts converge towards the areola beneath which they dilate to form lactiferous sinuses, that serve as small reservoirs of milk. After narrowing in diameter, each lactiferous sinus runs separately up through the nipple (or papilla) to open directly on the surface.

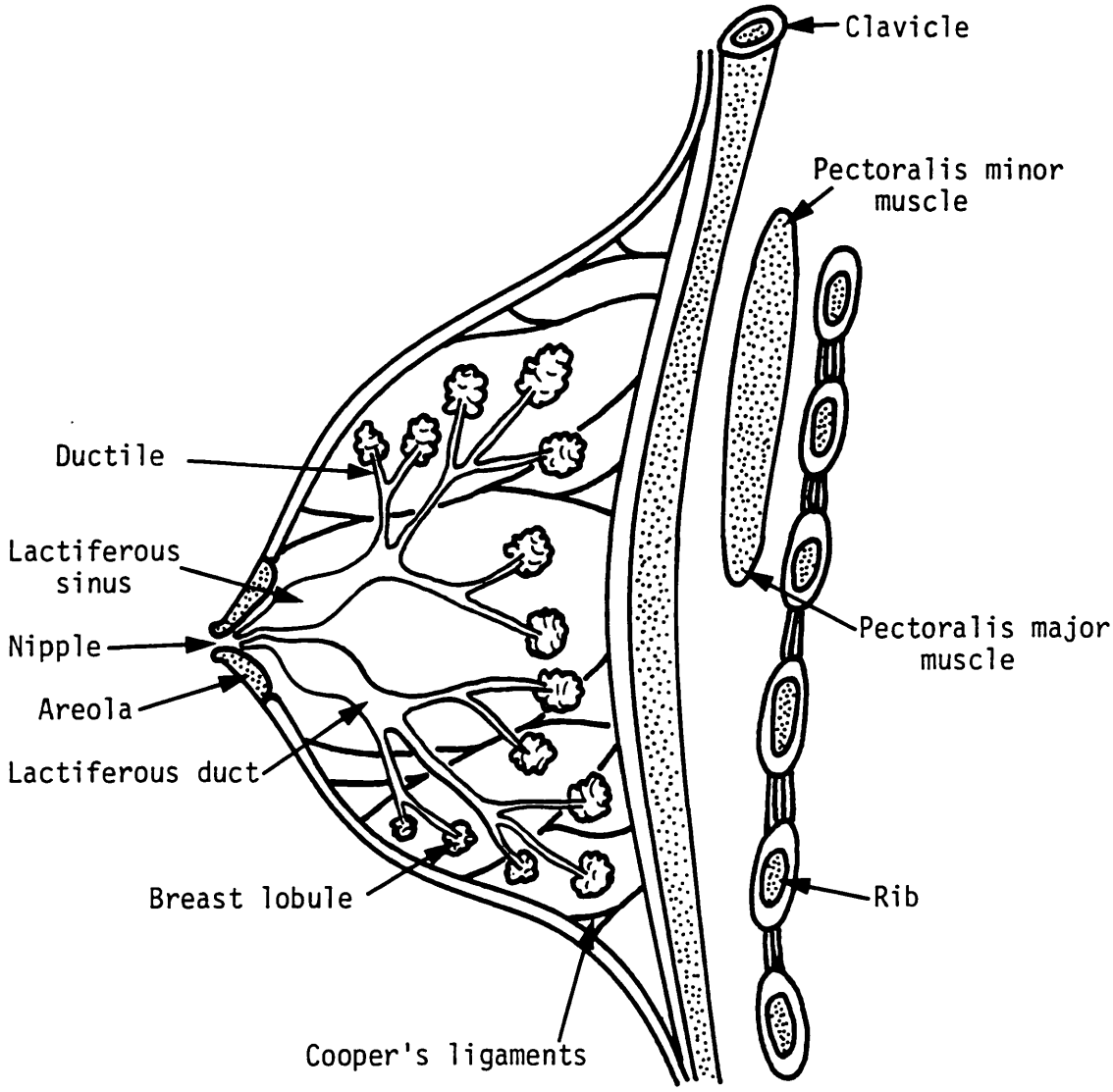


Fig. 3. Structure of the breast.

Lymphatic vessels drain the breast substance and the overlying skin, including the areola and the nipple (figure 4). They are arranged radially and end in surrounding groups of nodes. The lymphatics from the nipple and areola join the subareolar plexus of Sappey, which drain into the axillary nodes. Most lymph vessels from the glandular tissue of the breast lobules drain laterally inwards, parallel with blood vessels, into the axillary nodes. The subcutaneous lymphatics from the upper and medial breast pass backwards into the supraclavicular- and internal mammary nodes, while from the lower and inner quadrant they may enter the subperitoneal lymph plexus.

Function

The development of the breast at adolescence can be considered the most important secondary sexual characteristic in females. The primary biological function of the breast is as an organ of lactation, and its activity is influenced by changes in levels of several hormones.

Growth and development of the breast begins at adolescence with an increase in connective tissue and vascularity, and fat deposition. Oestrogens from the developing ovaries stimulate duct formation, enlargement of the nipple and pigmentation of the areolae. The cyclical exposure of the breast tissue to oestrogens and progesterone during the menstrual cycle induces additional, but limited growth of ducts, lobules and alveoli, while the increase in breast size is chiefly due to deposition of fat and growth of connective tissue. The glandular tissue of the breast undergoes cyclic changes throughout the menstrual cycle which are most marked premenstrually with transient oedema, fluid retention and perhaps some secretory activity.

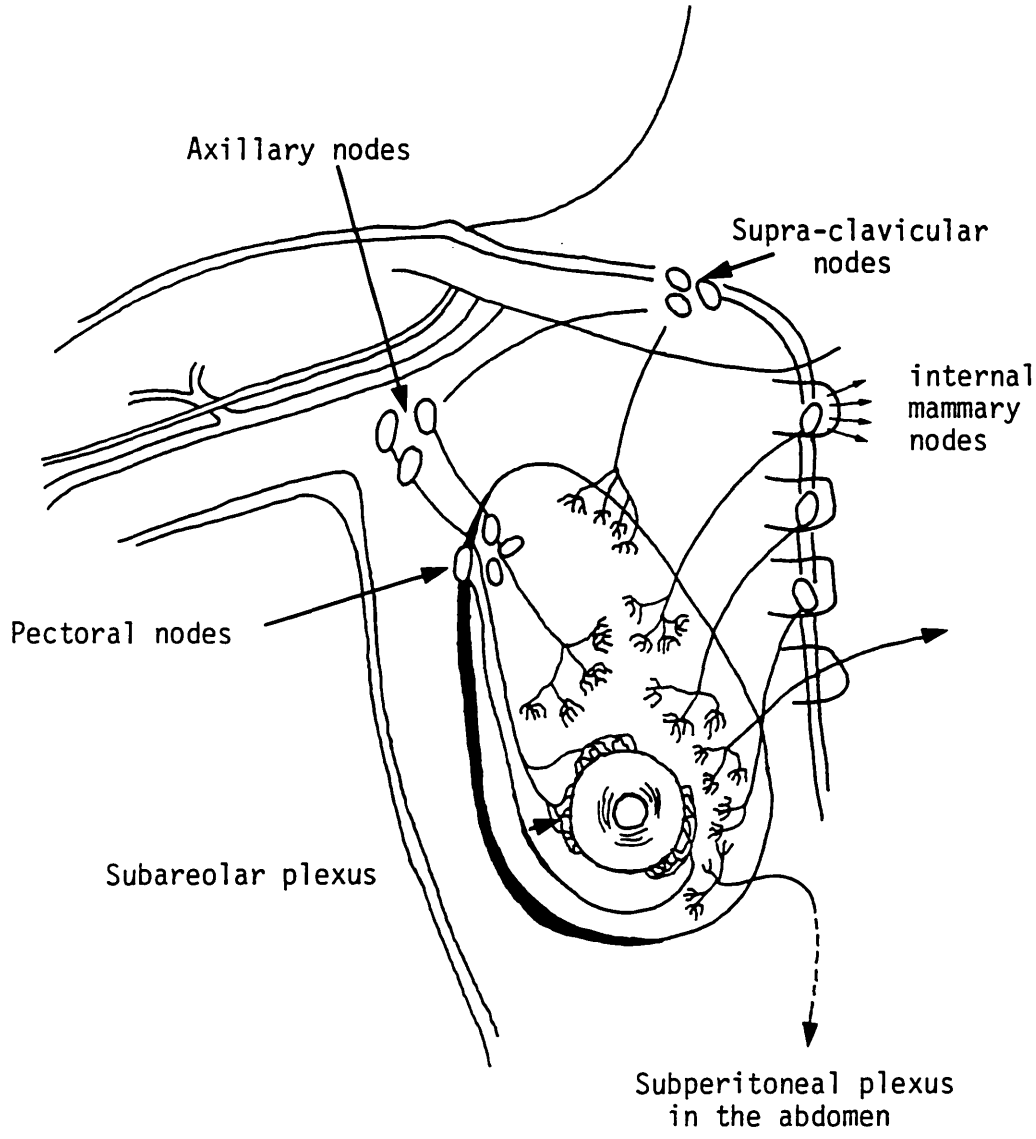


Fig. 4. Schematic diagram of the lymphatic drainage of the breast

The interaction of pituitary, ovarian, thyroid and adrenal hormones is essential for the complete growth and maturity of the mammary gland. The development of the duct system is dependent on oestrogen. Progesterone is required in synergism with oestrogen, in the presence of prolactin, for the development of the lobulo-alveolar system (Cowie et al., 1971). The processes of lactogenesis and milk secretion are regulated principally by prolactin and facilitated by corticosteroids.

During pregnancy, in addition to oestrogen and progesterone, the increasing levels of placental lactogen combine to stimulate extensive proliferation of the secreting structures of the breast, but lactogenesis is minimal and lactation absent. Evidence has accumulated to indicate that oestrogen and progesterone act via their intracellular receptors (see 'Oestrogen Action via Intracellular Receptors', pp. 56) to stimulate mammary development and to inhibit milk formation. Prolactin, the key requirement for lactogenesis, functions at the cellular level by increasing the number of both oestrogen and prolactin receptors (Kledzik et al., 1976). However, elevated oestrogen levels, as in pregnancy, inhibits prolactin binding to mammary tissue and under such circumstances lactation is supressed despite the presence of large numbers of prolactin receptors. Following parturition, oestrogen and progesterone levels fall rapidly and the lactogenic action of prolactin is unopposed. Since prolactin receptors are abundant in the secretory tissue of the breast, lactogenesis and milk secretion are established soon after delivery. The ovarian steroids, oestradiol and progesterone, seem to be of little importance in the maintenance of milk secretion. Episodic release of prolactin is induced by suckling which maintains milk secretion by promoting synthesis of lipids, milk and lactose. Stimulation of the nipple induces the release of oxytocin from the

neurohypophysis. This hormone stimulates contraction of the myoepithelial cells which surround the alveolus, forcing milk out of the alveoli and into the smaller lactiferous ducts. The resulting increase in intramammary pressure results in milk let-down, causing milk to spurt from the nipple and to be removed from the breast by the suckling infant (Johnson & Everitt, 1980). As long as the baby is suckling, the endocrine system is stimulated to produce milk. Once suckling ceases or as a result of artificial termination of lactation by hormone administration, (such as bromocriptine, a dopamine receptor agonist which markedly depresses prolactin and hence milk secretion), involution occurs whereby the lobules decrease in size and number and the breast tissue as a whole shrinks.

After the menopause involution occurs. Most of the glandular structures within the breast atrophy and are replaced by fatty or fibrous tissues. However, many postmenopausal women retain considerable amounts of major ducts and glandular tissue within the breast into old age.

Breast Cancer

Incidence

Breast cancer is the most common form of malignant disease in women over the age of 40 years in the Western world. In the United Kingdom the present incidence is about 50 cases per 100,000 of the female population per annum (Baum, 1981). The prevalence of the disease is reported to be low in developing countries and relatively uncommon in China and Japan (Doll et al., 1970). The incidence of the disease increases until about the time of menopause, when it levels off and then declines after the age of about 65 years.

Cancer of the breast is rare in men, the incidence being approximately 1% of that of women.

Aetiology

The precise causes of breast cancer remain undefined because of the many factors that contribute to its development (Bulbrook et al., 1980). A variety of interrelated factors, including genetic (Petrakis, 1977), hormonal (Kirschner, 1977), enviromental (Miller, 1977), physiologic (LiVolsi, 1978; Juret, 1976) and sociobiologic (Gjorgov, 1978) factors exert an influence on the development of this disease.

Hormonal regulation of the breast is important in the development of breast cancer. Early pregnancy (MacMahon et al., 1970) and premature induction of menopause lowers the incidence, whereas family history of the disease (Henderson et al., 1974) and late menopause are associated with increased incidence of breast cancer. There are conflicting reports on the relative risk associated with administration of exogenous hormones, such as use of oral contraceptives. The reports of Vessey et

al., (1981, 1982, 1983) found no increase in risk of developing breast cancer associated with oral contraceptive use at any period in life. A significantly increased risk of breast cancer in oral contraceptive users has been observed in only three studies (Fasal & Paffenbarger, 1975; Jick et al., 1980; Pike et al., 1983). The study of Pike et al., (1983) suggests that long term use of high progestagen pills, especially at a young age (before the age of 25) and before a first full term pregnancy increases the risk of breast cancer. However, a significantly reduced risk of benign disease (fibroadenoma) has been found in birth control pill users in all the epidemiological studies (Jick et al., 1980; Ramcharan et al., 1981; Kay, 1981; Ory et al., 1981; Fasal & Paffenbarger, 1975; Gambrell, 1984).

The incidence of male breast cancer may be related to hormonal changes and it is particularly high in patients with Kleinfelter's syndrome, 47XXY (Meiss et al., 1982).

Natural History and Metastasis

Breast cancer usually presents as a painless lump in the breast when it has to be differentiated from other breast lumps, such as benign cysts which are present in more than 50% of postmenopausal women (Frantz et al., 1951). Local signs such as deep fixation of the lump or palpable regional lymph nodes may help in the diagnosis of cancer.

Lymphatic spread is common and the majority of patients with apparently 'early' disease have disseminated disease involving the lymph nodes. The number of lymph nodes invaded by the tumour has an important relation to prognosis. Patients with less than four nodes involved have

a better five year survival rate than when four or more are invaded (Fisher et al., 1976). The extent of nodal involvement is a good indicator of the presence of distant metastases.

Tumour spread via the bloodstream is also common, often occurring early in the natural history of the disease. The most common sites of distant matastases include bone (pelvis and lumbar spine), lungs and liver (Sugarbaker, 1981).

Classification

Primary malignant disease of the breast arises usually from the epithelium of the ducts and occasionally from the epithelium of the alveoli in the lobules. Secondary tumours of the breast, as a result of metastatic deposits from a primary tumour at another site, are extremely rare

Benign tumours of the breast arise from the surface epithelium of the ducts (duct papilloma) or from the glandular epithelium (adenoma). Fibroadenomas of the breast consist of both epithelial and connective tissue elements. Intraduct carcinoma is non-invasive and is confined to the lumen of the ducts. Lobular carcinoma is relatively uncommon and arises in the lobules as an epithelial overgrowth showing cellular pleomorphism.

The most common malignant tumour arising in the breast is adenocarcinoma accounting for 80-90% of all tumours. These tumours have no particular distinctive characteristics. In the early stages there is a palpable lump with no fixation to either the deep structures or the overlying skin, and no clincally apparent lymph node involvement. If the tumour is diagnosed at this stage the survival (5yr or longer)

prospects of the patient are good. Locally advanced stages are indicated by fixation of the tumour to the skin or to the underlying structures with or without gross involvement of the axillary lymph nodes.

Medullary and mucoid carcinomas are rare, accounting for 2-5% of breast cancers. Squamous cell carcinoma is a rare tumour of the breast, but when present is usually well differentiated. Sarcomas, malignant tumours of connective tissue origin, are very rare (1%).

D: Management of Breast Cancer

There are a large number of possible therapeutic approaches for the optimum management of breast cancer. The treatment of advanced or metastatic breast cancer may involve a single or combination of the various therapies: endocrine manipulation, radiotherapy, chemotherapy and immunotherapy (Gallagher et al., 1978; Stoll, 1981). The precise choice of systemic therapy for an individual patient is guided by both clinical and biochemical indices such as size and location of the primary tumour, extent of nodal involvement, metastatic spread, menopausal status, receptor status etc. The presence of cytosol oestrogen receptors in human breast tumours is currently being used to select patients with the potential to respond to endocrine therapy (Leung et al., 1974; DeSombre et al., 1974). A generalised scheme for the treatment of patients with metastatic breast cancer is shown in Figure 5. There is as yet no cure for breast cancer. The treatments are essentially palliative, but much can be done to improve the quality of life and many patients enjoy prolonged survival.

Endocrine Manipulation

The mechanism of action of endocrine therapy is based on the reduction of steroid hormones of ovarian and adrenal origin capable of stimulating tumour growth. The hormone-receptor status of the patient's tumour is very important in predicting response to therapy (McGuire et al., 1977), since tumours that lack oestrogen receptors are unlikely to benefit from hormonal manipulation. Endocrine therapy is the treatment of choice for patients with hormone dependent tumours. It may involve endocrine ablation (removal of glands synthesising the steroid nucleus) or hormone additive therapy (competitive blocking of steroid action).

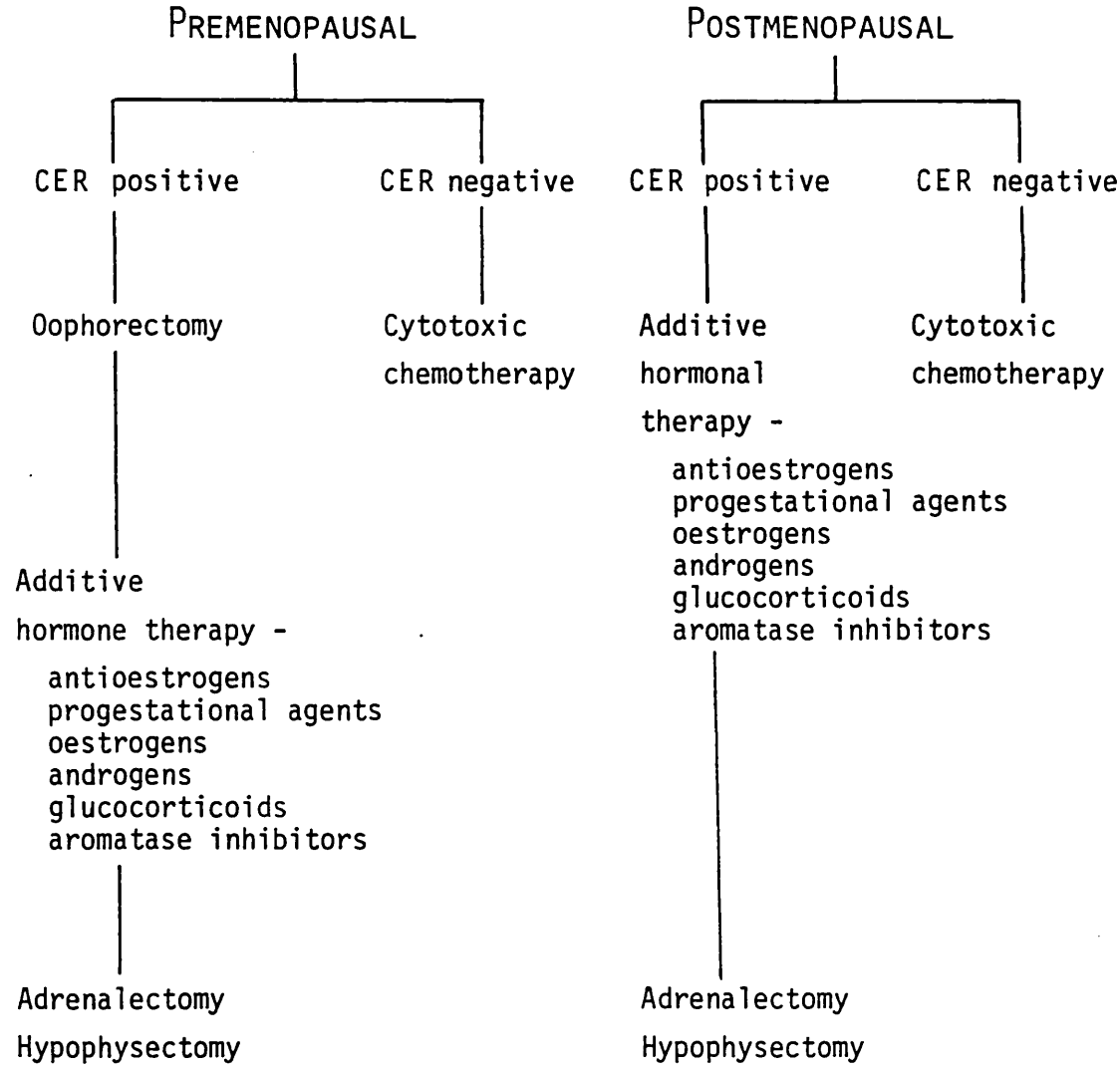


Fig. 5. Generalised treatment scheme dependent on receptor status for patients with advanced breast cancer. CER= cytosol oestrogen receptor.

Many studies have shown that 55-70% of patients with tumours containing cytosol oestrogen receptors respond to endocrine therapy (McGuire, 1975; Leake et al., 1981a; Hawkins et al., 1980).

Endocrine Ablative Therapy

Oophorectomy is the initial therapy in premenopausal women with hormone dependent tumours (Legha et al., 1981). Ovarian ablation results in the removal of oestrogens, progestins and androgens, all of which may stimulate tumour growth. Radiation ablation of ovaries has been shown to be as effective as surgical removal in these patients (Nissen-Meyer, 1975). However, removal of ovaries by surgery is preferable to ovarian irradiation because it acts rapidly and results in a more complete reduction in hormone levels.

Premenopausal patients who respond to oophorectomy and then relapse may be candidates for a variety of secondary endocrine treatments including ablative therapy (medical or surgical adrenalectomy or hypophysectomy) or hormonal administration (antioestrogens, progestins or androgens) (Legha et al., 1978; Santen et al., 1977; Newsome et al., 1977). Adrenalectomy can be accomplished surgically as well as chemically by the drug aminoglutethimide, which blocks synthesis of steroids at the desmolase conversion of cholesterol to pregnenolone with an additional effect on aromatisation (figure 6) (Newsome et al., 1977; Santen et al., 1980). Replacement therapy with glucocorticoids (hydrocortisone, fluorohydrocortisone and prednisone) is required after adrenalectomy and hypophysectomy, with associated side effects (Siiteri, 1982).

Postmenopausal patients who respond to primary hormonal additive therapy and subsequently relapse are candidates for ablative therapy,

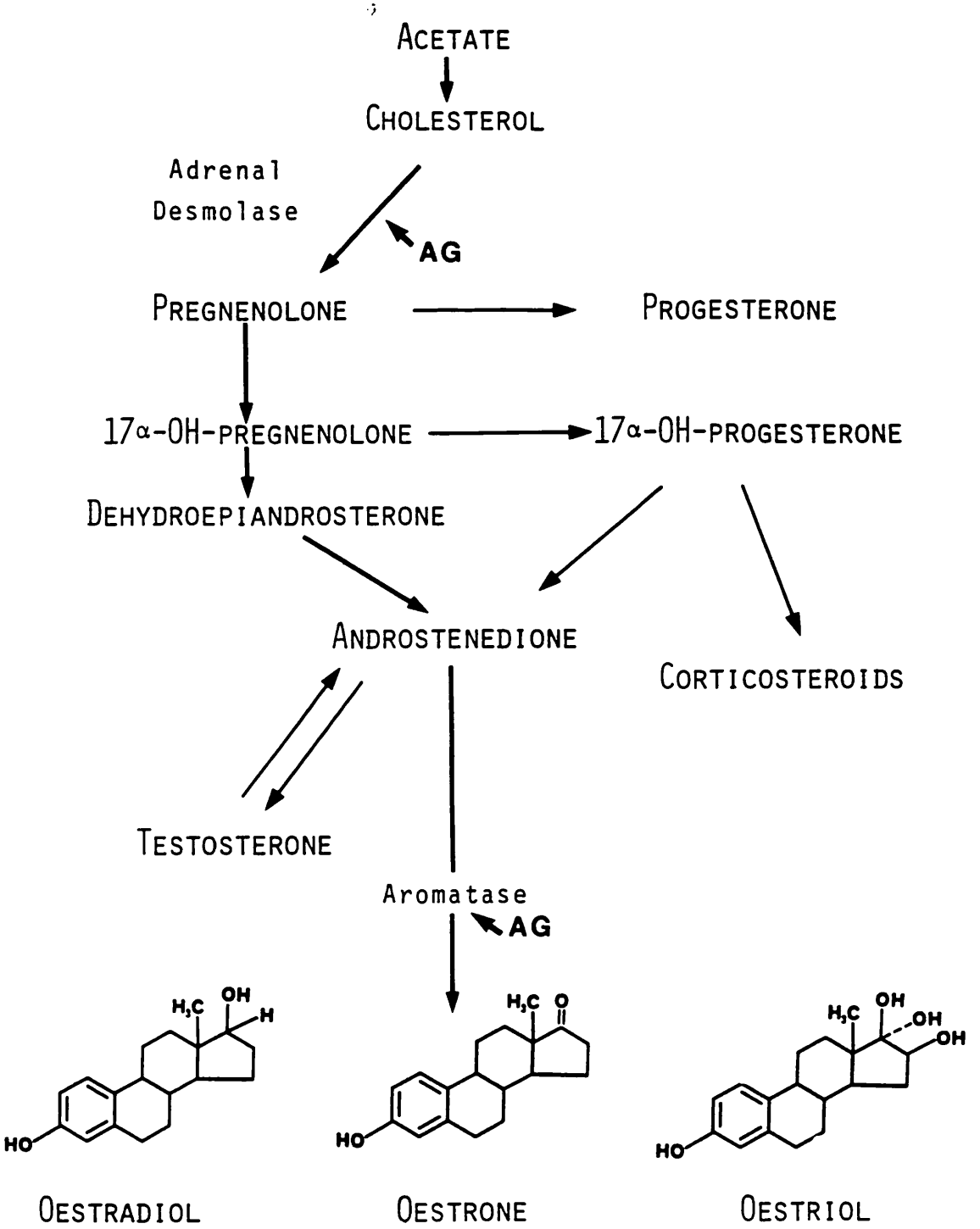


Fig. 6. Biosynthetic pathway for oestrogens. AG = aminoglutethimide.

either medically with aminoglutethimide, or surgically by adrenalectomy or hypophysectomy (Santen et al., 1977). This may replace or precede change of hormone administration.

Hormone Therapy

The mechanism of action of hormone additive therapy is far from clear. It may act by:

(1) depleting cytoplasmic receptors and preventing processing of the nuclear receptor, thus reducing sensitivity of the tumour to endogenous hormone, or

(2) the cytoplasmic receptor complex formed with the 'added' hormone may not be able to interact properly with the specific genomic site for the oestrogen receptor complex (Clark et al., 1973a).

Alternatively, it may act by an indirect pathway such as depression of pituitary function, resulting in depression of prolactin release (Djiane & Durrand, 1977).

Pharmacological doses of oestrogens, progestins, androgens or glucocorticoids are administered as additive therapy. The antioestrogen, tamoxifen has largely replaced the sex-hormones because of its lack of side effects (Mouridsen et al., 1978; Henningson et al., 1980). Newer agents such as Danazol and LHRH analogues are under trial and appear to act by inhibiting gonadal steroidogenesis (Barbieri & Ryan, 1981; Corbin, 1982). Recent evidence indicates that LHRH agonists have direct antitumour effects on the growth of the human breast cancer cell line, MCF-7 in culture (Miller et al., 1985), and thus application of this therapy could be effective in both premenopausal and postmenopausal women with breast cancer.

Antioestrogens

Antioestrogen therapy has been shown to be effective in some patients after initial endocrine additive therapy and, in particular, after ablative procedures such as ovariectomy, adrenalectomy and hypophysectomy. The mechanism by which antioestrogens inhibit tumour growth is not clearly defined. Antioestrogens interact with the oestrogen receptor promoting translocation and nuclear binding (Clark, 1973; Sutherland & Jordan, 1981). There is evidence that the difference between oestrogen and antioestrogen action lies subsequent to the translocation step (Horwitz & McGuire, 1978) (further discussed on pp. 77-82).

Administration of the antioestrogen tamoxifen is the initial therapy of choice for postmenopausal women with metastatic breast cancer (Kiang & Kennedy, 1977; Legha et al., 1978; Mouridsen et al., 1978). A response rate of more than 30% is observed in unselected patients, but this rate is approximately doubled in patients whose tumours contain oestrogen receptors. Diethylstilboestrol (DES) has also been used but is more toxic (Kennedy, 1974; Carter et al., 1977). Patients who respond to DES or tamoxifen and then relapse are sometimes treated with a change of hormone, instead of endocrine ablation. Medroxyprogesterone acetate (MPA) (Izuo et al., 1981), megestrol acetate (Legha et al., 1978; Ansfield et al., 1976; Alexieva-Figusch et al., 1984), tamoxifen or DES may be used, depending on the patient's prior exposure to hormones. Similarly, premoenopausal patients who initially respond to endocrine ablative therapy may be considered for secondary hormone additive therapy employing either tamoxifen or megestrol acetate (Legha et al., 1978).

Chemotherapy

Patients with disseminated breast cancer who fail to respond to hormonal manipulation, patients with rapidly advancing and widespread disease and those with metastatic tumours that lack oestrogen receptors are all candidates for chemotherapy (figure 5). A wide range of chemotherapeutic agents have been shown to induce regression of breast cancer metastases. These include: alkylating agents such as cyclophosphamide (C) and melphalan (L-PAM) which interfere with DNA replication by cross-linking DNA, antitumour antimetabolites such as doxorubicin (Adriamycin, A) and mitomycin C which bind to DNA causing breakages in the strands and interfere with RNA synthesis, general antimetabolites such as methotrexate (M) and 5-fluorouracil (F) which affect the synthesis of nucleic acids, and alkaloids such as vincristine (V) and vinblastine which are metaphase inhibitors and interfere with the mitotic spindle.

Tumours that lack oestrogen receptors have been shown to have a high thymidine labelling index (Meyer et al., 1977), suggesting aggressive growth (Mouridsen et al., 1978). Premenopausal disease is generally more aggressive (Allegra et al., 1977; Bonadonna et al., 1977; Knight et al., 1977). Single drugs will give a response in only 20-30% of the patients with metastatic disease. Adriamycin is considered most effective, especially if it is administered during the early phase of treatment (Carbone & Davis, 1978; Taylor & Gelber, 1982). However, responses with single agents are of short duration, usually less than 6 months, and patients with multiple sites of metastases may not respond as well as patients with single areas of involvement.

Combinations of up to five drugs with differing mechanisms of action and non-overlapping toxicity are now used in preference to single

agents (Carbone et al., 1977; Baum, 1981) in the hope of increasing response rates and minimising drug resistance (Brambilla et al., 1978). A recent approach is to use intermittent or cyclic drug therapy, using up to two different drug combinations to achieve the same effect (Pannuti & Creaven, 1979), and minimise side effects due to prolonged drug administration.

Breast cancer is known to be heterogeneous (Rosen et al., 1977; Heppner et al., 1978), composed of clones of oestrogen receptor-positive and negative cells (Nenci, 1978, 1981). This biologic heterogeneity may influence the response rate and may explain why complete response rates are low for any single mode of therapy. Studies using a combination of endocrine ablation and chemotherapy have observed additive benefit. Ahmann et al., (1977) observed improved response rates and prolonged survival times in ovariectomised premenopausal women treated with chemotherapy. Similarly adrenalectomy in combination with chemotherapy employing 5-fluorouracil improved response rates and disease free survival in postmenopausal women (Moore et al., 1974). Heuson (1976) showed that multi-agent chemotherapy with tamoxifen was more effective than either modality alone. However, with the available chemotherapeutic agents, failure rates are still unacceptably high.

Adjuvant Systemic Therapy

Adjuvant systemic therapy for primary breast cancer is based on the observation that many women presenting with apparently 'early' disease already have widespread undetectable micro-metastases (de Vita et al., 1975) and that 70-80% of patients die of disseminated disease (Mueller & Jeffries, 1975). Additionally, recurrent disease may represent clones of tumour cells with different biological

characteristics such as receptor status, hormone dependence or independence, and thus may not respond to treatment designed to influence the primary cancer. So, systemic therapy with drugs that have been shown to be effective in advanced breast cancer, given as an adjuvant to primary local therapy theoretically represents the best chance of improving prognosis of primary breast cancer.

Adjuvant Chemotherapy

The studies of Fisher et al., (1977) and Bonadonna et al., (1978) involving randomised clinical trials comparing mastectomy alone with a group of patients recieving a systemic chemotherapeutic agent or agents following mastectomy observed similar trends in respect of recurrence-free survival (RFS) for both modalities. The only significant differences in disease-free survival were seen in premenopausal women (Rossi et al., 1981). There are not many series ' of long-term follow-up data in the literature for any firm conclusions to be made.

The available data demonstrate a therapeutic effect measured by prolongation of RFS and preliminary data also indicate a survival gain in some subsets of patients. The optimal duration of therapy is not defined, but there is an indication that the maximum cytotoxic effect is achieved within the first few months after mastectomy. In this respect, preoperative chemotherapy is now being considered. Several studies comparing the efficacy of single- and multiple- drug therapy as adjuvants indicate that combinations are superior to single drug treatments (Fisher et al., 1981; Davis et al., 1981; Cooper et al., 1981; Gluksberg et al., 1982).

Adjuvant Endocrine Therapy

Endocrine ablative and additive therapy in advanced breast cancer gives an overall response rate of 30-40% (Taylor, 1962; Stoll, 1969). When patients are selected according to receptor status, it has been shown that 50-60% of the oestrogen receptor positive and approximately 10% of the oestrogen receptor negative tumours respond to endocrine therapy in advanced disease (McGuire et al., 1975; Engelsman et al., 1973; Jensen et al., 1973). Several studies have also shown that the oestrogen receptor status of the primary tumour and that of the recurrent tumour remains unchanged in about 85% of the cases (Allegra et al., 1980b; Webster et al., 1978). Therefore, the role of adjuvant endocrine therapy with or without chemotherapy has been investigated extensively during the last decade. Generally the results are very similar to chemotherapy. Ovarian irradiation and the addition of prednisone has been shown to improve survival and prolong RFS (Meakin et al., 1979). Tamoxifen, either as a single agent or in combination with chemotherapy prolongs RFS. The combination of this agent with chemotherapy is superior to chemotherapy alone (Mouridsen et al., 1978, 1979; Wallgren et al., 1981). The efficacy of tamoxifen appears to be mediated through the oestrogen receptor system, although biochemical mechanisms independent of the oestrogen receptor system cannot be ruled out (Sutherland et al., 1983a, 1983b).

Immunotherapy

The efficacy of adjuvant immunestimulation in the treatment of advanced breast cancer remains to be demonstrated. Several randomised trials have failed to show the benefit of bacillus Calmette-Guerin (BCG) when given in combination with chemotherapy (Hubay et al., 1980; Cohen

et al., 1982), or after completion of chemotherapy (Buzdar et al., 1982). However, intralesional injection of BCG used alone or in combination with hormonal therapy appears to be effective in treating selected patients with chest wall recurrence following radiation therapy (Sparks et al., 1977).

Interestingly, a randomised trial using adjuvant weekly injections of polyadenylic-polyuridylic acid (PPA) for six weeks has demonstrated a significant increase in RFS after 5 years (Lacour et al., 1982) and a recent update at 8 years confirms the earlier conclusions (Lacour et al., 1984). The authors suggest that the induction of interferon and natural killer (NK) cells may be involved in the PPA mechanism of action. However, these results have to be confirmed by other investigators.

E: Oestrogen Action via Intracellular Receptors

A major advance in understanding the mechanism of oestrogen action was made when Jensen and Jacobson (1962) demonstrated the selective uptake and retention of radiolabelled oestradiol in target organs of the rat, the uterus and vagina. Non-target tissues, such as muscle, failed to retain the radiolabelled oestradiol. The difference between target and non-target tissues was in the ability to retain steroid and not in the uptake (Peck et al., 1973). Target tissues have the ability to preferentially bind and retain hormone against a concentration gradient in the circulating blood (King & Mainwaring, 1974).

Mechanism of Steroid Hormone Action

In 1968 two independent groups put forward a general 'two-step' model of steroid hormone action (Jensen et al., 1968; Gorski et al., 1968).

Steroid enters the target cell and binds to specific cytoplasmic proteins, termed 'receptors', resulting in an increased affinity for the nucleus. The steroid-receptor complex translocates to the nucleus where it interacts with chromatin resulting in the modulation of transcriptional activity (O'Malley & Means, 1974). The activity of ribonucleic acid (RNA) polymerase enzymes is increased (Gorski, 1964), resulting in specific messenger RNA (mRNA) induction (Borthwick & Smellie, 1975) and ultimately synthesis of new cellular proteins (Notides & Gorski, 1966; Wira & Baulieu, 1971; Edwards et al., 1980), and cell growth and division.

The general concept that steroid binding increases the affinity of the receptor for chromatin still holds true, although modifications in

the overall scheme have been suggested. Martin and Sheridan (1980) proposed that unbound receptors are in equilibrium between the nucleus and the cytoplasm according to the free-water content of these intracellular compartments. Steroids enter both the cytoplasm and nucleus of a target cell and bind to unoccupied receptors. Binding of steroid to the unoccupied receptors in the nucleus results in stabilisation of the receptor complex, which then becomes non-diffusible. The cytoplasmic steroid-receptor complexes move into the nucleus to re-establish the equilibrium (translocation).

Recently, the cytoplasmic localisation of oestrogen receptor has been questioned (Sheridan et al., 1979; Martin & Sheridan, 1982; King & Greene, 1984; Welshons et al., 1984). These workers have observed, using autoradiographic, immunohistochemical and biochemical techniques, that the intracellular receptor is located within the nucleus with no evidence of a cytoplasmic form. These reports are analogous to the nuclear localisation of vitamin D and thyroid hormone receptors (Walters et al., 1980; Spindler et al., 1975). The suggestion that the cytoplasmic localisation of receptor is an artefact of tissue preparation has therefore been proposed (Sheridan et al., 1979; Martin & Sheridan, 1980; Williams & Gorski, 1971; Gorski & Gannon, 1976; Martin & Sheridan, 1982; Welshons et al., 1984). However, the two-step model can be accommodated by these recent suggestions. The initial binding of steroid to the nuclear receptor increases the affinity of the receptor complex for chromatin, resulting in subsequent binding to chromatin proteins. The very extensive literature describing the cytoplasmic oestrogen receptor has demonstrated, <u>in vitro</u>, that a variety of manipulations (for example, elevated temperature, 25-30 C for 30 minutes or increasing the ionic strength of the buffer) can be used to generate a form of receptor that is indistinguishable from the nuclear (5S)

receptor (Brecher et al., 1970; Jensen et al., 1971b; Mohla et al., 1972). Autoradiographic analysis of receptor distribution in tissues prepared under conditions of minimal cellular damage also demonstrated a temporal sequence of events progressing from the cytoplasm to the nucleus (Sar & Stumpf, 1976). The use of cytosol receptor preparations, which may or may not be derived from a nuclear pool not occupied by steroid, to study receptor action is a meaningful experimental tool.

Entry of Oestrogen into the Cell

It is generally accepted that steroids enter the target cell by passive diffusion, figure 7 (Gorski & Gannon, 1976; Higgins & Gehring, 1978). This may explain why steroids enter both target and non-target cells (Jensen & Jacobson, 1962). The cell membrane, which is composed of a lipid bilayer, offers very little resistance to the diffusion of steroids, which are lipophilic, into the cell. However, the mechanism of passive diffusion has been challenged by Milgrom et al., (1973) and Baulieu, (1978). They suggested that some membrane-bound proteins containing sulphydryl groups may facilitate steroid uptake into the target cell. Studies using various sulphydryl blocking agents have been unable to repeat these results (Peck et al., 1973). Affinity chromatography techniques have enabled Pietras and Szego, (1977) to demonstrate the existence of specific oestradiol-17β binding sites on the surface of oestrogen responsive cells. These sites were not detected in non-target intestinal cells. Similar findings have been reported by others (O'Malley & Means, 1974).

In contrast, Terayama et al., (1976) have failed to demonstrate these plasma-membrane binding sites in neoplastic cells. Zanker et al., (1981) have reported a similar loss of high affinity plasma-membrane

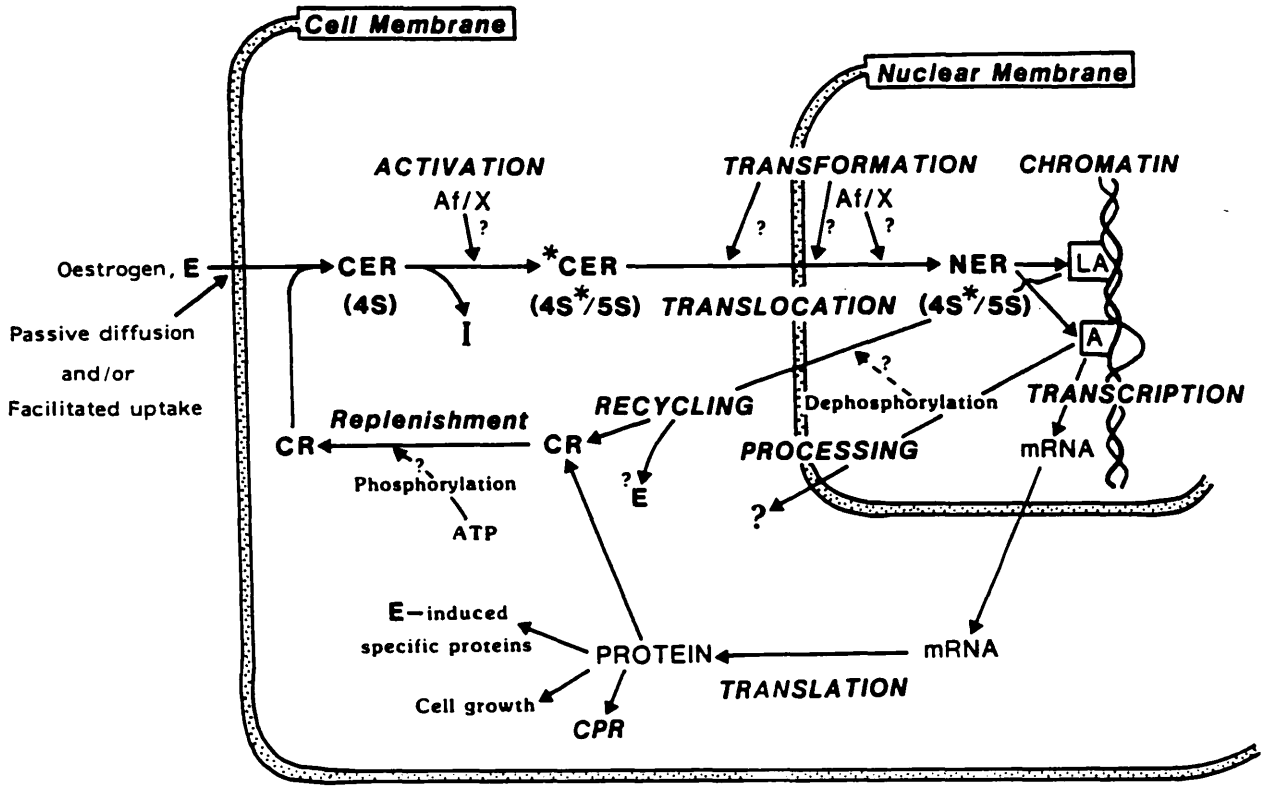


Fig. 7. Mechanism of oestrogen receptor action. CR, cytosol oestrogen receptor; CER, cytosol oestrogen-receptor complex; *CER, activated oestrogen-receptor complex; NER, nuclear oestrogen-receptor complex (activated and/or transformed); CPR, cytosol progesterone receptor; I, low molecular weight inhibitor of activation; Af/X, activating factor/ addition of a subunit; A, high affinity nuclear acceptor site(s); LA, low affinity nuclear acceptor sites; ? steps requiring further elucidation.

oestrogen binding sites (Kd = 6.4 X 10^{-10}M) in neoplastic breast tissue compared to normal tissue. They suggested that in normal cells the plasma-membrane sites may serve as modulators of intracellular hormone levels, protecting the cells from excessive exposure. The loss of plasma-membrane receptors may be a reflection of the process of cellular dedifferentiation.

Studies using fluorescent oestradiol as a probe have also indicated the involvement of plasma membranes in the initial uptake of the steroid (Nenci et al., 1980). However, these studies should be interpreted with caution due to the very high concentration of oestradiol used (Chamness et al., 1980; Penney & Hawkins, 1982), which may result in general non-specific binding. Techniques such as dry-mount high resolution autoradiography have been unable to identify these membrane-bound receptors. These contrasting results may be due to the failure to distinguish between binding of steroids to high-affinity specific receptors and to other non-specific proteins (Panko et al., 1982). Based on various studies, Rao (1982) has suggested that perhaps both passive and facilitated processes may be involved in steroid uptake by the cell.

Cytosol Oestrogen Receptor

The studies of Toft and Gorski (1966) demonstrated, using radiolabelled oestradiol, that the specific hormone binding molecules, termed 'receptors', were present in the soluble fraction of the cell homogenates as well as in the nuclei. This confirmed the earlier results of Talwar et al., (1964) who had demonstrated the presence of macromolecular bound [^{3}H]-oestradiol by sephadex chromatography. The receptor-oestradiol interaction was found to exhibit stereospecificity for oestrogens in that testosterone was unable to inhibit oestradiol

uptake (Noteboom & Gorski, 1965). Autoradiographic studies of the intracellular localisation of [^{3}H]-oestradiol in target tissues also confirmed the presence of oestrogen binding sites, both in the nucleus and cytoplasm (Stumpf, 1969).

Much of the present knowledge concerning the oestrogen receptor has been derived from the rat uterus. The proteinaceous nature of the oestrogen receptor was demonstrated by Toft and Gorski (1966) by its sensitivity to proteolytic enzymes but not to nucleases. The receptor-oestradiol interaction was found to be pH sensitive. It was irreversibly destroyed at acidic pH (Toft & Gorski, 1966; Puca et al., 1971), but was stable between pH 7 and 9. A requirement for sulphydryl groups for receptor-oestradiol interaction has been demonstrated in the rat uterus (Jensen et al., 1967; Terenius, 1967; Muldoon, 1971), rabbit uterus (Ikeda, 1982), human myometrial tissue (Ratajczak et al., 1982) and human breast cancer (McGuire & De La Garza, 1973).

Receptors are characterised by their specificity, high affinity and low capacity of binding (Shyamala & Gorski, 1969; Giannopolous & Gorski, 1971). A variety of methods have been used to assess the affinity (equilibrium dissociation constant, Kd) of the receptor-oestradiol interaction. These include sephadex gel filtration (Puca & Bresciani, 1969; Ginsburg et al., 1974; Singhagowinta et al., 1975), charcoal adsorption (Mester et al., 1970), equilibrium dialysis (Erdos et al., 1969), hydroxylapatite adsorption (Erdos et al., 1970; Pavlik & Coulson, 1976), polyacrylamide gel electrophoresis (Ritzen et al., 1974), and protamine sulphate precipitation (Steggles & King, 1970). Despite differences in methodology, the affinities of the receptor-oestradiol interaction generally agree and fall in the range 10^{-9} to 10^{-10} M (King & Mainwaring, 1974; O'Malley & Means, 1974). These

high affinity sites are thought to be involved in the physiological response. Low affinity sites are also detected, with affinities in the range 10^{-5} to 10^{-7} M (McGuire & Julian, 1971); they probably represent non-specific binding to other cytoplasmic proteins.

It has been shown that only phenolic steroids having a 17β-hydroxyl group compete with oestradiol for oestrogen receptor binding (Korenman, 1969; Tseng & Gurpide, 1976). Non-steroidal antioestrogens have low binding affinities for the oestrogen receptor (Ruh & Ruh, 1974). However, some synthetic non-steroidal oestrogens such as diethylstilboestrol (DES) and selected triphenylethylenes compete effectively with 17β-oestradiol for the steroid binding sites (Puca & Bresciani, 1968, 1969b; Duax & Weeks, 1980).

The receptor-oestradiol interaction can be reversibly dissociated by heating to 45^{0}C for 5 minutes, but higher temperatures result in the degradation of the receptor (Baulieu et al, 1971; Puca et al., 1971; Myatt et al., 1979b).

The metal ion chelating agent, ethylene diamine tetra-acetic acid (EDTA), is required to optimise the receptor-oestrogen interaction and prevent its aggregation *in vitro* (King & Mainwaring, 1974). The presence of reduced sulphydryl groups is essential for the binding of oestradiol to the receptor, and they can be protected by sulphydryl reducing agents such as dithiothrietol (DTT) and thioglycerol (Gorski et al., 1968; King & Mainwaring, 1974; Sando et al, 1979a,b). These agents are thought to prevent receptor aggregation (Puca & Bresciani, 1969b).

Receptor Forms and Interconversions

Steroid receptors have been analysed in vitro by sucrose density gradient analysis to define their hydrodynamic parameters, such as molecular Stokes radius, axial ratios and sedimentation coefficients (Puca et al., 1971). The rate of sedimentation of the protein per unit of gravitational field is expressed in Svedberg (S) units ($1S=10^{-13}$ seconds) (Svedberg & Pederson, 1940). The cytosol oestrogen receptor exists in vitro$_5$with a sedimentation coefficient of 8-9S in low salt (McGuire & Julian, 1971; Shyamala & Nandi, 1972; Jensen et al., 1974), and can be distinguished from the non-specific binding to serum albumin, which sediments at 4-5S, or in the immature rat from α-foetoprotein which sediments at 4S in low salt (Plapinger et al., 1973). Variations in the sedimentation coefficient of the ~8S receptor in low salt may be due to the various degrees of aggregation of the receptor with itself or with non-specific proteins (Mueller et al., 1972; Stancel et al., 1973). The 8S receptor has an estimated molecular weight in the range 200-238,000 daltons; Stokes radius, 67 Å; frictional ratio (f/fo), 1.65; homogeneous electrofocussing with a single isoelectric point at pH 6.2 (Erdos, 1968; Puca et al., 1971; Stancel et al., 973). Under isotonic conditions (0.15 M salt) the sedimentation coefficient is approximately 4S (Stancel et al., 1973; Yamamoto & Alberts, 1974). The 4S receptor has an estimated molecular weight in the range 60-80,000 daltons; Stokes radius, 33-44 Å; frictional ratio (f/fo); 1.25-1.45; heterogeneous electrofocussing with two major components, isoelectric points at pH 6.6 and 6.8 (Puca et al., 1971; Notides & Nielsen, 1974). It has been suggested that the oestradiol receptor complex slowly aggregates with other proteins present in the homogenate. This can be minimised by dilution, implying that, in vivo, the receptor may exist as 4S and that 8S may be an aggregate of several

proteins formed as a result of homogenisation (Stancel et al., 1973). Increasing the salt concentration above 0.4M salt does not further disaggregate the receptor. There is no loss of binding sites or change in the dissociation constant on conversion of the receptor from 8S to the 4S form by salt. Removal of salt by dialysis causes re-aggregation of the 4S receptor to give the 8S form (Baulieu et al., 1971). Proteolytic enzymes, such as trypsin, convert the 8S receptor to the 4S form (Puca et al., 1971) without loss of oestradiol binding capacity (Notides et al., 1973). This 4S receptor does not bind to DNA or chromatin (Andre & Rochefort, 1973), but binds to crude nuclei (Rochefort & Baulieu, 1973). A protease mediated conversion of the 8S receptor to the 4S form has been reported in human breast tumour cytosol (Schneider & Dao, 1977). Similar findings have also been reported for the human myometrium (Notides et al., 1973). The presence of both 8S and 4S oestrogen receptor has been described in various target tissues in animals as well as in human breast tumours (King & Mainwaring, 1974; Muldoon, 1978; Wittliff & Savlov, 1978).

Smaller fragments of the oestrogen (Shermann et al., 1978, 1980), glucocorticoid (Carlstedt-Duke et al., 1983; Sherman et al., 1981), androgen (Lea et al., 1979) and progesterone (Sherman et al., 1974; Vedeckis et al., 1980) receptors have been detected in a wide range of healthy and malignant tissues. These fragments, designated the 'mero-receptor' have a sedimentation coefficient of approximately 3S and appear to be the smallest form of the receptor still capable of binding steroid but has no affinity for nuclei or DNA (Holbrook et al., 1983a; Vedeckis et al., 1983).

Type II Oestrogen Receptors

In addition to the classical oestrogen receptor, Clark et al., (1978) have described the presence of secondary cytoplasmic oestrogen binding sites (designated type II receptors) in the rat uterus with a Kd of 10^{-8} M (Clark et al., 1978; Clark & Markaverich, 1981). This cytoplasmic type II oestrogen receptor does not translocate to the nucleus and may be more abundant in the target cell than the classical receptor sites. The presence of type II oestrogen binding sites has also been demonstrated in mouse mammary tumours (Watson & Clark, 1980) and in human breast tumours (Panko et al., 1981). These sites may have a role in sequestering steroid from the circulation.

Nuclear type II oestrogen binding sites have also been detected (Eriksson et al., 1978), and they appear to be distinct from the cytoplasmic type II sites. They are not present in tissues which do not proliferate in response to oestrogenic stimulation, such as hypothalamus and pituitary which are secretory organs (Kelner & Peck, 1981). Recently a specific endogenous inhibitor of nuclear type II sites has been demonstrated (Markaverich et al., 1983). These authors suggested that this inhibitor is a component of all tissues; it is complexed with type II oestrogen binding sites in tissues which are not proliferative, that is, hypothalamus and pituitary, or tissues that do not respond to oestrogen. Markaverich et al., (1981) also observed that the presence of nuclear type II oestrogen binding sites correlated with true growth response and that progesterone and dexamethasone, which inhibit uterine growth, blocked the increase in nuclear type II sites without interfering with nuclear oestrogen receptor binding and retention. Therefore, it is thought that the nuclear type II sites play an important role in the response pathways which are involved in uterine growth (Clark & Markaverich, 1981).

Antioestrogen Binding Sites

Recently there have been reports of specific antioestrogen binding sites distinct from the oestrogen receptor (Sutherland et al., 1980; Gullino & Pasqualini, 1982). There is no evidence that these sites mediate the effects of antioestrogens in target tissues. It is possible that these antioestrogen binding sites have a natural ligand (Clark et al., 1983), and it is fortuitous that they are so 'named', since they also bind triphenylethylene antioestrogens. It has been suggested that these sites may be involved in the control of cholesterol metabolism (Clark et al., 1983) because of their abundance in the low density lipoprotein fraction of serum.

Translocation of the Cytosol Oestrogen Receptor.

The nuclear accumulation of receptor occurs with a concomitant depletion of cytosol receptor (Giannopolous & Gorski, 1971; Jensen et al., 1968; Williams & Gorski, 1972). Translocation occurs as a result of the cytoplasmic oestradiol-receptor complex acquiring increased affinity for nuclear acceptor sites; a process termed 'activation' (Buchi & Villee, 1976). Receptor extracted with 0.4M salt after translocation had a sedimentation coefficient of 5S (Jensen et al., 1968; Jensen & DeSombre, 1972). Transformation of the receptor from 4S to the 5S form can be facilitated *in vitro* by elevated temperature (Jensen et al., 1968; Gorski et al., 1968; Notides, 1978). This cytoplasmic 5S form is indistinguishable from the nuclear 5S receptor from oestrogen treated animals (Brecher et al., 1970; Jensen et al.,1971a; Mohla et al., 1972). The exact site of receptor transformation from 4S to 5S, whether it is in the cytoplasm or nucleus, has not been elucidated (see figure 6). It has been suggested that the 4S to 5S transformation occurs in the nucleus (Linkie & Siiteri, 1978). This is supported by the demonstration that nuclear entry of cytoplasmic proteins with molecular weights much greater than 60,000 daltons is restricted (Paine & Feldherr, 1972). Since the extranuclear receptor can be transformed *in vitro* to the nuclear form, the evaluation of this process using cytosol preparations *in vitro* is meaningful for studying steroid hormone action and extrapolation to the *in vivo* situation.

The mechanism of intracellular translocation is not clearly understood (figure 6). It was proposed that the formation of the nuclear receptor complexes was a 'two-step' mechanism; a temperature sensitive (activation) step, followed by translocation and nuclear binding. Autoradiographic analysis of receptor distribution in tissues

prepared under conditions of minimal cellular damage also demonstrated a temporal sequence of events progressing from the cytoplasm to the nucleus (Sar & Stumpf, 1976). The data of Nenci (1976, 1981) using fluorescent oestradiol, lends support to such a thesis.

However, Linkie (1977) demonstrated the presence of both 4S and 5S receptor complexes in nuclei of immature rat uterus, hypothalamus and pituitary. Thus, the 4S receptor found in the cytoplasm may be able to freely diffuse across the nuclear membrane. Sheridan et al., (1979) and Martin & Sheridan (1982) have suggested that unbound cytoplasmic and nuclear receptors are in equilibrium and translocation occurs as a result of this equilibrium being disrupted due to steroid binding to nuclear receptors which then binds to nuclear components and becomes nondiffusible (see pp. 57).

Nuclear Oestrogen Receptor

The appearance of the 5S receptor in the nucleus is dependant on the availability of oestradiol to the target cell (Musliner et al., 1970), which binds to the cytosol receptor and becomes activated before nuclear localisation. Further evidence for this temporal sequence of events comes from Sarff and Gorski (1971) and Giannopolous and Gorski (1971) who demonstrated that following oestrogen treatment, the steroid binds to the cytoplasmic receptor and then moves into the nucleus with a concomitant depletion of cytosol oestrogen-receptor complex.

Size of the Receptor

Nuclear 5S receptor complexes can be extracted from oestrogen-treated rats with 0.4M KCl (Jensen & DeSombre, 1972). The 5S nuclear oestrogen-receptor complex has an estimated molecular weight in the range 110-140,000 daltons. The nuclear receptor binds to several oestrogens *in vitro* with affinities in the range 1.2-2.6 X 10^{-9} M in mature rat uterus (Clark et al., 1972b). Puca et al., (1971) demonstrated that purification of the nuclear receptor resulted in disaggregation of the 5S form to give a 4.5S species, with a molecular weight of approximately 60,000 daltons, without loss of steroid binding capacity. The moiety associated with the 4S receptor to give the 5S form may have been added as a result of receptor activation (discussed on pp. 84-91).

Nuclear Binding of Receptor

Steroid receptor complexes, following translocation to the nucleus, are thought to bind to specific nuclear sites, termed 'acceptor' sites (Pooley & King, 1969; King et al., 1971), to induce the physiological response. This may represent a secondary level of specificity of hormone action, the primary one being the specific binding to cytoplasmic receptor (figure 6). All components of the nucleus, such as nuclear membrane (Jackson & Chalkey, 1974a,b), ribonucleoproteins (Liao et al., 1973), histone proteins (Kallos et al., 1981), basic non-histone proteins (Puca et al., 1974, 1975), acidic non-histone proteins (Spelsberg et al., 1971, 1972; Steggles & King, 1971; O'Malley et al., 1972), DNA (Toft, 1972; King & Gordon, 1972; Yamamoto & Alberts, 1974, 1975), and salt-insoluble nuclear fractions (nuclear matrix) (Barrack & Coffey, 1980; Buttyan et al., 1983) have been proposed as acceptor sites for the steroid receptor complexes.

Acceptor Site Hypothesis

The 'acceptor' site hypothesis suggests that nuclear binding is a saturable process with a limited number of binding sites. Chamness et al., (1974) and Andre & Rochefort (1975) found no evidence of saturation of nuclear sites by cytoplasmic oestrogen receptor, however, Buller et al., (1975) were able to demonstrate saturation. Similarly, saturable binding of oestrogen receptor to DNA alone has also been reported (King & Gordon, 1972; Toft, 1972; Yamamoto & Alberts, 1972). The apparent saturability may be due to varying concentrations of total cytosol protein, which at higher levels depresses receptor binding (Chamness et al., 1974), but Buller et al., (1975) demonstrated saturable nuclear binding under conditions of constant protein concentration and varying receptor concentrations. Yamamoto & Alberts (1974) showed that the oestrogen-receptor complex bound to DNA with low affinity and suggested that a large excess of these nuclear binding sites were present, with a few high affinity sites (figure 6). The interaction of the receptor complex with the high affinity sites ($Kd=10^{-12}M$) are thought to be involved in the promotion of physiological responses (Spelsberg, 1976; Clark & Peck,1979; Mulvihill & Palmiter, 1977). It has been observed that the fractional quantity of oestrogen receptors remaining bound to the nucleus (possibly to the high affinity sites) for more than 6 hours correlated with the uterotrophic response (Anderson et al., 1973; Clark & Peck, 1979). However, it has been suggested that the low affinity sites which are abundant may serve to maximise the probability of nuclear localisation of the receptor complexes and the small numbers of high affinity sites are involved in the long term retention (>6h) of the oestrogen-receptor complexes (Clark & Peck, 1976).

Chromatin Binding Sites

Steroid receptor complexes have been demonstrated to bind to both transcriptionally active (Starthling & O'Malley, 1976) and inactive (Sala-Trepat et al., 1977; de Boer et al., 1978) chromatin. However, Davies et al.,(1980) found that the acceptor sites for the androgen receptor were present in the transcriptionally active fraction, but due to the relative proportions of the two chromatin fractions, the majority of the binding appeared to be present in the inactive chromatin. It has been suggested that these discrepencies in intra-chromatin distribution of acceptor sites may be due to methodological artefacts (Davies et al., 1980). The binding of oestrogen- and androgen- receptor complexes to chromatin was found to be more frequently associated with nucleosome units (Massol et al., 1978; Davies et al., 1980).

Since the initial demonstration that receptors could bind to chromatin (O'Malley et al., 1971), components of chromatin have been studied to identify the putative acceptor. The studies of Kallos et al., (1981) revealed that the oestrogen receptor from the rabbit uterus bound with differing affinities to histone protein fractions. Using controlled proteolytic digestion they were able to distinguish the stable histone-oestrogen-receptor binding sites from the protease-sensitive DNA binding sites. Furthermore, the binding to the H2B histone fraction had the most stabilising effect on the oestrogen-receptor-DNA interaction. Thus, they concluded that there were distinct sites on the oestrogen receptor for hormone, histone protein and DNA binding. They suggested that histones stabilise the binding of oestrogen receptor to high affinity sites on the DNA by providing additional specificity determinants on the chromosome.

There is also evidence in the literature suggesting that non-histone proteins of chromatin may be the nuclear acceptor sites (Spelsberg et al., 1972; King & Gordon, 1972; Puca et al., 1974, 1975; Mainwaring et al., 1976). The non-histone acidic proteins were demonstrated to have the acceptor capacity and their removal decreased progesterone receptor binding to chick oviduct chromatin to levels seen in non-target tissue chromatin (Spelsberg et al., 1972, 1976a). They also showed that removal of histone proteins did not unmask acceptor sites. From subfractionation and reconstitution experiments they identified the AP3 fraction of the acidic proteins of oviduct chromatin to have the acceptor capacity, that is, high affinity binding (O'Malley et al., 1972; Spelsberg et al., 1972, 1976b). This acceptor activity is probably completely masked in non-target tissues(O'Malley et al.,1972; Spelsberg & Toft, 1976). Similar acidic-protein acceptor activity has also been demonstrated for the human breast tissue oestrogen receptor (Charreau & Baldi, 1977). However, chromatin reconstitution, on which these studies are based, may give rise to artefactual distribution of acceptor sites (Fulmer & Fasman, 1979; Stein et al., 1975).

Receptor-DNA Interaction

Evidence for the involvement of DNA in the acceptor activity has come from studies in which nuclease treatment destroyed acceptor activity (Shyamala, 1971; King & Gordon, 1972). King and Gordon (1972) further demonstrated that de-histoned chromatin exposed additional acceptor sites confirming the results of Spelsberg et al.,(1971). Therefore, both DNA and chromatin proteins appear to be involved in acceptor activity and their precise three-dimensional conformation may be important for the interaction with receptor.

Recently, it has been demonstrated that oestrogen- and androgen-receptor complexes can bind to specific acceptor sites on the DNA associated with the nuclear matrix, a salt-insoluble and nuclease resistant nuclear fraction, of their target tissues (Buttyan et al., 1983). There is a suggestion that active hormone regulated genes seem to be located on the nuclear matrix (Ciejek et al., 1973). Other studies have shown that receptors for progesterone and glucocorticoids can recognise specific DNA sequences in vitro (Pfahl, 1982; Compton et al., 1983). In binding studies using nitrocellulose filter adsorption of [^{32}P]DNA, the progesterone receptor complex shows a preference for A,T (adenine,thymine) rich regions of DNA (Hughes et al., 1981; Compton et al., 1983). Using synthetic DNA with different sequences, Kallos and Hollander (1978) have demonstrated that the oestradiol receptor from the rabbit uterus binds preferentially to A,T rich segments of double-stranded DNA. Kumar et al.,(1980, 1983) and Thanki et al., (1978) have shown a rank-order of binding of uterine cytosol oestradiol-receptor to oligodeoxynucleotides covalently linked to cellulose; the relative binding order being oligo(dG) > oligo(dT) > oligo(dC) >> oligo(dA) > oligo(dI). Similar results have been obtained in our laboratory with the relative binding order being oligo(dG) > oligo(dT) > oligo(dC) > oligo(dI) > oligo(dA) (Hyder et al., 1984). These studies suggest that steroid receptors may act in a manner analogous to some procaryotic regulatory proteins which recognise specific DNA sequences in or near the promoter regions of genes under their control, and by binding to those regions they modulate the activity of the adjacent promoter. It has been demonstrated that the nuclear oestrogen-receptor complex from the chick oviduct preferentially binds to a DNA fragment (containing A,T rich sequences) situated upstream from the 5' end of the gene containing the oestrogen-dependant

hypomethylation site (Jost et al., 1984). There is also some evidence that the progesterone-mediated induction of transcription in untransformed oviduct cells depends on the progesterone receptor binding to the promoter region (5' flanking sequence) of the ovalbumin gene (Dean et al., 1983).

In conclusion, the role of the acceptor site ascribed to various chromatin fractions may be to locate the receptor complexes around specific genes under their control, while the nucleotide specificity of the receptor-complex (Andre et al., 1976; Kallos & Hollander, 1978; Thanki et al., 1978; Kumar et al., 1980, 1983) may be involved in the recognition of specific DNA sequence.

'Unoccupied' Nuclear Oestrogen Receptors

Unoccupied nuclear receptors have been demonstrated in the rat uterus (Thrower et al., 1981), hypothalamus (White & Lim, 1980a) and in human breast tumours (Zava & McGuire, 1977; Garola & McGuire, 1977; Panko & McLeod, 1978). Zava and McGuire showed that these unoccupied sites were present in the nucleus despite growing the MCF-7 cells in medium without oestradiol. Clark et al., (1972a) demonstrated that one hour after oestradiol injection into rats (which would result in maximal nuclear accumulation of receptor), there was a 20% rise in unoccupied nuclear sites in the hypothalamus and pituitary. Similar observations have been made at 6h post-injection in the rat hypothalamus by White & Lim (1980b). The functional significance of these unoccupied receptors remains to be established, however, their nuclear localisation suggests an involvement in the physiological response. Interestingly the proportion of unoccupied nuclear oestrogen receptor increases during progesterone antagonism of oestradiol stimulation (White et al., 1982; MacDonald et al., 1983).

Fate of the Nuclear Oestrogen Receptor

The fate of the oestrogen-receptor complex after its interaction with nuclear components is not known. It has been suggested that the complex is released from chromatin (Clark & Peck, 1976) and that free oestradiol is released (Puca & Bresciani, 1968) which would ensure propagation of the hormonal responses. It is possible that the steroid is released with the inactivated receptor from the nucleus to prevent recycling of the receptor.

Receptor Processing

After oestradiol binds to its cytoplasmic receptor and is translocated to the nucleus, there is a progressive loss of nuclear receptor without a concomitant restoration of cytoplasmic sites, before a steady state is established. Horwitz & McGuire (1978a) have described this nuclear event as 'processing' of oestrogen receptor (figure 6). They showed that induction of progesterone receptor in the human breast cancer cell line, MCF-7, correlated with oestrogen receptor processing during oestradiol stimulation and when processing stopped, the level of progesterone receptors fell. Processing does not involve protein synthesis (Horwitz & McGuire, 1980). The mechanism of processing is not understood. It may be a dynamic equilibrium between degradation and de novo synthesis of receptor. Kassis and Gorski, (1983) have suggested that processing is not a disappearance of the nuclear receptors, but rather a receptor modification to a less KCl-soluble and less-exchangeable form characterised by a slow rate of dissociation of the oestrogen-receptor complex and more rapid sedimentation in sucrose density gradients.

Receptor Recycling and Replenishment

The initial depletion of cytoplasmic receptor following translocation of the oestradiol-receptor complex to the nucleus is followed by a gradual replenishment of receptors in the cytoplasm (figure 6). Using protein and RNA synthesis inhibitors, Clark & Peck (1976) have demonstrated, following oestradiol injection in immature rats, that a small proportion (approximately 40%) of the receptor was recycled. Similar observations have been made by other workers (Mester & Baulieu, 1975; Cidlowski & Muldoon, 1978). Receptor replenishment has therefore been proposed to involve both recycling and de novo synthesis of receptor.

Kassis and Gorski (1981) have shown that injection of the 'short-acting' oestrogen, 16α-oestradiol, stimulated the early responses and that complete recycling occurred within 4h in the presence of cycloheximide. They suggested that receptor processing was not mandatory for uterine growth. An alternative explanation for these observations could be that 16α-oestradiol has a shorter biological half life than oestradiol and probably does not bind to transcriptionally active chromatin sites in the nucleus, and that only receptor complexes bound to transcriptionally active chromatin may be processed.

In conclusion, binding of steroid-receptor complex to nuclear acceptor sites and the subsequent replenishment of cytosol receptor may involve recycling as well as de novo synthesis of receptor.

Inactivation of the Nuclear Oestrogen Receptor

A possible mechanism to control the intracellular levels of active receptor has been proposed by Auricchio et al., (1980). They demonstrated the presence of a phosphatase enzyme, in the nuclei of the mouse uterus and mammary gland, capable of inactivating the cytosol oestrogen receptor *in vitro*. They showed that the same nuclear phosphatase also inactivated the nuclear oestrogen receptor (Auricchio et al., 1980, 1981a, 1981b) and is only present in oestrogen target tissues. This enzyme has a high affinity both for the oestradiol-receptor complex and the unbound receptor. Its inactivating activity was inhibited by phosphatase inhibitors as well as 4-nitrophenyl phosphate, a phosphatase substrate (Auricchio et al., 1981a, 1981b). This suggests that nuclear inactivation of receptor occurs via a dephosphorylation process. Auricchio et al., (1981, 1984) and Migiliaccio et al., (1982) also demonstrated that the receptor inactivated by the nuclear phosphatase could be reactivated (to a hormone binding state) by an ATP-dependent cytoplasmic enzyme. Therefore, they have concluded that the oestrogen receptor is a phosphoprotein and the hormone binding activity of the receptor is regulated by a phosphorylation-dephosphorylation process. Recently these authors demonstrated that the oestrogen receptor was phosphorylated on the tyrosine residues (Migiliaccio et al., 1984). There is also evidence for such a mechanism of regulation for the glucocorticoid receptor (Pratt et al., 1979).

Nuclear receptor-antioestrogen complexes exhibit long-term nuclear retention in MCF-7 cells (Horwitz & McGuire, 1978b) and rat uterus (Jordan et al., 1980), and have been shown to be stable *in vitro* towards nuclear phosphatases (Auricchio et al., 1981). The latter proposed that

the antioestrogens act by protecting the receptor in the nuclear compartment against dephosphorylation.

Progesterone modifies and/or antagonises oestrogen action (Lerner, 1964; Hsueh et al., 1975). It does not interfere with the initial binding of oestrogen to its cytoplasmic receptor, or the subsequent translocation of the oestrogen-receptor complex to the nucleus (Toft & Gorski, 1966; Anderson et al., 1972). It has been suggested that progesterone antagonism of oestrogen action results from an inhibition of cytosol receptor replenishment which would decrease the availability of cytosol receptor for binding and subsequent translocation (Clark et al., 1977). Recent evidence suggests that progesterone reduces nuclear levels of oestrogen receptor, possibly by the induction of a factor, termed 'oestrogen-receptor regulatory factor', (ReRF) (Evans & Leavitt, 1980; Okulicz et al., 1981). This ReRF may be involved in the processing of the nuclear oestrogen receptor. MacDonald et al., (1982) have demonstrated that ReRF action on oestrogen receptor is mediated by an acid phosphatase. This suggests that progesterone antagonism of oestrogen action may be through a dephosphorylation of the nuclear receptor complex. This view would be in agreement with the nuclear phosphatase activity involved in inactivating the nuclear oestradiol-receptor complex described by Auricchio et al.,(1981, 1984). It is not clear whether the ReRF itself is a phosphatase involved in the inactivation of the receptor, or that it modulates the activity of this acid phosphatase.

Mechanism of Antioestrogen Action

Antioestrogens have been shown to be effective in suppressing the growth and development of hormone-dependant mammary tumours in the rat

(Katzenellenbogen et al., 1979; DeSombre & Arbogast, 1974; Jordan & Allen, 1980; Nicholson & Golder, 1975), inhibiting the growth of some human breast cancer cell lines that contain oestrogen receptors (Lippman et al., 1976; Edwards et al., 1980), and evoking the regression of some human breast cancers (Horwitz & McGuire, 1978a,b; Bloom & Boesen, 1974; Heuson, 1976; McGuire et al., 1978). Antioestrogens interact with the cytoplasmic oestrogen receptor, promote translocation and nuclear binding of the receptor-complex (Clark & Peck, 1976; Sutherland & Jordan, 1981; Coezy et al., 1982), and induce some, but not all, oestrogen-specific responses (Ruh & Baudendistel, 1978). However, the precise mechanism by which antioestrogens evoke tumour regression remains unclear.

Several hypotheses are available to explain the antagonistic activity of these non-steroidal antioestrogens. The first is that the binding of antioestrogens is less efficient than oestradiol at activating the oestrogen receptor and triggering biological responses. One of the criteria for activation of oestrogen receptor is an increased stability of the ligand receptor-complex as evidenced by a decrease in the dissociation rate constant. There is evidence in the literature which indicates that differences exist between oestrogen receptor activation in the presence of oestrogen and antioestrogens (Rochefort & Borgna, 1981; Rochefort et al., 1983). Rochefort & Borgna (1981) have shown that heating of cytosol preparations decreased the dissociation rate of oestradiol, but not that of antioestrogens from the oestrogen receptor. Studies from our laboratory, using mature rat uterine cytosol, show that the dissociation kinetics of the oestrogen receptor complexed with oestradiol, Tamoxifen (Tam) or 4-hydroxy-tamoxifen (OH-Tam) (an active metabolite of Tamoxifen) is biphasic, with a fast and a slow dissociating form (White et al., 1984). The data of

Rochefort & Borgna (1981) suggest that, in lamb uterine cytosol, antioestrogen-oestrogen receptor complexes follow single component dissociation kinetics. White et al., (1984) have also observed that a greater proportion (40-50%) of the antioestrogen-oestrogen receptor complexes dissociate with slow dissociation kinetics compared to oestradiol receptor complexes (25%). The antioestrogens probably act as allosteric (Bullock et al., 1978) ligands which stabilise the oestrogen receptor in an inactive or partially activated form, whereas oestrogens stabilise the receptor either in the fully activated form, or a form which can become fully functional.

A second criterion for oestrogen receptor activation, in vitro, is the ability of the receptor complex to bind to nuclei, DNA or polyanions. Evans et al., (1982) have shown that the oestrogen receptor complexed to the antioestrogen, OH-Tam, had a lower affinity for double stranded DNA than the oestradiol receptor complex, although the maximum proportion of oestrogen receptor complexes able to bind to DNA were similar. Using oligo(dT)-cellulose binding ability of oestrogen receptor to determine the extent of activation, results from our laboratory show a time dependent increase in activation of oestrogen receptor complexed with oestradiol (~6% at 1hX4^{0}C, compared to ~25% at 24hX4^{0} C) (Myatt et al., 1984). However, antioestrogen-oestrogen receptor complexes did not exhibit this time dependent increase in activation, but the initial proportion of OH-Tam-oestrogen receptor complexes bound to oligo(dT)-cellulose (~40%) was higher than the oestradiol-receptor complexes; only about 6% of the Tam-oestrogen receptor complexes were activated.

The interaction of oestradiol with its receptor may facilitate the binding of a second subunit, described by Yamamoto (1974), or the

putative activating factor proposed by Myatt et al., (1979b) and Thapman and Clark, (1981) which activates the receptor. However, interaction of antioestrogens with the oestrogen-receptor may not facilitate binding of this second subunit, thus resulting in aberrant activation of the receptor complex. It is noteworthy in this context that the B16 monoclonal antibody developed against the calf uterine oestrogen receptor (Greene et al., 1980) could discriminate between oestrogen receptor complexed with oestradiol and OH-Tam (Borgna et al., 1982). The absence of the second subunit in the OH-Tam-receptor complex may form the basis for this distinction.

Horwitz and McGuire (1978b) have suggested that impaired nuclear processing may be the basis for antioestrogen action. They showed that nuclear receptors complexed to the antioestrogen, Tam, were only partly (30%) processed compared to the nuclear-oestradiol-receptor complexes (70%), while the nafoxidine-bound nuclear receptors were not processed at all. Auricchio et al., (1981) have shown that nuclear receptor-antioestrogen complexes are not inactivated by nuclear phosphatases in vitro. It has been shown in MCF-7 cells and rat uterus that nuclear receptor-antioestrogen complexes exhibit atypical long-term nuclear retention (Horwitz & McGuire, 1978b; Jordan et al., 1980). Unlike the nuclear oestradiol-receptor complex, replenishment of the oestrogen receptor in the cytosol does not occur with antioestrogen-bound nuclear receptors suggesting that antioestrogen-oestrogen receptor complexes are dysfunctional (Katzenellenbogen et al., 1981). Differences in the salt-extractability of nuclear receptor-oestrogen complexes versus nuclear receptor-antioestrogen complexes (Baudendistel & Ruh, 1976; Ruh & Baudendistel, 1977; Katzenellenbogen et al., 1978) may be a

manifestation of genuine differences in chromatin interaction of these compounds.

A plausible mechanism of oestrogen action at the nuclear level could be that the oestradiol-receptor complex interacts with the genome in a manner similar to the 'ping-pong' mechanism of enzyme action. Oestradiol-receptor complexes bind to regulatory sites on the genome, initiate certain events and are released and/or processed before the next set of receptor complexes bind and initiate subsequent events; these in turn are released and/or processed to make way for the next set of receptor complexes, etc. A sequence of such events may be necessary to evoke a complete oestrogenic response and may explain why there is a discontinuous requirement of oestrogen for a complete oestrogenic response (Harris & Gorski, 1978). It has been proposed that continual 'processing' of nuclear receptor may be an active step required for continued oestrogen response (Clark et al., 1973; Edwards et al., 1979). However, antioestrogen-oestrogen receptor complexes which may be non-activated or only partially activated are initially able to interact with the genome, but are not subsequently released and/or processed thus blocking the genomic sites for further binding of receptor complexes to propagate the response. This view would be supported by the observations that oestrogen receptors complexed with antioestrogens are only partially processed (Horwitz & McGuire, 1978b), stable <u>in vitro</u> towards inactivation by nuclear phosphatases (Auricchio et al., 1981) and are therefore retained for much longer in the nucleus compared to the oestradiol-receptor complex. This would perturb the subcellular distribution of the oestrogen receptor, maintaining over 90% in the nucleus and very low levels in the cytoplasm, and the endogenous oestrogens are thus unable to initiate subsequent rounds of receptor translocation and sustain sensitivity to support growth of the tumour.

F: Regulation of Receptor Activation

Assessment of Activation of Cytoplasmic Oestrogen Receptor

Activation is defined as the 'increase in the affinity of the oestrogen receptor complex for nuclei, DNA or other polyanions' (Toft, 1972; Buller et al., 1975; Parchman & Litwack, 1977; Miller & Toft, 1978). It can be assessed *in vitro* by the ability of the cytosol oestradiol-receptor complex to bind to nuclei (Buchi & Villee, 1976; Haselbacher & Eisenfeld, 1976), chromatin (Steggles et al., 1971), DNA (Rousseau et al., 1975) and to synthetic oligonucleotide matrices such as oligo(dT)-cellulose or DNA-cellulose (Mainwaring & Irving, 1973; Yamamoto & Alberts, 1974; Thrower et al., 1976; Park & Wittliff, 1977), ATP-Sepharose (Miller & Toft, 1978) and phosphocellulose (Fleischmann & Beato, 1979). Activation can also be assessed by analysis of the dissociation kinetics of the oestrogen-receptor complexes (Weichman & Notides, 1977, 1979; Myatt et al., 1982a). The dissociation of [^{3}H]-oestradiol from the receptor follows biphasic (two component) kinetics generated by the non-activated and the activated forms of the receptor (Weichman & Notides, 1977, 1979). These investigators showed that the non-activated, 4S form of the receptor gave the first rapid exponential dissociating component, while the activated 5S receptor exhibited the second, slower dissociating component. Heat activation of the receptor resulted in the loss of the fast dissociating component (Weichman & Notides, 1977). Oestradiol binding and activation of the oestrogen receptor shifts the equilibrium to a high affinity, activated, slower-dissociating phase (Notides et al., 1981). Myatt et al., (1982a) demonstrated that dilution and dialysis of rat uterine oestrogen receptor promoted binding of oestrogen-receptor complexes to

oligo(dT)-cellulose and resulted in an increase in the proportion of the slow-dissociating component.

Activation and Transformation

The terms 'activation' and 'transformation' have been used interchangeably in the literature and this has led to much confusion. Weichman and Notides (1977) referred to the alteration in sedimentation coefficient from 4S to 5S (transformation) as 'activation' of the receptor. However, Bailly et al., (1980) have shown that activation preceeds transformation of the oestrogen receptor, confirming the earlier results of Buchi and Villee (1976). They described 'activation' as an increase in the affinity of the receptor for nuclear acceptor sites, which is distinct from 'transformation' which results in an increase in size from 4S to the 5S form. The studies of Muller et al., (1983), Milgrom et al., (1981), and Gschwendt & Kittstein, (1980) have come to similar conclusions. A variety of manipulations such as salt treatment, dilution, aging or alkaline pH activate oestrogen receptors (Milgrom, 1981; Grody et al., 1982). All receptors show a temperature sensitive activation step, which results in an increased affinity for binding to nuclear acceptor sites. However, the accompanied transformation process appears to be unique to the oestrogen receptor (Notides & Nielsen, 1975; Yamamoto & Alberts, 1975; Gorski & Gannon, 1976; Chan & O'Malley, 1976; Bailly et al., 1980; Jensen & DeSombre, 1973). The transformation process has also been shown to be temperature sensitive *in vitro*, but it requires much longer incubation at elevated temperature (15-25^{o}C) compared to activation (Buchi & Villee, 1976).

Structure of the 5S Receptor

The activation of the oestrogen-receptor complex is accompanied by transformation of the 4S receptor to the 5S form, resulting in a two-fold increase in molecular weight (King & Mainwaring, 1974; Notides & Nielsen, 1974; Thrower et al., 1976). It is generally agreed that the 5S receptor is a dimer, but there is disagreement as to whether it is a homodimer (composed of two 4S monomers) (Notides et al.,1981), or a heterodimer (composed of a 4S monomer associated with a non-steroid binding protein of identical molecular size) (Yamamoto, 1974; Clark & Peck, 1979; Myatt et al., 1979b; Thrower et al., 1976; Thapman & Clark, 1981).

Formation of the 5S receptor complex may not occur in neoplastic breast tissue. Fazekas and Mcfarlane (1980) observed a 2.5S receptor on incubation of breast tumour cytosol with [^{3}H]-oestradiol at 30^{0}C for 30 min. (conditions that normally promote activation). However, they did not assess the 'activation' of the receptor. Park and Wittliff (1977) demonstrated that the temperature-induced activation of the cytosol oestrogen receptor from rat mammary gland did not show the 4S to 5S transformation. This suggests that there are differences in the 'activation' of the oestrogen receptor and that the 4S receptor can exist in the non-activated and activated forms. It is possible that both 4S and 5S receptor forms can be activated, according to their ability to bind to oligo(dT)- or DNA- cellulose, but only the 5S receptor complex is capable of inducing the complete biological response.

Low Molecular Weight Inhibitor(s) of Activation/Transformation

Evidence for the involvement of a low molecular weight inhibitor of activation of the oestrogen receptor in rat uterus and mouse leydig cell tumours has been reported (figure 6) (Sato et al., 1978, 1979; Shen et al., 1979; Myatt et al., 1979b). Similarly, the presence of a low molecular weight inhibitor of glucocorticoid receptor activation in rat liver (Bailly et al., 1977; Goidl et al., 1977) and androgen receptor activation in rat ventral prostate (Sato et al., 1980) has also been reported. Sato et al., (1980) showed that heating, dialysis or gel filtration of rat uterine cytosol containing prelabelled oestradiol-receptor complexes resulted in an increase in activation and transformation of the 4S to the 5S receptor. They concluded that these manipulations removed a low molecular weight inhibitor(s) probably bound to the receptor. Myatt et al., (1982a) have also shown that dilution and dialysis promoted activation of the rat uterine cytosol oestradiol-receptor complexes. Enhanced activation of glucocorticoid receptors as a result of dilution has also been demonstrated in rat liver cytosol (Bailly et al., 1977; Goidl et al., 1977). Shyr and Liao (1978), using the androgen receptor system in ventral prostate of rats, have also described an inhibitor in the cytosol which impedes binding to nuclear chromatin. Fishman (1981) demonstrated that electrolysis of the rat uterus resulted in an increase in nuclear binding of oestrogen receptor, a result he attributed to the removal of an inhibitor. Notides and Nielsen (1975) suggested that physiological salt concentration promotes dissociation of the inhibitor from the 4S receptor and that rapid desalting or dialysis facilitated the interaction of the 4S receptor to the second subunit to form the 5S receptor.

The presence of a low molecular weight inhibitor may explain the temperature dependence of 5S formation and the temperature- sensitive activation observed by Buchi and Villee (1976). Removal of this inhibitor may facilitate the interaction of subunit X (Yamamoto, 1974) or a putative activating factor (Thrower et al., 1976; Myatt et al., 1979b; Thapman & Clark, 1981) with the 4S receptor to give the 5S form. Alternatively, loss of this inhibitor may result in steric modification of the 4S receptor allowing the interaction of the subunit X or putative activating factor resulting in the formation of the 5S receptor. The steric modifications induced by binding of steroid and loss of the inhibitor may be very important in determining the extent of the physiological response to oestrogen. Thus, oestrone, which has a lower affinity for receptor than oestradiol, and does not promote 4S to 5S transformation, does not give a complete oestrogenic response (Yamamoto & Alberts, 1972; Weichman & Notides, 1980). Collectively, these data suggest that activation and transformation are intimately connected and that the role of the effector ligand is to generate a receptor species which is capable of being transformed by interaction with a second subunit.

The presence of a low molecular weight inhibitor of activation appears to be a common feature of steroid receptor function. The requirement of steroid for the permanent dissociation of this moiety suggests that it may be serving a regulatory function in the receptor mediated action of steroids such that in the absence of ligand, activation of the receptor does not occur and therefore there is no translocation or transformation of the receptor.

Modulation of Activation/Transformation

Evidence derived from the use of the phosphoprotein phosphatase inhibitor, sodium molybdate, has suggested that activation of receptor may involve a phosphorylation-dephosphorylation mechanism. Molybdate affects several properties of steroid receptors that are related to the activation/transformation process. Under incubation conditions (25-30°C for 30 min.) that normally promote activation (and transformation), the presence of molybdate inhibited transformation of the non-activated 4S receptor to the activated 5S form (Shyamala & Leonard, 1980; Mauck et al., 1982) and markedly increased the magnitude of the fast [^{3}H]-oestradiol dissociating component of the receptor indicating inhibition of receptor activation (Mauck et al., 1982; Myatt et al., 1982a). However, a small proportion of the receptor (10-20%) consistently exhibited slow dissociation in the presence of molybdate, equivalent to the amount bound to oligo(dT)-cellulose (Myatt et al., 1982a). This may represent receptor that was already activated *in vivo*.

Chong and Lippman (1982) found that molybdate only partially inhibited the ATP induced transformation of the non-activated oestrogen receptor in MCF-7 cell cytosol. Similar results have been reported for the rat liver glucocorticoid receptor (John & Moudgil, 1979).

The precise mechanism of molybdate inhibition of receptor activation is unknown. However, molybdate is an effective phosphatase inhibitor and this mode of action has been suggested for its effects on activation of glucocorticoid (Nielson et al., 1977a, 1977b) and oestrogen (Auricchio et al., 1980) receptors. Therefore, molybdate appears to act by inhibiting dephosphorylation of the receptor or a factor associated with the receptor which is required for activation (Sando et al., 1979a, 1979b). However, only phosphatase inhibitors

chemically similar to molybdate, such as tungstate and vanadate, are able to inhibit activation of the chick oviduct progesterone receptor; other phosphatase inhibitors, levamisole,sodium fluoride, arsenate and chromate have no effect (Nishigori & Toft, 1980). Moncharmont et al., (1982) have shown that molybdate protects against heat inactivation (37°C for 5 min.) of the cytosol oestrogen receptor from calf uterus, but fluoride, p-nitrophenyl phosphate and arsenate which are classical inhibitors of phosphatases have no protective effect. However, inactivation of the glucocorticoid receptor from a steroid binding state to a non-binding state is sensitive to all major phosphatase inhibitors (Nielson et al., 1977a, 1977b). Therefore, these results argue against molybdate acting as a phosphatase inhibitor of oestrogen- and progesterone- receptor activation.

Chong and Lippman (1982) demonstrated that the non-metabolisable analogues of ATP and GTP, adenylylimidodiphosphate (AIDP) and guanylylimidodiphosphate (GIDP), promoted receptor activation. Similar observations have been made in our laboratory (Myatt et al., 1985). This suggests phosphorylation of the receptor complex during activation is unlikely.

Receptor activation has been proposed to involve loss of a low molecular inhibitor from the receptor (Sato et al., 1978, 1979; Myatt et al., 1982a). The inhibitory action of molybdate on receptor activation may be by preventing the dissociation of the inhibitor from the receptor (Noma et al., 1980), whereas the ribonucleotide triphosphates may interact with the receptor or the inhibitor to facilitate dissociation of the low molecular weight inhibitor from the receptor. An ionic effect of ATP on activation of both glucocorticoid- and oestrogen-

receptors has been suggested (Holbrook et al., 1983a; Myatt et al., 1985).

Other possibilities include molybdate complexing with sulphydryl and/or phosphate moieties of the receptor (Leach et al., 1979; Sando et al., 1979b; Nishigori & Toft, 1980) which can block receptor activation. Molybdate has been shown to interact with thiol groups of cysteine and imidazole groups of histidine (Weathers et al., 1979). The interaction of molybdate with heavy metals has been postulated in its inhibitory effect on particulate phosphoprotein phosphatase from mouse liver (Paigen, 1958); this kind of heavy metal interaction could be involved in a variety of protein activities and there is some evidence that receptors may be metalloproteins (Shyamala, 1975; Lohmar & Toft, 1975). In the avian progesterone receptor system, molybdate inhibits activation by elevated temperature, but not by high salt (Nishigori & Toft, 1980). It is, therefore possible that the action of molybdate involves ionic interactions that are diminished in the presence of high salt concentration.

Pyridoxal-5'-phosphate (pyridoxal-P) reacts with free amino groups, such as the e-amino groups of lysine residues to form a schiff base (Martial et al., 1975). It has been shown that pyridoxal-P inhibits activation of the oestrogen (Muller et al., 1980), progesterone (Nishigori & Toft, 1979) and androgen (Mulder et al., 1980) receptors. In the hepatic glucocorticoid receptor system, Cake et al., (1978) showed that pyridoxal-P not only inhibited activation of the receptor, but also inhibited the DNA binding ability when added immediately after heat activation. However, the results of Traish et al., (1980) suggested that pyridoxal-P had direct inhibitory effects on the activation of the uterine oestrogen receptor. Moreover, Henrikson et

al., (1981) showed that the mouse uterine oestradiol-receptor binding to various oligonucleotides was competitively inhibited by pyridoxal-P. This suggests that pyridoxal-P interacts with the polynucleotide binding domain of the steroid receptor complex and inhibits activation, although it is possible that allosteric effects induced as a result of interaction with a lysine residue in other regions may also be important.

CHAPTER TWO

METHODS AND MATERIALS

A: Materials

All general purpose reagents were of Analytical Reagent (AR) grade and were supplied by British Drug Houses (BDH) Ltd., Poole, Dorset unless otherwise stated.

Radioactive steroids were purchased from Radiochemical Centre, Amersham, Bucks., with the following specific activities:

[2,4,6,7n-^{3}H]-oestradiol	85-114 Ci/m.mol
[1,2,6,7n-^{3}H]-progesterone	80-104 Ci/m/mol

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

Diethylstilboestrol, (DES)

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

Hydrocortisone, [11B,17,21-Trihydroxypreg-4-ene-3,20-dione]

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

Calf thymus deoxyribonucleic acid (DNA), type 1

Dithiothreitol, (DTT) or Cleland's reagent

Ethylenediamine tetra acetic acid, (EDTA)

Phenyl methyl sulphonyl fluoride, (PMSF)

Adenosine-5'-triphosphate, (ATP)

Molybdic acid (sodium salt)

Oligo(dT)-cellulose was supplied by Collaborative Research Inc., Mass. U.S.A. (obtained through Uniscience Ltd., Cambridge).

Dextran T-70 was obtained from Pharmacia (G.B.) Ltd., Milton Keynes.

Liquid scintillation fluid, Unisolve I, was purchased from Koch-Light Laboratories Ltd., Haverhill, Suffolk.

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

Cryotubes were purchased from Gibco Ltd., Uxbridge, Middlesex.

Bovine serum albumin (BSA) fraction IV was obtained from Miles Laboratories Ltd., Slough.

Calibration proteins for high performance size exclusion gel chromatography were obtained from Boeringer Mannheim GmBH, Mannheim, W.Germany.

Radioactive, molecular weight marker, protein standards were purchased from New England Nuclear (NEN), Dreieich, W.Germany, with the following specific activities:

Ovalbumin, [methyl-^{14}C] methylated-	0.012 mCi/mg
Albumin (bovine serum), [methyl-^{14}C] methylated-	0.016 mCi/mg
Phosphorylase B, [methyl-^{14}C] methylated-	0.012 mCi/mg
Globulins, [methyl-^{14}C] methylated-	0.0088 mCi/mg
Myosin, [methyl-^{14}C] methylated-	0.013 mCi/mg

B: Apparatus

Tumour tissue was pulverised after freezing in liquid nitrogen using a Mikro-Dismembrator II from B.Braun Melsungen AG, W.Germany (supplied by F. T. Scientific Instruments Ltd., Tewkesbury, Glos.).

Centrifugation procedures were performed using the MSE 'coolspin' and Prepspin 65 centrifuges (Fisons, Crawley, Sussex).

Spectrophotometric determinations were made using a Gilford 252 Spectrophotometer (Gilford Instruments Ltd., Teddington, Middx.)

High performance size exclusion chromatography was performed using spherogel-TSK G3000 SW columns fitted with a guard column (supplied by Anachem Ltd., Luton, Bedfordshire) connected to a Model: 6000A solvent delivery pump (Waters Associates (Instruments) Ltd., Hartford, Northwich, Cheshire) and fractions collected using an automatic fraction collecter, FRAC-100 obtained from Pharmacia (G.B.) Ltd., Milton Keynes.

Radioactivity was monitored using an Intertechnique SL 3000 Liquid Scintillation counter (Intertechnique Ltd., Uxbridge, Middx.).

All weighing procedures were carried out using either a model H16 Mettler Analytical Balance (supplied by A. Gallenkamp & Company Ltd., London), or a Sartorius (model 1204 MP) top-pan balance (Sartorius-Werke GmBH, Gottingen, W.Germany).

C: Methods

Measurement of Radioactivity

Radioactivity extracted into ethanol (2ml) was counted in 10ml toluene containing 4.5g/l butyl-PBD [2-(4'-tert.-Butylphenyl)-5 (4''-biphenyl)-1,3,4-oxadiazole 'Scintran']. Aqueous samples were counted in Unisolve I scintillation fluid (0.4-2.0ml aqueous phase per 4.0ml scintillant). Activity was expressed in counts per minute (c.p.m.) using external standard ratio counting coupled to quench correction curves, counting at an efficiency of approximately 50%.

Breast Tumour Specimens

Tumour samples were obtained from patients undergoing surgical biopsy of breast lumps or mastectomy in the Breast Unit of the Department of Surgery at Hammersmith Hospital. Excised tissue was collected on ice and taken to histopathology where a representative section of the tumour adjacent to that taken for histology (i.e., a mirror image) was provided by the histologist. Back at the laboratory, excess fatty and necrotic tissue and blood was removed and the tumour tissue was frozen and stored in liquid nitrogen prior to assay. Routine analysis of stored specimens was carried out within two weeks of sample reciept.

Homogenisation of Tumour Tissue

Tumour tissue (approximately 0.3 - 0.5g) was placed in a 7ml teflon vial together with a steel ball and sealed. After freezing in liquid nitrogen for 3 min, the sealed teflon vial was removed and fastened onto the mikro-dismembrator and vibrated at optimum amplitude

(13 mm displacement) for 1 min to pulverise the tissue. The powderised tissue was then transferred to a weighed polystyrene tube which was re-weighed. The tissue powder was suspended in 9 volumes (w/v) of ice cold PEDG buffer [10 mM sodium phosphate buffer containing 1.5 mM EDTA, 1 mM DTT and 30% (v/v) glycerol, pH 7.4 at 4°C]. The resulting homogenate was then fractionated.

Subcellular Fractionation

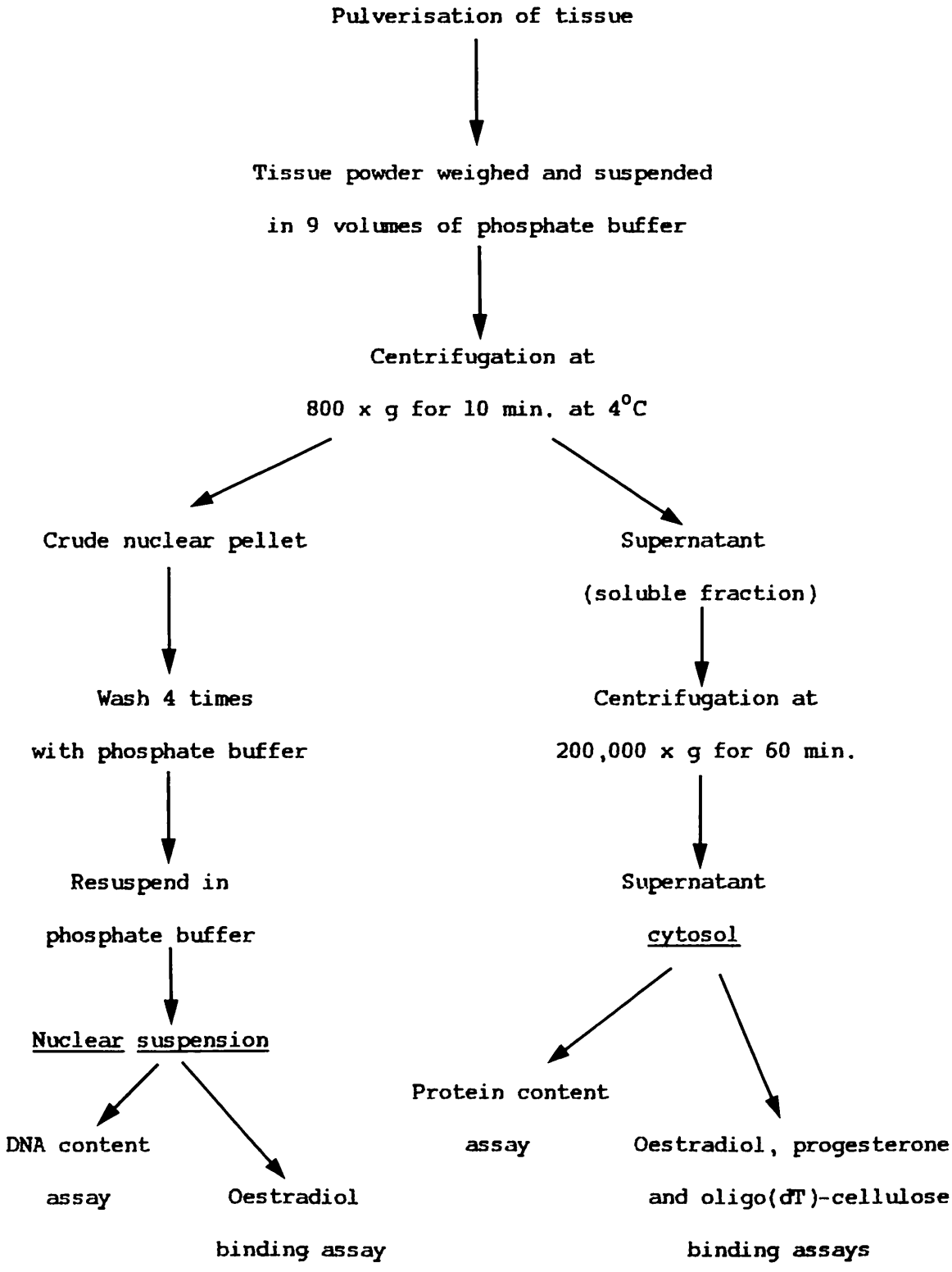
All procedures were carried out at 0-4°C unless otherwise stated. The homogenate was centrifuged at 800 x g for 10 min in a MSE coolspin to yield a crude nuclear pellet (see figure 8). The supernatant was removed and further centrifuged in a MSE Prepspin 65 at 200,000 x g for 60 min to give a cytosol and a microsomal pellet. The supernatant was carefully removed, leaving any fat behind and used for oestradiol, oligo(dT)-cellulose and progesterone binding assays.

The crude nuclear pellet was dispersed in the original homogenisation volume of PEDG buffer and centrifuged at 800 x g for 10 min. The resulting supernatant was removed and discarded. This washing procedure was repeated three times. After the final wash, the pellet was reconstituted in the original volume of homogenisation buffer and mixed thoroughly. The resulting nuclear suspension was used for oestradiol binding assay.

Protein Estimation

The total protein concentration of cytosol preparations was measured using the method of Lowry et al., (1951). A standard reference solution of bovine serum albumin (BSA) was prepared at a concentration of 1mg/ml in distilled water, aliquoted into polystyrene tubes and kept

Figure 8: Subcellular Fractionation of Tissue Specimens



at -20°C. On the day of assay, an aliquot was thawed and diluted with phosphate buffer to give a range of standard protein concentrations from 0 to 140 ug/ml.

Reagents

1: A copper tartarate carbonate solution was prepared containing 2ml of 1% (w/v) sodium tartarte and 0.2ml of 5% (w/v) copper sulphate per 100ml of 2% (w/v) sodium carbonate in 0.1M NaOH.

2: Folin & Ciocalteau's phenol reagent (Folin's reagent) was diluted 1 in 3 volumes prior to assay.

Assay procedure

The copper tartarate carbonate solution (2.5ml) was added to 0.5ml of each protein solution (standards or cytosols) and the mixture allowed to stand for 15 min at ambient temperature. Then, 0.25ml of dilute Folin's reagent was added, the mixture shaken and allowed to stand at room temperature for 30 min before reading the absorbance at 720nm. A standard curve was constructed for the known protein standards and the protein concentration of the cytosols was derived from this standard curve. Routinely, the cytosols were diluted 10-fold and 100- fold and the protein content estimated in duplicate aliquots.

DNA estimation

The amount of DNA in the crude nuclear pellets was measured using the method of Burton (1956). A stock solution (400 ug/ml) of calf thymus DNA was prepared in 5mM NaOH and kept at 0-4°C. Prior to use, equal volumes of this solution and 1.0M perchloric acid (PCA) were mixed and hydrolysed at 70°C for 15min. From this hydrolysed stock solution a range of standard reference solutions, from 0 to 200 ug/ml, were prepared in 0.5M PCA.

The crude nuclear pellets (1ml of the nuclear suspension pelleted by centrifugation and the supernatant discarded) were similarly hydrolysed with 1ml of 0.5M PCA in a water bath at 70°C for 15 min before cooling to 4°C and centrifugation to isolate the hydrolysate.

Reagents

1: A solution of 1.5% (w/v) diphenylamine in glacial acetic acid, containing 1.5% (v/v) concentrated sulphuric acid was prepared on the day of assay. The solution was stored in a dark bottle, away from direct sunlight.

2: From a stock acetaldehyde solution of 0.78 g/ml, an aqueous solution of acetaldehyde with a final concentration of 16.2 mg/ml was prepared.

A reagent mixture containing 0.1ml of aqueous acetaldehyde per 20ml of diphenylamine solution was prepared and used for assay.

Assay procedure

One volume of the hydrolysate (usually 0.5ml) from either the reference standard or the crude nuclear pellets was mixed with two volumes of diphenlyamine solution and incubated at 30°C for 18h in a shaking water bath. Following incubation, the assay tubes were removed from the water bath and the absorbance monitored at a wavelength of 600nm and a standard curve constructed from the known DNA concentrations. The concentration of DNA in the crude nuclear pellets was derived by reference to this standard curve.

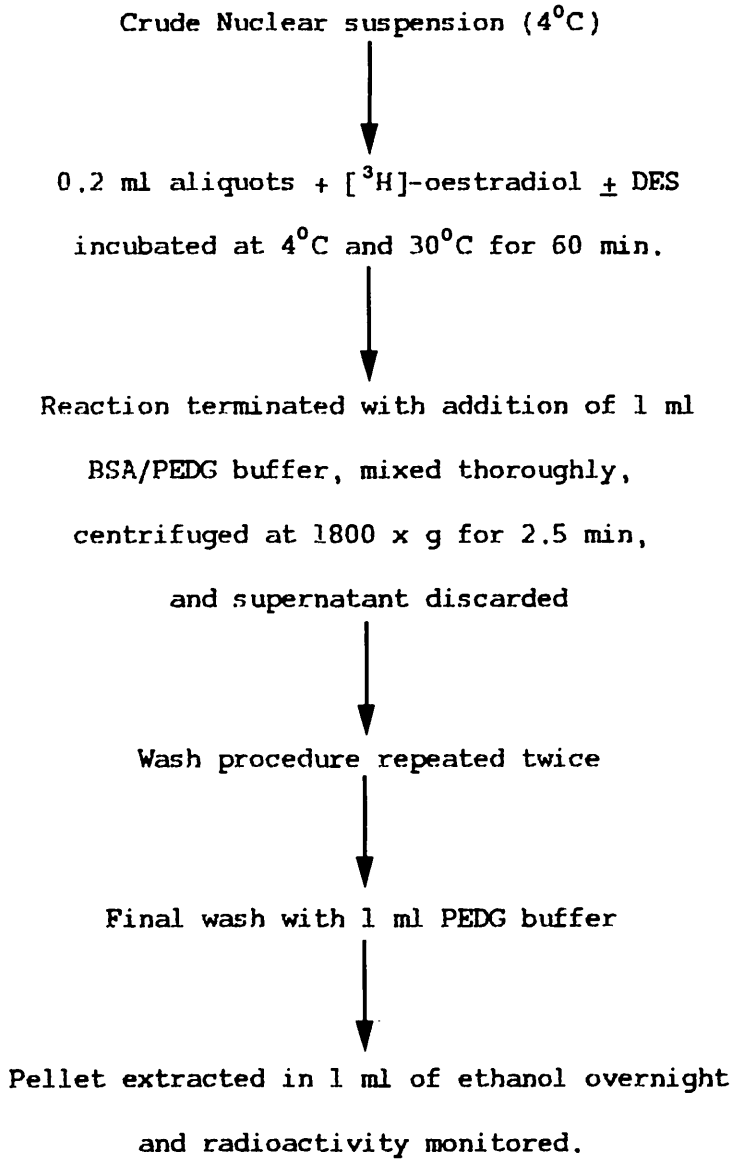
Receptor Assays

Nuclear Receptor Assay

Routinely, assays were carried out in triplicate: 0.2ml aliquots of the nuclear suspension were incubated with 5nM [^{3}H]-oestradiol, in the absence and presence of 1μM DES. During sampling, the nuclear suspension was constantly agitated. Binding studies were performed at 30^{0}C for 60 min to assess the total number of nuclear binding sites (i.e.,occupied and unoccupied) and at 4^{0}C for 60 min to estimate only the unoccupied sites (figure 9).

At the end of the incubation period, the nuclear binding assay was terminated by the addition of 1ml of ice-cold PEDG buffer containing 1% BSA to adsorb the free, unbound activity. After centrifugation in a MSE coolspin at 1800 x g for 2.5 min, the supernatant was removed and discarded. The pellet was resuspended in 1ml of ice-cold BSA/PEDG buffer, mixed thoroughly and re-centrifuged. This washing procedure was repeated two more times, followed by a final wash with PEDG buffer only. The [^{3}H]-oestradiol activity in the final pellet was extracted with 2ml of ethanol overnight at room temperature and transferred to a scintillation vial. The ethanol extracts were mixed with 10ml of toluene containing butyl-PBD and the radioactivity determined. Specific [^{3}H]-oestradiol binding to oestrogen receptor was determined from the difference in activity between samples without DES, which measures total binding, and that with DES which gives a measure of the nonspecific binding to nuclear components.

Figure 9: Nuclear Receptor Assay



Cytosol Oestrogen Receptor Assay

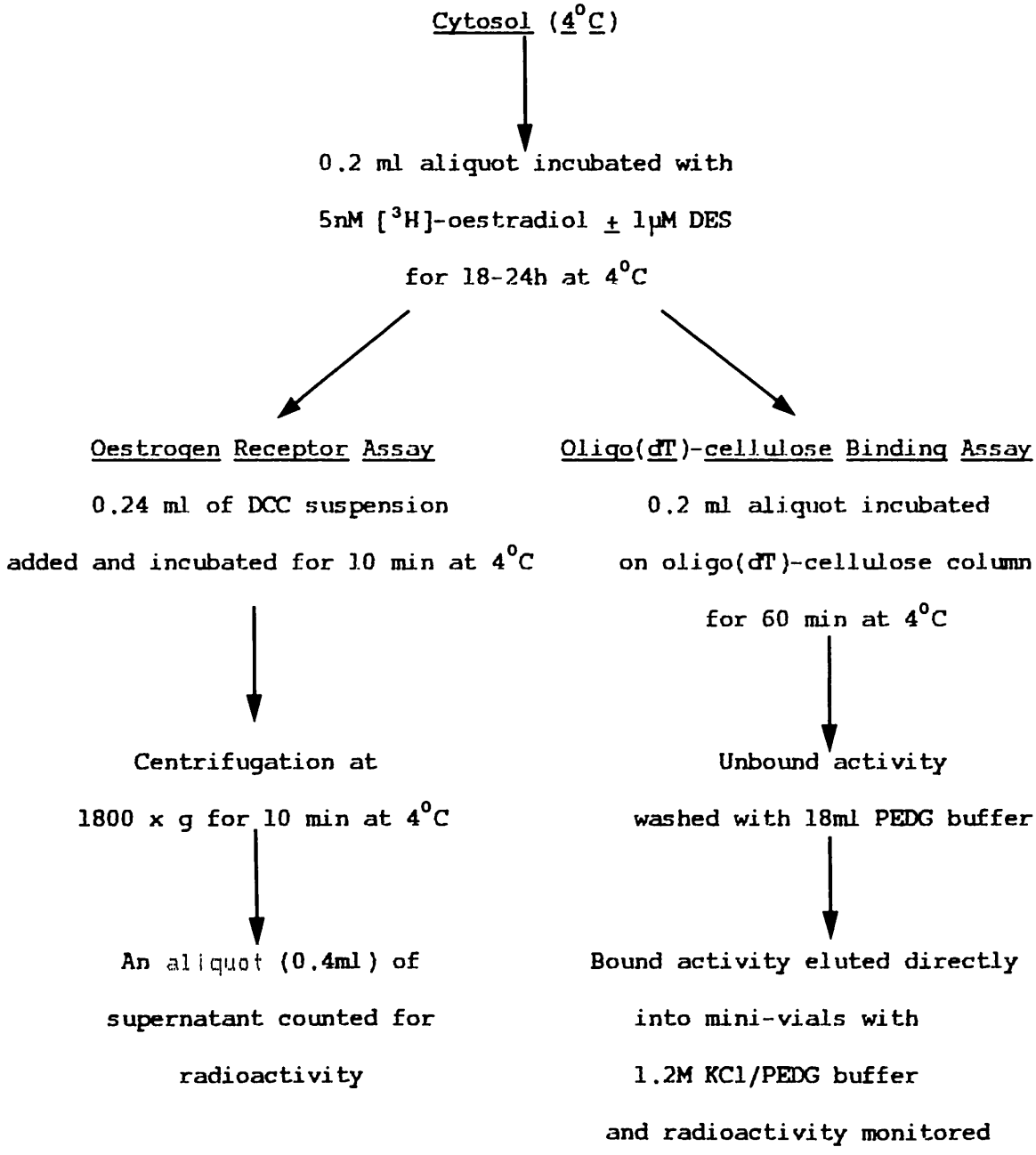
Routine assays were performed in duplicate 0.2ml aliquots of cytosol, incubated with 5nM [^{3}H]-oestradiol in the absence and presence of 1μM DES in a final volume of 0.2ml for 18-24h at 0-4^{0}C (figure 10). In preliminary experiments, saturation analysis was performed using a range of [^{3}H]-oestradiol concentrations from 0.1 to 10 nM, each either in the absence or presence of a 200-fold molar excess of DES to determine the equilibrium dissociation constant for the oestradiol-receptor interaction.

After overnight incubation (18-24h), the free [^{3}H]-oestradiol was separated from the macromolecular bound activity by charcoal adsorption. An equal volume (0.24ml) of ice-cold dextran-coated charcoal (DCC) suspension was added to the incubation mixture, giving a final concentration of 0.5% activated charcoal and 0.05% dextran T-70, thoroughly mixed and incubated at 0-4^{0}C. During the 10 min incubation, the assay tubes were intermittently shaken. The tubes were then centrifuged in a MSE coolspin at 1800 x g for 10 min at 4^{0}C. An aliquot (400μl) of the resulting supernatant, containing only the bound activity, was transferred into a scintillation vial containing 4ml of Unisolve I and the radioactivity determined. The difference in activity between samples without and with DES gave a measurement of specific binding of oestradiol to its receptors.

Cytosol Progesterone Receptor

Aliquots (0.2ml) of cytosol were incubated for 18-24h at 0-4^{0}C in duplicate with 10nM [^{3}H]-progesterone in the absence and presence of a 100-fold excess (1μM) of unlabelled progesterone. All assay tubes also

Figure 10: Cytosol Oestrogen Receptor and Oligo(dT)-cellulose Binding Assay.



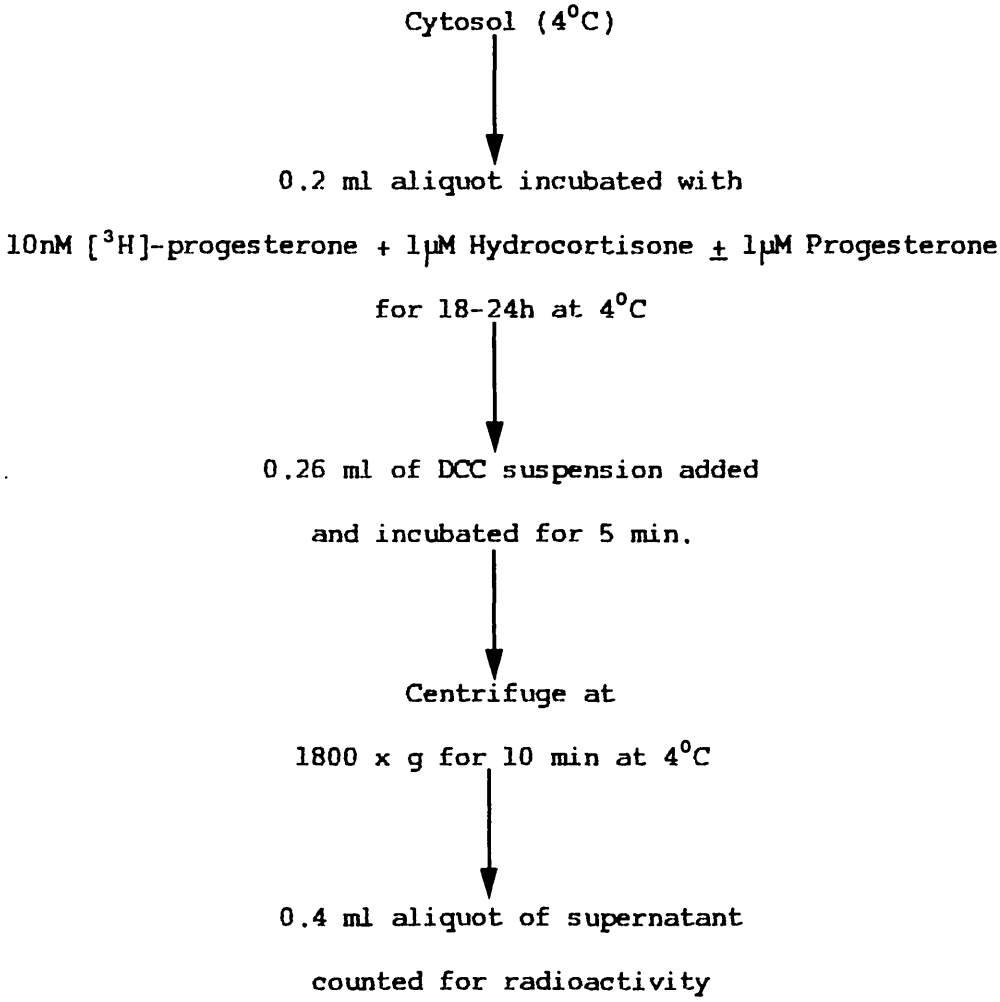
contained a 100-fold excess (1μM) of hydrocortisone to eliminate nonspecific binding of tritiated progesterone to contaminants such as corticosteroid binding globulin (figure 11). In preliminary experiments, saturation analysis was performed as described for the oestradiol-receptor assay, using a range of [^{3}H]-progesterone concentrations from 0.1 to 20 nM with a 100-fold molar excess of hydrocortisone in the absence and presence of a 100-fold molar excess of unlabelled progesterone to determine the equilibrium dissociation constant for the progesterone-receptor interaction.

At the end of the incubation period, the free [^{3}H]-progesterone was removed by DCC adsorption as described for the oestradiol receptor assay. However, initial experiments had shown that the maximal exposure time of the progesterone binding reaction to charcoal was critical for the optimal measurement of progesterone receptors. An equal volume (0.26ml) of the DCC suspension was added to the assay tubes, mixed thoroughly and incubated for 5 min at 4^{o}C before centrifugation. An aliquot (400μl) of the supernatant was carefully removed to a scintillation vial containing 4ml of Unisolve I and the radioactivity determined. Specific binding of progesterone was calculated from the difference between the total binding activity and the binding activity in the presence of excess unlabelled progesterone

Binding of Oestrogen Receptor to Oligo(dT)-Cellulose

Aliquots of cytosols incubated overnight with [^{3}H]-oestradiol were used for binding studies to oligo(dT)-cellulose by a column method, described by Myatt (1979). Columns were prepared using borosilicate glass pipettes, plugged with glass wool containing 200mg of oligo(dT)-

Figure 11: Cytosol Progesterone Receptor Assay



-cellulose and placed in perspex tanks. Oligo(dT)-cellulose chromatography was carried out either with the columns in the perspex tanks surrounded by ice or columns maintained at 0-4^{0}C in a cold room.

Prior to chromatography of samples, the columns were equilibrated with 10ml of ice-cold PEDG buffer. Aliquots (0.2ml) of prelabelled cytosol were loaded onto each oligo(dT)-cellulose column and allowed to bind (figure 10). Following incubation for 1h at 4^{0}C, the material that had not bound to the column was washed off with 20ml of PEDG buffer. Preliminary experiments had shown that 18-20ml of PEDG buffer was sufficient to remove all of the unbound activity from the column. The last 2ml of this buffer wash was routinely collected directly into scintillation vials containing 4-10ml of Unisolve I and the radioactivity determined. Macromolecular associated [^{3}H]-oestradiol bound to the oligo(dT)-cellulose column was then eluted with 2 x 2ml of PEDG buffer containing 1.2M KCl. The eluates were collected directly into scintillation vials containing 4-10ml of Unisolve I and the radioactivity monitored. Initial experiments had shown that 90-95% of the bound activity was eluted by 0.4M KCl/PEDG buffer, but that 1.2M KCl/PEDG buffer was required to remove the remaining activity. No further bound material could be removed if the volume of 1.2M KCl/PEDG buffer was increased beyond 4ml. Specific oestradiol-receptor binding to oligo(dT)-cellulose was calculated from the difference in accumulated activity in KCl-eluted samples between aliquots without and with DES. This gave a measurement of the binding of oestradiol-receptor complexes to oligo(dT)-cellulose and hence the extent of activation of the receptor complex. The level of binding to oligo(dT)-cellulose was expressed as a percentage of the total specific oestradiol binding capacity measured in an aliquot of the same cytosol.

Immediately after use, the oligo(dT)-cellulose columns were washed with 8ml of 0.1M NaOH to remove any residual activity and other proteins, before re-equilibrating with 10ml of PEDG buffer. The columns were kept at 0-4^{0}C between experiments and each time they were washed and re-equilibrated before re-use.

Effect of the Protease Inhibitor, PMSF, on the Measurement of Breast Tumour Cytosol Oestrogen Receptor

Breast tumour cytosol was prepared from a 40% (w/v) homogenate and divided into two; one half was diluted with an equal volume of PEDG buffer, while the other half was diluted with an equal volume of PEDG buffer containing increasing amounts of the protease inhibitor, PMSF, to give concentrations in the range 0 to 12mM PMSF in cytosol. The cytosols were incubated with 5nM [^{3}H]-oestradiol in the absence and presence of 1μM DES. After incubation for 1h at 4^{0}C, equal volumes of buffer containing the appropriate concentration of PMSF were added to the series of samples not containing the inhibitor, while PEDG buffer only was added to the rest of the samples. After a further incubation for 1h at 4^{0}C, macromolecular bound [^{3}H]-oestradiol was separated from free steroid by DCC adsorption. The concentrations of cytosol oestrogen receptor measured in the presence of the protease inhibitor, PMSF, were related to that measured in a duplicate aliquot without the inhibitor (100%).

D: Characterisation of Oestrogen Receptor Activation

Optimal Buffer Conditions for Oligo(dT)-cellulose Binding Assay

Breast tumour cytosol was prepared from a concentrated homogenate [20% (w/v)] in PED buffer and diluted with equal volumes of buffer containing 0%, 20%, 60% glycerol and/or 0.30, 0.8M KCl to give final concentrations of 0%, 10% and 30% glycerol and/or 0.15M and 0.4M KCl in cytosol. Aliquots (0.4ml) of each cytosol were incubated with 5nM [^{3}H]-oestradiol with and without 1μM DES for 18-24h at 0-4^{0}C. At the end of the incubation period, the receptor content was estimated by DCC adsorption and the extent of activation was assessed by oligo(dT)-cellulose chromatography of aliquots of the same cytosol. The receptor concentration was determined from the difference in activity counts between samples without and with DES, and the extent of activation was expressed as a percentage of the total specific counts bound to the receptor.

Stability of the Activated Cytosol Oestrogen Receptor During Long-Term Incubation

Tumour cytosol was prepared as described and was labelled with 5nM [^{3}H]-oestradiol in the absence and presence of 1μM DES. The cytosol was incubated at 0-4^0 C and at various times after the addition of [^{3}H]-oestradiol (18,42,66 and 90 hours), duplicate aliquots of cytosol were assayed for receptor content and the ability to bind to oligo(dT)-cellulose.

Effect of the Sulphydryl Reducing Agent, DTT, on Oestrogen Receptor Activation

Breast tumour cytosol was prepared from a 20% (w/v) homogenate in PEG buffer (10mM phosphate buffer containing 1.5 mM EDTA and 30% (v/v) glycerol) and aliquots were diluted to 10% with a range of DTT concentrations from 0.2 to 200mM in PEG buffer to give final concentrations of DTT in the range 0.1 to 100mM in cytosol. Aliquots of each sample were incubated with 5nM [^{3}H]-oestradiol either in the absence or presence of a 200-fold excess of DES for 18-24h at 4^{0}C. The extent of oligo(dT)-cellulose binding of receptor was then measured and related to the cytosol receptor content of each duplicate aliquot.

Effect of Cytosol Dilution on Oestrogen Receptor Activation

Cytosol was prepared from a 10% (w/v) homogenate of breast tumour tissue and serially diluted with equal volumes of PEDG buffer. Aliquots of each cytosol dilution were incubated with 5nM [^{3}H]-oestradiol in the absence and presence of 1μM DES for 18-24h at 4^{0}C. The concentration of cytosol oestrogen receptor and the extent of oligo(dT)-cellulose binding of the receptor complexes was then measured in duplicate aliquots of each cytosol dilution.

Time Course of Oestrogen Receptor Activation

The effect of time of exposure of oestradiol-receptor complexes to oligo(dT)-cellulose was studied following 1h and 18h incubation of cytosol with 5nM [^{3}H]-oestradiol at 4^{0}C. Following each incubation period, aliquots (0.2ml) of cytosol were further incubated on oligo(dT)-cellulose columns for varying time periods (0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, and 8 hours) before eluting the bound receptor

complexes. The cytosol receptor content was also estimated in duplicate aliquots (0.2ml) at each time point by DCC adsorption. Specific binding of oestradiol to its receptor was calculated from the difference in activity counts between total binding and the binding in the presence of excess DES. Binding of oestrogen receptor to oligo(dT)-cellulose was assessed by the difference in activity in KCl-eluted samples in the absence and presence of DES and expressed as a percentage of the total specific counts bound to the receptor.

Effect of Temperature on Cytosol Oestrogen Receptor Activation

Tumour tissue cytosol was prepared and incubated with 5nM [^{3}H]-oestradiol in the absence and presence of 1μM DES for 1h at 4^{0}C. Aliquots of the cytosol were then heated at various temperatures (25 ,30 and 37^{0}C) for various time periods (10 ,15 ,20 and 30 min) before cooling to 4^{0}C. This was immediately followed by the measurement of receptor concentration and the ability of the receptor complex to bind to oligo(dT)-cellulose.

The effect of heat treatment on cytosol oestrogen receptor levels and oligo(dT)-cellulose binding was studied in a series of tumour samples. Aliquots of cytosol were incubated with 5nM [^{3}H]-oestradiol for 1h at 4^{0}C. Nonspecific binding was determined in duplicate aliquots containing 1μM DES. For each sample, an aliquot was maintained at 4^{0}C, while a duplicate aliquot was activated by heating and then cooled to 4^{0}C. Cytosol oestrogen receptor concentration and the extent oligo(dT)-cellulose binding were then measured in both the heat treated samples and those maintained at 4^{0}C.

Effect of Molybdate on Activation of Breast Tumour Cytosol Oestrogen Receptor

A concentrated cytosol was prepared from a 20% (w/v) homogenate and diluted with equal volumes of either PEDG buffer or buffer containing 40mM sodium molybdate to give a final concentration of 20mM molybdate in cytosol. The cytosols were incubated for 18h at 4°C with 5nM [^{3}H]-oestradiol in the absence or presence of 1μM DES and the macromolecular bound [^{3}H]-oestradiol was separated from free steroid by DCC adsorption. Activation was assessed in duplicate aliquots by oligo(dT)-cellulose chromatography.

Effect of ATP on Cytosol Oestrogen Receptor Activation

A 20% (w/v) homogenate was prepared from tumour tissue and divided into two; one half was diluted with PEDG buffer and the other half was diluted with PEDG buffer containing 20mM ATP. Cytosol was prepared from these homogenates and incubated with 5nM [^{3}H]-oestradiol in the absence and presence of 1μM DES. Following incubation for 1h at 4°C, duplicate aliquots of each cytosol (with and without ATP) were activated by heating at 25°C for 30 min and cooled to 4°C, while duplicate aliquots were maintained at 4°C. The extent of oestrogen receptor activation was assessed by oligo(dT)-cellulose chromatography and related to specific counts associated with the cytosol oestrogen receptor.

E: High Performance Size Exclusion Gel Chromatography (HPSEC) of Oestrogen Receptors in Breast Tumours

Cytosols incubated with 5nM [^{3}H]-oestradiol with and without 1μM DES for 1h at 4^{0}C were subjected to DCC adsorption to remove free steroid prior to analysis by HPLC size exclusion chromatography (figure 10). Each sample was applied with a syringe-loaded valve injector (Rheodyne, model 7125) fitted with a 200μl sample loop and eluted isocratically on spherogel-TSK G3000 SW size exclusion columns by solvent delivery with a solvent metering pump. An additional aliquot was taken at this time to estimate the specific binding capacity and recovery. Elution was carried out at a flow rate of 0.6 ml/min using ice-cold PDG buffer (10mM phosphate buffer containing 1mM DTT and 20% (v/v) glycerol, pH 7.4 at 4^{0}C) either in the presence or absence of 0.4M KCl and 0.33 min fractions (0.2ml) of the column effluent were collected directly into mini-scintillation vials using an automatic fraction collector. All solvents (HPLC grade) and buffers used for HPLC were filtered through a 0.2μm filter to remove particulate contaminants before thorough degassing under vacuum. When buffer containing 400mM KCl was used, the columns were purged immediately after use with distilled water. At the end of a day of analyses, the columns were routinely purged with at least three column volumes of PDG buffer followed by a similar volume of distilled water. To remove build-up of organic material, the guard column was flushed with a filtered solution of 20% (v/v) dimethylsulphoxide in aqueous methanol [20% (v/v) methanol in distilled water]. The columns were stored in filtered distilled water.

Chromatography fractions were mixed with 4ml of Unisolve I and the radioactivity determined. The extent of activation was also assessed in duplicate aliquots by oligo(dT)-cellulose chromatography.

Calibration of HPSEC columns

The spherogel TSK G3000 SW columns were calibrated using standard molecular weight protein markers - ferritin, catalase, BSA, ovalbumin, chymotrypsinogen A and cytochrome c - prepared at a concentration of 1mg/ml in the elution buffer. Samples of these standard proteins, either individually or as a mixture in a final volume of 0.2ml, were injected onto the columns and separated at a flow rate of 0.6ml/min. The elution pattern of the protein standards was monitored at 280nm using an absorbance monitor, ISCO model UA-5, connected to the end of the columns. Similarly, carbon-14 labelled protein standards were also used for calibration as described above, and the activity monitored in 0.33 min fractions.

F: Statistical Analysis of Data

All analysis of receptor data was performed on the PE3220 computer at the Royal Postgraduate Medical School Computer Center using the 'Minitab' statistical package. Relationships among nominal scale variables were examined using Chi-square analysis, contingency tables or the Fisher exact test. Because distributions of receptor protein concentrations were heavily skewed, statistical analyses were performed using non-parametric tests. Summary statistics are given either in terms of mean $\pm$ S.E.M or mean $\pm$ S.D. Further statistical analysis was also performed using Student's 't' test. Correlations were assessed via the Pearson product moment correlation test and significance levels derived using the standard error estimation. All neccessary underlying statistical assumptions were checked prior to application of a given test. Results are reported as statistically significant if $p \leq 0.05$, two-tailed test.

CHAPTER THREE

SEX STEROID RECEPTORS IN HUMAN BREAST CANCER

A: RECEPTOR BINDING

Saturation Analysis of the Cytosol Oestrogen Receptor

The interaction of oestradiol with cytosol oestrogen receptor was studied in six human breast tumour specimens using a range of [^{3}H]-oestradiol concentrations from 0.1 to 10nM, each in the absence and presence of a 200-fold molar excess of DES to determine the non-specific binding. The cytosol oestrogen binding sites were apparently saturated at a concentration of 5nM [^{3}H]-oestradiol (see figure 12a). Plots of the ratio of bound to free ligand versus the concentration of bound ligand, according to Scatchard (1949), revealed a single class of oestrogen binding sites (figure 12b) and allowed the calculation of the apparent dissociation constant (Kd) from the slope of the curve, and the number of binding sites by extrapolation of the curve to the abcissa. Analysis of the Scatchard plots yielded Kd's in the range 1.6-5.4 x 10^{-10}M, demonstrating high-affinity binding between [^{3}H]-oestradiol and its receptors. These values are in general agreement with those found by other investigators (Feherty et al., 1971; Wittliff et al., 1971; Leclerq et al., 1973; McGuire et al., 1973; Wittliff et. al., 1978) and are in the same range as those found in the rat uterus (Sanborn et al., 1971) and in the DMBA-induced rat mammary tumours (McGuire & Julian, 1971).

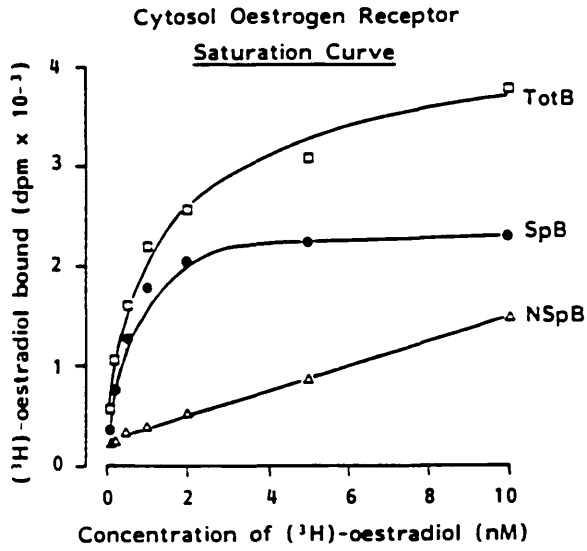


Fig. 12a. Saturation analysis of oestrogen receptors in human breast carcinoma. Aliquots (0.2 ml) of cytosol prepared from a human breast tumour biopsy were incubated with increasing concentrations of ^{3}H-oestradiol-17β either in the absence (□) or presence (△) of a 200 fold molar excess of DES for 18-24h at 0-4°C. Specific binding (●) was determined from the difference between the total binding and binding in the presence of the competitor.

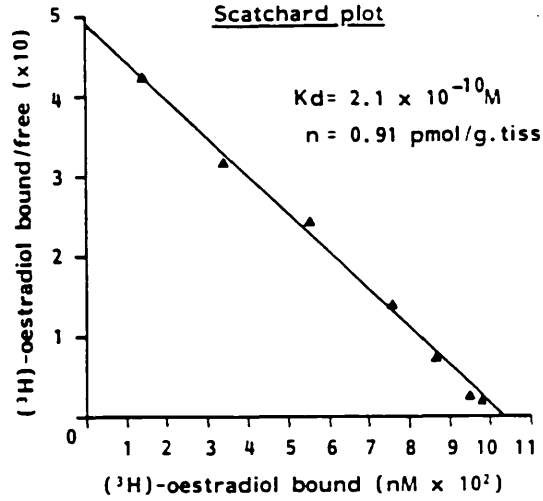


Fig. 12b. Scatchard plot. The specific binding data from curve A was plotted according to the method of Scatchard (1949). The dissociation constant (Kd), determined from the slope of the curve was 2.1 X 10^{-10} M for this preparation. The binding capacity was determined from the intercept on the abscissa and gave a value of 0.91 p.mol/g wet weight of tissue.

Saturation Analysis of the Cytosol Progesterone Receptor

Saturation analysis was carried out on four human breast tumour specimens with a range of [^{3}H]-progesterone concentrations from 0.1 to 20nM in the presence of a 100-fold molar excess of hydrocortisone to eliminate non-specific binding of labelled progesterone to contaminants such as corticosteroid binding globulin. Low-affinity, non-specific binding was determined by adding a 100-fold molar excess of unlabelled progesterone. The cytosol progesterone binding sites were approaching saturation at a concentration of 10nM [^{3}H]-progesterone (see figure 13a). Scatchard analysis of the specific binding data revealed a single progesterone binding site and yielded Kd's in the range 0.99-10.36 x 10^{-9}M (figure 13b), in agreement with those found by other investigators (Raynaud et al., 1977; Barnes et al., 1977; Wittliff et al., 1978) and are in the same range as those found in the lactating mammary gland of the rat and experimental rat mammary tumours (Wittliff et al., 1978).

Analysis of Multiple Receptor Parameters

Limitation of the amount of biopsy material (usually 100-400mg) and a requirement for multiple receptor analyses in each specimen meant that there was insufficient cytosol to analyse receptor binding by Scatchard analysis. A single concentration of ligand was therefore used (King et al., 1979). The measurement of oestrogen receptor was carried out using 5nM [^{3}H]-oestradiol in the absence and presence of a 200-fold molar excess (1μM) of DES. Of 291 primary tumours analysed, oestrogen binding capacity ranged from 0 to 23.09 picomoles per gram wet weight of tissue (0 to 737.6 femtomoles per milligram of cytosol protein). In a series of six tumour specimens, the oestrogen binding values obtained

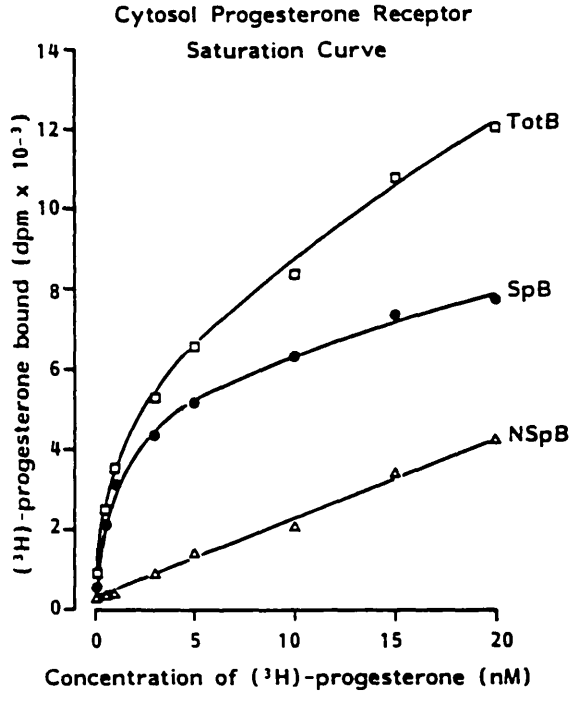


Fig. 13a. Saturation analysis of progesterone receptors in human breast carcinoma. Aliquots (0.2 ml) of cytosol prepared from a human breast tumour biopsy were incubated with increasing concentrations of ^{3}H-progesterone with a 100 fold molar excess of hydrocortisone either in the absence (□) or presence (△) of a 100 fold molar excess of unlabelled progesterone for 18-24h at 0-4^{o}C. Specific binding (●) was determined from the difference between total binding and binding in the presence of the competitor.

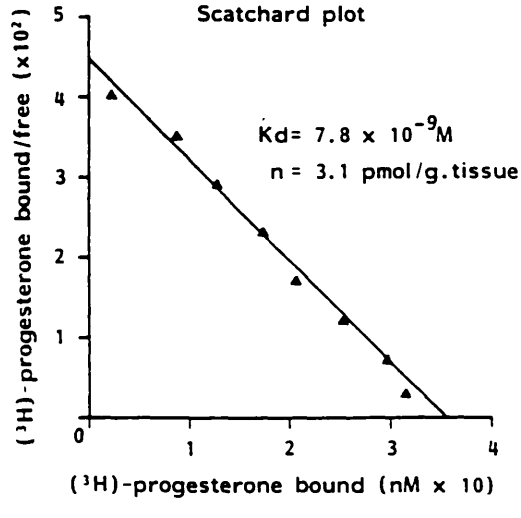


Fig. 13b. Scatchard plot. The specific binding data from curve A was plotted according to the method of Scatchard (1949). The dissociation constant (Kd), determined from the slope of the curve was 7.75 X 10^{-9} M for this preparation. The binding capacity was determined from the intercept on the abscissa and gave a value of 3.1 p.mol/g wet weight of tissue.

with a single point assay, using 5nM [^{3}H]-oestradiol, were not significantly different from the number of oestrogen binding sites determined by Scatchard analyses (see Table 1). Similar observations have been made by other investigators (Leclercq et al., 1973; Mobbs & Johnson, 1976; McGuire et al., 1977a; Garola & McGuire, 1978).

Assay of cytosol progesterone receptors was carried out using 10nM [^{3}H]-progesterone with a 100-fold molar excess (1μM) of hydrocortisone either in the absence or presence of a 100-fold molar excess (1μM) of unlabelled progesterone. In the series of tumours analysed, the progesterone binding capacity ranged from 0 to 85.1 picomoles per gram wet weight of tissue (0 to 750.0 femtomoles per milligram cytosol protein). As with the oestrogen receptors, progesterone binding capacity determined using a single point assay was not significantly different from that derived from Scatchard analysis (Table 1).

Effect of the Protease Inhibitor, PMSF, on the Measurement of Breast Tumour Cytosol Oestrogen receptor.

Addition of the protease inhibitor, PMSF, at homogenisation or post-incubation with [^{3}H]-oestradiol had no significant effect on the concentration of oestrogen receptors measured (figure 14). The mean values for six separate experiments were within two standard deviations of the control values (in the absence of inhibitor) at all concentrations of the inhibitor indicating that there was no significant change in receptor levels measured either in the absence or presence of PMSF. The protease inhibitor does not compete with oestradiol for binding to oestrogen receptor.

TABLE 1: COMPARISON OF SATURATION ANALYSIS WITH SINGLE-POINT ASSAY IN HUMAN BREAST TUMOUR SPECIMENS.

	SATURATION ANALYSIS		SINGLE-POINT ASSAY
	K_d ($\times 10^{-9}$M)	n (p.mol/g.wet weight)	n (p.mol/g.wet weight)
CER	0.54	9.98	9.56
	0.14	2.63	2.60
	0.21	0.91	0.88
	0.16	1.82	2.07
	0.43	0.28	0.29
	0.16	1.98	2.08
CPR	7.75	3.10	2.93
	0.99	0.29	0.30
	3.66	2.83	3.00
	10.36	1.13	4.47

K_d = affinity constant; n = number of binding sites

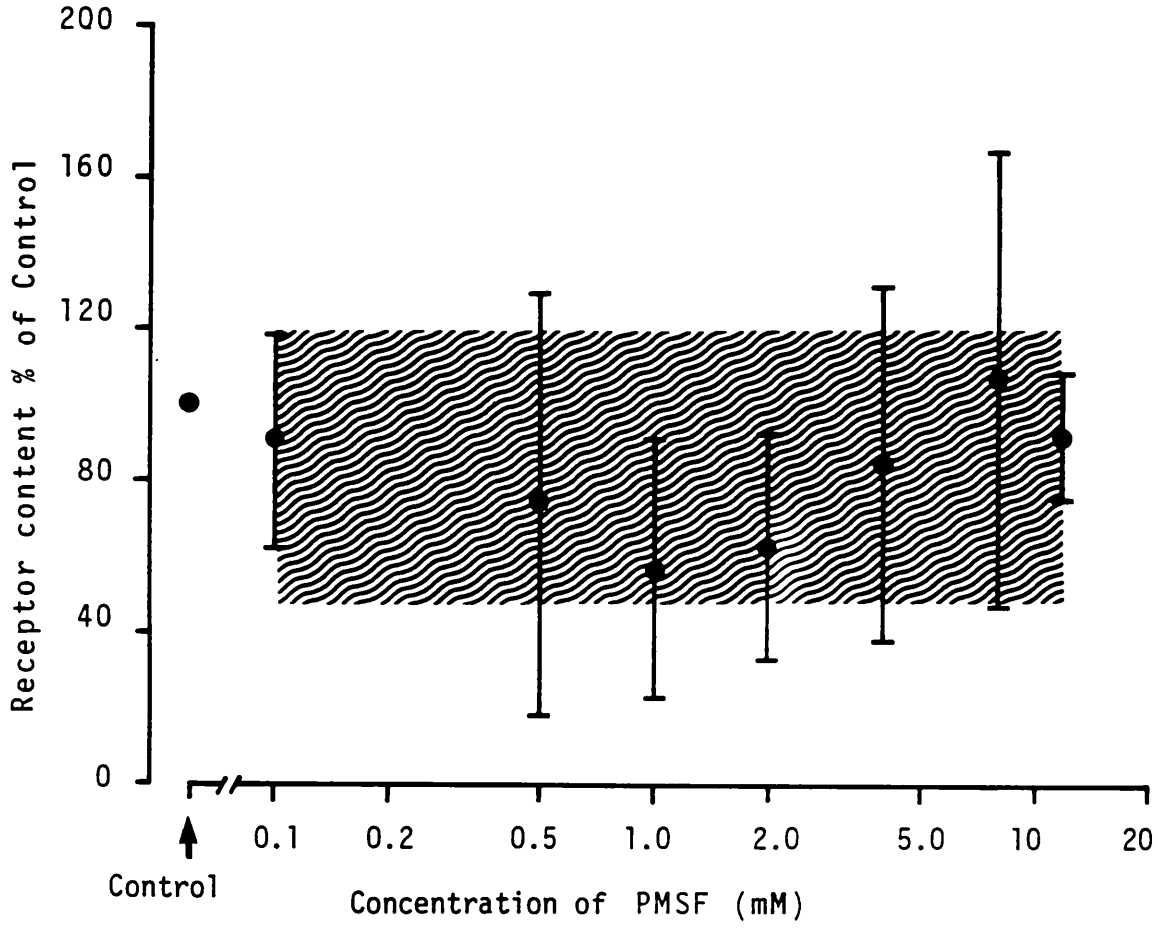


Fig. 14. Influence of the protease inhibitor, PMSF on the measurement of cytosol oestrogen receptor. Shaded area represents mean ± 2SD for five separate determinations.

Quantitative Expression of Receptor Binding Data

Receptor concentrations expressed as femtomoles per milligram cytosol protein (f.mol/mg.protein) fall into the ranges observed by most investigators, both for the cytosol oestrogen and progesterone receptors (Barnes et al., 1977; Garola & McGuire, 1978; Koenders et al., 1980; Wittliff et al., 1984). A comparison of cytosol receptor concentrations expressed per gram wet weight of tissue (p.mol/g.tissue) with per milligram cytosol protein (f.mol/mg.protein),or for the nuclear oestrogen receptor concentration with per milligram DNA (p.mol/mg.DNA) gave very good correlations which were highly significant (see figures 15,16): CER, $r=0.880$ ($n=286$), $p<<0.001$; CPR, $r=0.634$ ($n=285$), $p<<0.001$; NER4, $r=0.528$ ($n=37$), $p<0.001$; NER30, $r=0.869$ ($n=37$), $p<0.001$. All analysis of data is therefore carried out on receptor concentrations expressed as p.mol/g.wet weight of tissue which allows direct comparisions of concentrations of receptor in the cytosol and nucleus.

Definition of a Positive Receptor Assay

Oestrogen receptor concentrations of >10 f.mol/mg.protein are generally reported in the literature as the cut-off values for a positive receptor assay. From figures 15 and 16, this value corresponds to 0.5 p.mol/g.tissue. A receptor concentration of ≥ 0.5 p.mol/g.wet weight of tissue was taken as positive for all parameters except for oligo(dT)-cellulose binding of oestrogen receptors where any level of binding constituted a positive assay. Using the value of ≥ 0.5 p.mol/g.wet weight of tissue to distinguish receptor-positive from receptor-negative tumours for cytosol oestrogen and progesterone receptors resulted in the distribution of receptor phenotypes that was not significantly different from that generated using ≥ 10 f.mol/mg. cytosol protein as the discriminant (Table 2). Similar observations have been reported by Lippman (1980).

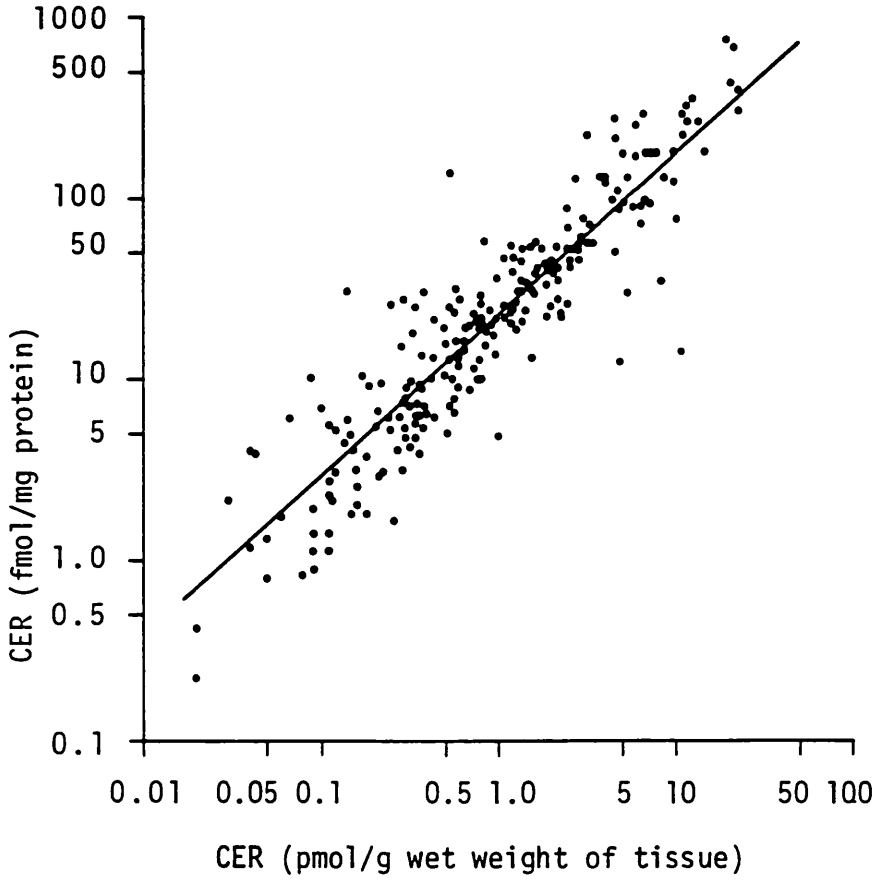


Fig. 15a. Correlation of cytosol oestrogen receptor concentrations expressed as pmol/g wet weight of tissue vs. fmol/mg protein in individual patients.

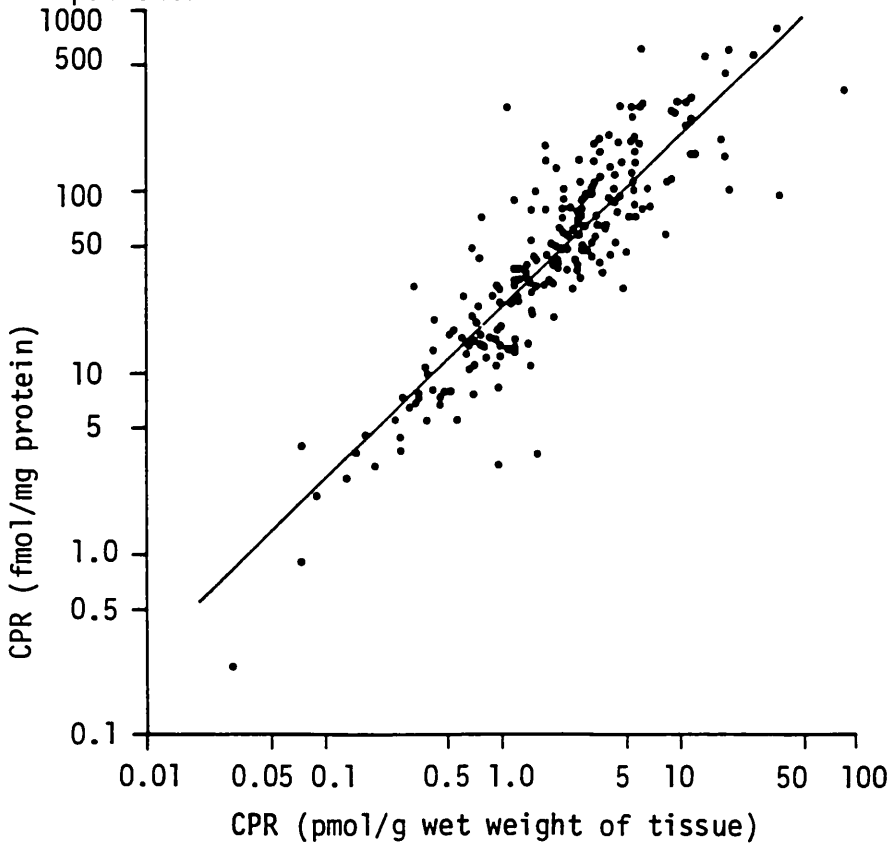


Fig. 15b. Correlation of cytosol progesterone receptor concentrations expressed as pmol/g wet weight of tissue vs. fmol/mg protein in individual patients.

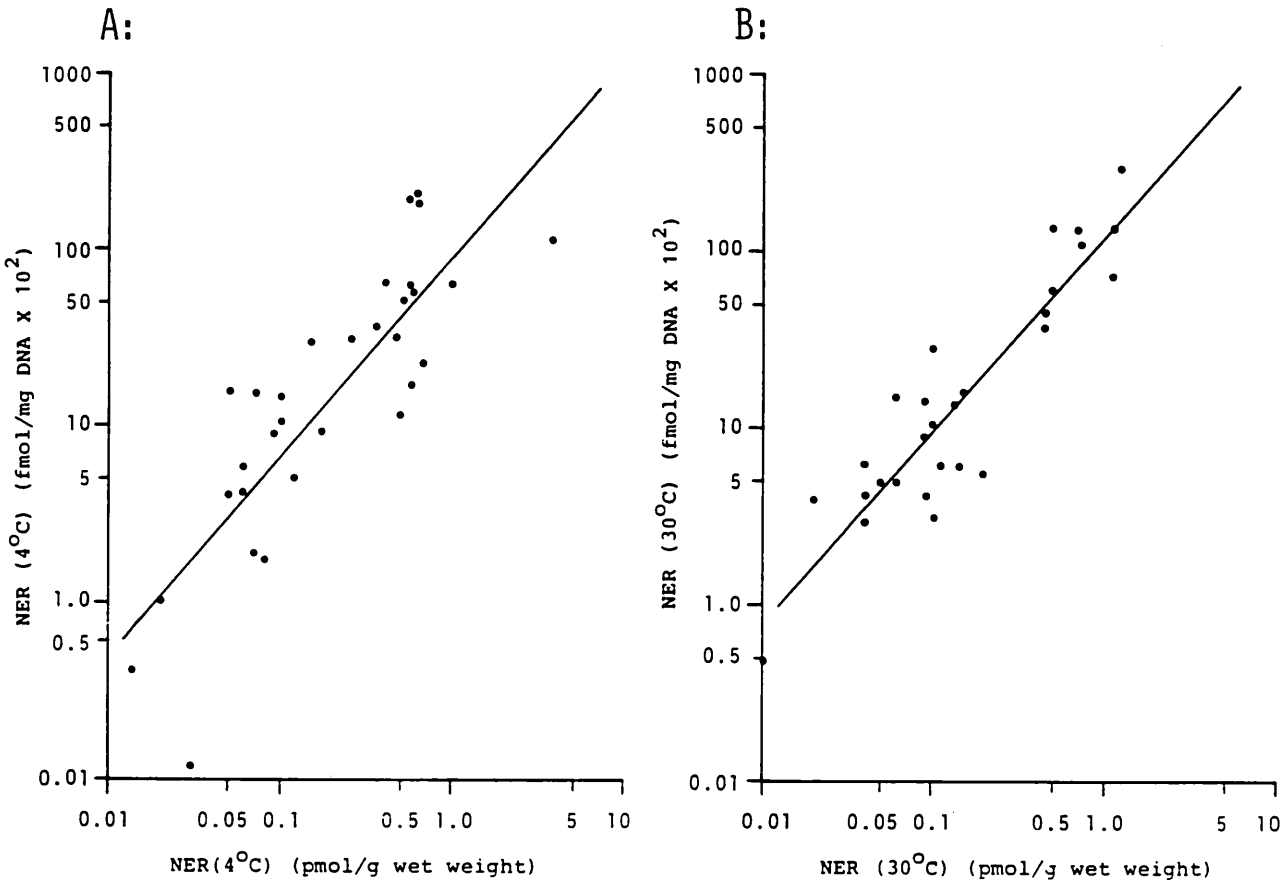


Fig. 16a. Correlation of 'unoccupied'nuclear oestrogen receptor (NER4) concentrations expressed as pmol/g wet weight of tissue vs. fmol/mg DNA; r = 0.528.

Fig. 16b. Correlation of 'total' nuclear oestrogen receptor (NER30) concentrations expressed as pmol/g wet weight of tissue vs. fmol/mg DNA; r = 0.869.

B: RECEPTORS IN BREAST CANCER I

A series of 291 patients, aged between 19 and 90 years, presenting at the Hammersmith Hospital and the local hospitals in the area with histologically confirmed primary carcinoma of the breast has been studied. The patients were grouped according to their menopausal status and amounted to 117 (40.2%) premenopausal and 174 (59.8%) postmenopausal.

Steroid Receptor Status and Incidence

Of all tumours (n=291) analysed, 52% were positive for cytosol oestrogen receptor and 64% of the tumours were positive for cytosol progesterone receptor. The incidence of receptor-positive tumours in relation to the menopausal status is shown in Table 2. There was no significant difference in the incidence of either the cytosol progesterone receptors in agreement with Koenders et al., (1980) or nuclear oestrogen (both total and unoccupied, i.e.,NER30 and NER4) receptors between the two groups. However, the frequency of oestrogen receptor positive tumours was significantly higher in post- than in pre-menopausal group: CER positive - 60.34%, post- vs. 40.18%, pre-menopausal, $p<0.001$. These findings are in agreement with other investigators (Hawkins et al., 1980; Lippman, 1980; Lesser et al., 1981).

The incidence of oestrogen receptor binding to oligo(dT)-cellulose was significantly higher in postmenopausal tumours(Table 2): DT binding - 42.3%, pre- vs. 60.6%, post- menopausal, $p<0.003$.

TABLE 2: INCIDENCE OF OESTROGEN AND PROGESTERONE RECEPTORS IN HUMAN BREAST TUMOURS.

	ALL (% positive)	PREMENOPAUSAL (% positive) n	POSTMENOPAUSAL (% positive) n
CER	52.23	40.18 (47/117)	60.34 (105/174)*
DT	53.14	42.34 (47/111)	60.63 (97/160)**
CPR	63.92	64.10 (75/117)	63.79 (111/174)
NER4	26.38	19.60 (11/56)	29.91 (32/107)
NER30	30.06	19.60 (11/56)	35.51 (38/107)
$CER_{\#}$	52.4	41.7 (48/115)	59.6 (102/171)
$CPR_{\#}$	63.9	64.9 (74/114)	63.2 (108/171)

Significantly different from corresponding premenopausal value (Chi square analysis): *$p<0.001$, **$p<0.003$. Receptor data was analysed using either $\geq$0.5 p.mol/g.wet weight of tissue as the discriminant or $\geq$10 f.mol/mg. protein (#).

Concentration of Sex Steroid Receptors in Human Breast Cancer

Of 291 primary breast tumours analysed, the cytosol oestrogen receptor concentrations ranged from 0 to 8.41 p.mol/g.tissue (0 to 136.0 f.mol/mg.protein) for premenopausal women and from 0 to 23.09 p.mol/g.tissue (0 to 737.6 f.mol/mg.protein) for postmenopausal women. The concentration of cytosol progesterone receptors ranged from 0 to 85.1 p.mol/g.tissue (0 to 750.0 f.mol/mg.protein) and from 0 to 170.41 p.mol/g.tissue (0 to 591.2 f.mol/mg.protein) in pre- and post-menopausal women respectively. In figure 17a, the cytosol oestrogen receptor binding capacity determined in tumours is related to patients' ages. The scatter diagram indicates a trend towards higher oestradiol binding capacity with increasing age. The correlation coefficient is statistically significant: $r=0.263$ ($n=291$), $p<0.001$. The progesterone receptor binding capacity in tumours does not appear to correlate with patient's ages [$r=-0.067$ ($n=291$), NS.] (figure 17b).

The mean concentrations of oestrogen and progesterone receptors grouped according to the menopausal status are shown in Table 3. The mean concentration of cytosol oestrogen receptor and its capacity to bind to oligo(dT)-cellulose was significantly higher in the postmenopausal tumours. There was no significant difference in the concentration of cytosol progesterone receptors or nuclear oestrogen receptors, NER4 or NER30, between the two groups. When a subset of receptor positive tumours were analysed (Table 4), in addition to the concentration of cytosol oestrogen receptor and its capacity to bind to oligo(dT)-cellulose, the concentration of unoccupied nuclear oestrogen receptors (NER4) were significantly higher in the postmenopausal group. This may be related to the endocrine environment in the postmenopausal women. However, there was still no significant difference in the

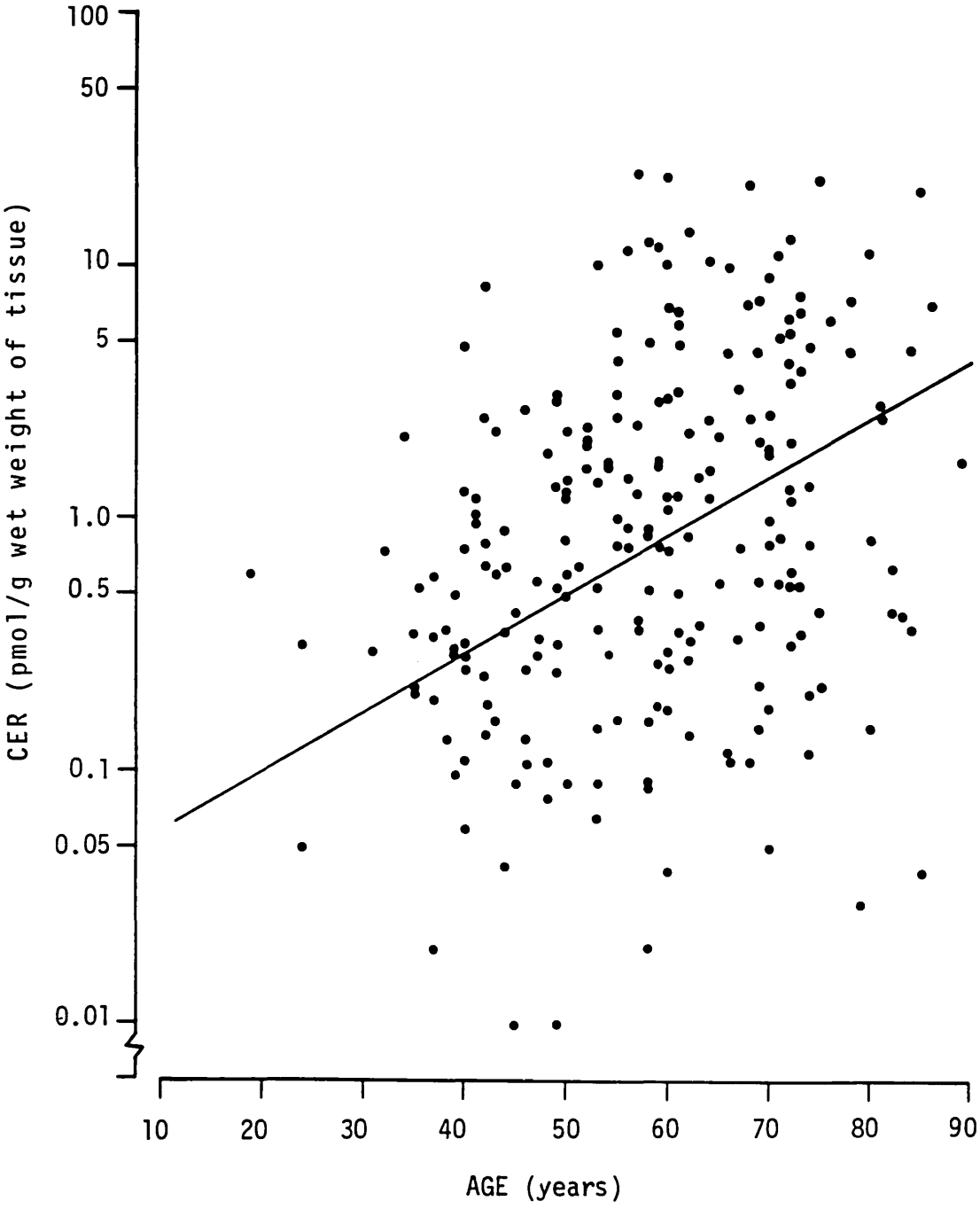


Fig. 17a. Influence of age on cytosol oestrogen receptor (CER) concentration in breast tumours from individual patients; correlation coefficient r= 0.263, n= 291, p<0.001.

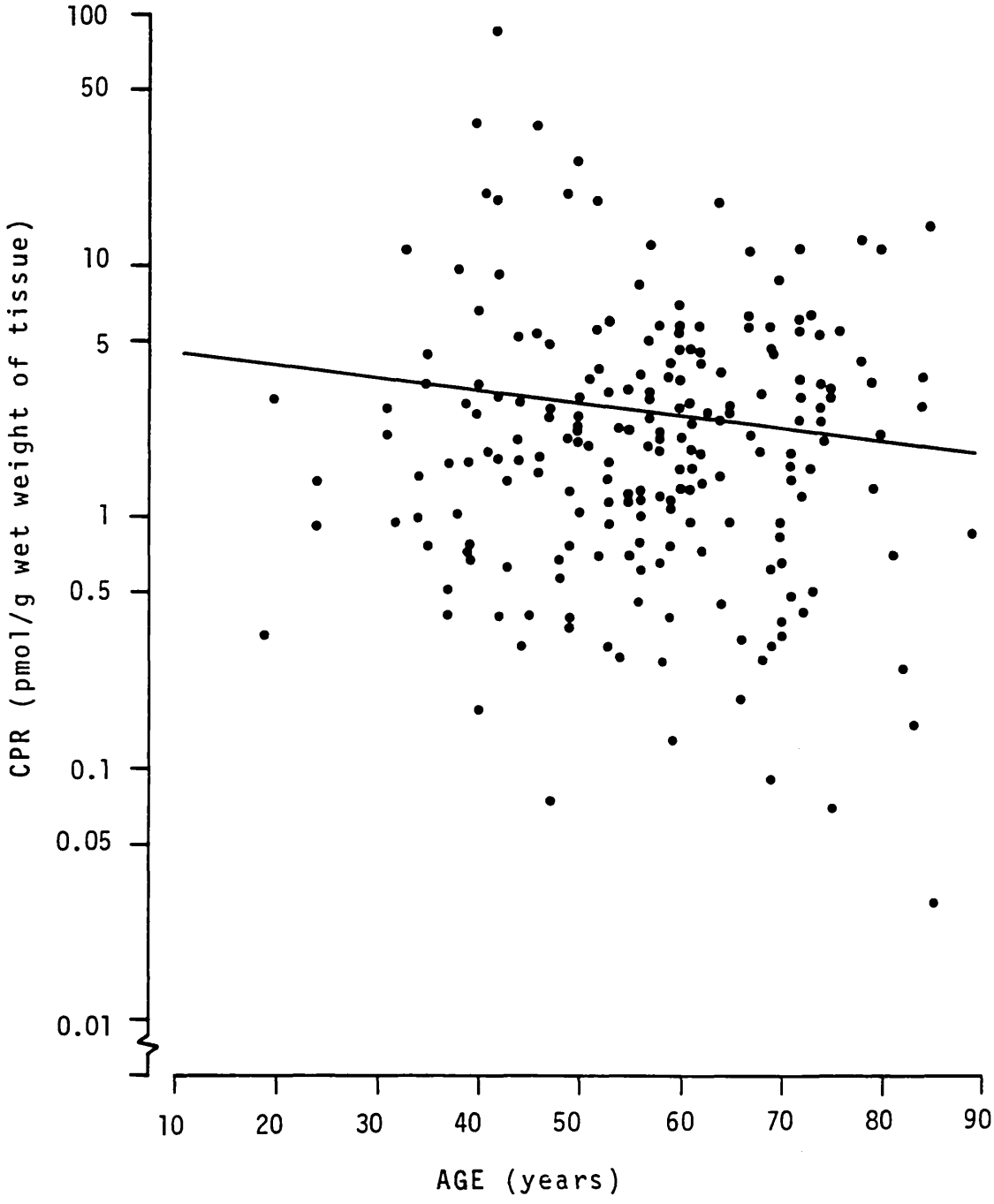


Fig. 17b. Influence of age on cytosol progesterone receptor (CPR) concentrations in breast tumours from individual patients; correlation coefficient r= -0.067, n= 291, NS.

TABLE 3: OESTROGEN AND PROGESTERONE RECEPTOR CONCENTRATIONS IN HUMAN BREAST TUMOURS.

	PREMENOPAUSAL (Mean ± S.E.M) (n)	POSTMENOPAUSAL (Mean ± S.E.M) (n)
CER	0.712 ± 0.105 (117)	2.814 ± 0.348 (174)*
DT	0.113 ± 0.023 (111)	0.819 ± 0.138 (160)**
CPR	3.739 ± 0.902 (117)	2.289 ± 0.227 (174)
NER4	0.274 ± 0.051 (56)	0.585 ± 0.098 (107)
NER30	0.402 ± 0.087 (56)	0.620 ± 0.096 (107)

Steroid receptor concentrations in pmol/g wet weight of tissue. Significantly higher than corresponding premenopausal value (Student's t test):*$p<0.0005$, **$p<0.0001$.

TABLE 4: OESTROGEN AND PROGESTERONE RECEPTOR CONCENTRATIONS IN HUMAN BREAST TUMOURS - RECEPTOR POSITIVES.

	PREMENOPAUSAL (Mean ± S.E.M) (n)	POSTMENOPAUSAL (Mean ± S.E.M) (n)
CER	1.599 ± 0.200 (47) *(0.50 - 8.42)*	4.576 ± 0.507 (105)** *(0.50 - 23.09)*
DT	0.266 ± 0.041 (47) *(0.01 - 1.59)*	1.351 ± 0.210 (97)** *(0.02 - 16.65)*
CPR	5.796 ± 1.351 (75) *(0.52 - 85.10)*	3.540 ± 0.295) (111) *(0.51 - 17.41)*
NER4	0.855 ± 0.158 (11) *(0.55 - 2.31)*	1.594 ± 0.246 (32)* *(0.50 - 6.90)*
NER30	1.395 ± 0.283 (11) *(0.52 - 3.67)*	1.502 ± 0.201 (38) *(0.53 - 5.47)*

Steroid receptor concentrations in pmol/g wet weight of tissue. Figures in italics represent the range of values observed. Significantly higher than corresponding premenopausal value (Student's t test): *$p<0.02$, **$p<0.0001$.

concentrations of cytosol progesterone and total nuclear oestrogen receptors (NER30) between the two groups.

The presence of cytosol and nuclear oestrogen receptors and cytosol progesterone receptors is indicative of a functional oestrogen receptor pathway (Barnes et al., 1979). There was no significant difference in the ratio of the concentration of either unoccupied nuclear oestrogen receptor to total cellular oestrogen receptors or the total nuclear oestrogen receptors to total cellular oestrogen receptors between the two groups [NER4/CER+NER30 : 0.716 $\pm$ 2.22 (n=99), post- vs. 0.799 $\pm$ 1.56 (n=52), pre- menopausal, t = 0.2671 (DF=149), NS; NER30/CER+NER30 : 0.338 $\pm$ 0.328 (n=99), post- vs. 0.488 $\pm$ 0.369 (n=52), pre- menopausal, t = 1.8071 (DF=149), NS]. This suggests that the functionality of a hormone-dependent tumour is independent of the menopausal status. However, the ratio of the total nuclear oestrogen receptors to cytosol progesterone receptors was significantly higher in the postmenopausal subjects [NER30/CPR: 0.252 $\pm$ 0.053 (n=56), pre- vs. 0.482 $\pm$ 0.081 (n=107), post- menopausal, $p < 0.02$], as was the ratio of unoccupied nuclear oestrogen receptors to cytosol progesterone receptors [NER4/CPR: 0.37 $\pm$ 0.088 (n=56), pre- vs. 2.116 $\pm$ 1.567 (n=107), post-menopausal, $p < 0.05$].

Assessment of the functionality of the oestrogen receptor pathway.

The simultaneous presence of either two or three receptor parameters in individual tumours has been investigated to evaluate which components contribute to the functionality of the oestrogen receptor pathway (Leake et al., 1981b; Barnes et al., 1979).

Distribution of Two Receptor Parameters in Individual Tumours

The presence of cytosol oestrogen receptor in individual tumours was significantly correlated with its ability to bind to oligo(dT)-cellulose, as well as the presence of cytosol progesterone receptors (Table 5) in both premenopausal and postmenopausal patients. The co-presence of cytosol and nuclear oestrogen receptors was significant only in postmenopausal tumours. The lack of such a correlation in premenopausal tumours may be due to the influence of high progesterone concentrations in the luteal phase of the menstrual cycle in these patients which would perturb the dynamics of the receptor states.

In tumours that contained cytosol oestrogen receptors, the incidence of the activated oestrogen receptor (i.e. oestrogen receptors capable of oligo(dT)-cellulose binding) was significantly higher in postmenopausal (87/99) patients than in premenopausal (30/44) patients (X^2 =7.94, DF=1, $p<0.01$). The mean capacity of oestrogen receptor binding to oligo(dT)-cellulose in these oestrogen receptor positive tumours was also significantly higher in postmenopausal patients (24.79 $\pm$ 16.2, n=87) compared to premenopausal patients (15.27 $\pm$ 20.1, n=30: t=2.69, $p<0.01$).

Of all oestrogen receptor positive tumours, 72.4% also contained progesterone receptors while 27.6% were negative. This indicates that the presence of cytosol oestrogen receptor does not neccessarily pertain to being progesterone receptor positive in all tumours. A possible explanation may be that at the time of biopsy, there was not sufficient endogenous oestrogen to stimulate progesterone receptor synthesis (Edwards et al., 1979). However, the presence of cytosol progesterone receptors was quantitatively correlated with the concentration of oestrogen receptors in both groups: r=0.728 (n=47), $p<0.001$, pre-;

TABLE 5: CORRELATION OF TWO-RECEPTOR PARAMETERS IN INDIVIDUAL BREAST CANCER PATIENTS.

		PREMENOPAUSAL (no. of patients)		POSTMENOPAUSAL (no. of patients)	
		CER		CER	
		+	-	+	-
NER4	+	3	8	27	5 ***
	-	14	31	35	40
NER30	+	5	6	30	8 **
	-	12	33	32	37
CPR	+	36	39	74	37 *
	-	11	31	31	32
DT	+	30	17	87	10 ***
	-	14	50	12	51

Significant correlation between the simultaneous presence or absence of the two receptor parameters (Chi square analysis): *$p<0.05$, **$p<0.005$, ***$p<0.001$.

r=0.448 (n=105), p<0.001, post- menopausal. Primary tumours with high oestrogen receptor levels were more likely to be positive for cytosol progesterone receptor than tumours with low oestrogen receptor levels.

Distribution of Three Receptor Parameters in Individual Tumours

In normal target cells responding to oestrogen, cytosol and nuclear oestrogen receptors should be found together with cytosol progesterone receptors as a functional end-point of oestrogenic stimulation.

The numbers of patients with nuclear oestrogen receptor data were too small for a satisfactory three-way analysis. The simultaneous presence of cytosol oestrogen receptor and its ability to bind to oligo(dT)-cellulose was apparently correlated with the presence of cytosol progesterone receptor in both groups (Table 6) [76.7% (23/30), pre-; 73.6% (64/87), post- menopausal]; this correlation being statistically significant in postmenopausal tumours (X^2= 4.234 (DF=1), $p < 0.05$).

In tumours containing cytosol oestrogen receptors capable of binding to oligo(dT)-cellulose, there was no significant difference in the distribution of cytosol progesterone receptors in the two groups (Table 6). However, the distribution of oligo(dT)-cellulose binding ability of oestrogen receptors in tumours containing both cytosol oestrogen and progesterone receptors was significantly different between premenopausal and postmenopausal tumours (CER+,CPR+,DT+: CER+,CPR+,DT- 23:10 pre-, vs. 64:7 post- menopausal, X^2 =8.39, DF=1, p<0.01). The lack of a correlation between the absence of oligo(dT)-cellulose binding ability of cytosol oestrogen receptor and the absence of cytosol progesterone receptor may be attributed to all the activated oestrogen

TABLE 6: FREQUENCY OF THE SIMULTANEOUS OCCURRENCE OF THREE RECEPTOR PARAMETERS IN INDIVIDUAL BREAST CANCER PATIENTS.

		PREMENOPAUSAL (no. of patients)		POSTMENOPAUSAL (no. of patients)	
		CPR		CPR	
CER	DT	+	-	+	-
+	+	23	7	64	23
+	-	10	4	7	5

Distribution of CPR positives in premenopausal patients (23/33) significantly different from postmenopausal (64/71); (Chi square analysis): $p<0.05$.

receptors being used up during nuclear transfer *in vivo* and generation of cytosol progesterone receptors.

Receptor Status Related to Histological Grade of the Tumours

In a small number of tumours, histological grading has been compared with the receptor status of an adjacent specimen of tumour in order to evalute the relationship between the receptor status and the extent of cellular differentiation. All tumours were graded according to Bloom & Richardson (1957) in the Histopathology Department of Hammersmith Hospital; the majority of the tumours were grade II (moderately differentiated, n=44) or III (poorly differentiated, n=22). There was a highly significant association between the grade of tumour and binding of cytosol oestrogen receptors to oligo(dT)-cellulose (Table 7) (X^2= 11.99 (DF=1), $p < 0.001$). The incidence of cytosol oestrogen receptor was significantly higher in grade II (84.0%) than in grade III (59.0%) (X^2=6.08, DF=1, $p<0.02$). A similar association was apparent between grade II tumours and the presence of cytosol progesterone receptors (X^2= 5.24 (DF=1), $p < 0.05$). There was a significantly higher incidence of cytosol oestrogen receptors binding to oligo(dT)-cellulose in grade II (34/37) tumours compared to grade III (7/13) tumours (X^2 =7.033, DF=1, $p<0.01$). It appears that the better-differentiated tumours are more frequently positive for cytosol oestrogen receptor, its ability to bind to oligo(dT)-cellulose and cytosol progesterone receptor (all determinants of a functional oestrogen response pathway).

C: RECEPTORS IN BREAST CANCER II

In a separate series of 145 patients, aged between 22 and 80 years, presenting at the Charing Cross Hospital with histologically

TABLE 7: CORRELATION OF RECEPTOR STATUS AND HISTOLOGICAL GRADE OF THE TUMOUR IN A SERIES OF PRIMARY BREAST TUMOURS.

		TUMOUR GRADE	
		II	III
CER	+	37	13*
	-	7	9
NER4	+	16	10
	-	5	7
NER30	+	14	11
	-	7	6
DT	+	34	7***
	-	9	15
CPR	+	37	12**
	-	7	10

Grade II: moderately differentiated, and Grade III: poorly differentiated. Significantly different from the corresponding distribution in Grade III tumours (Chi square analysis): *p<0.02, **p<0.05, ***p<0.001. Incidence of DT positive in tumours containing oestrogen receptors (CER+); Grade II (34/57), significantly different from corresponding Grade III (7/13), (Chi square analysis): p<0.01.

confirmed primary carcinoma of the breast, the extent of activation, that is, the ability of the oestrogen receptor to bind to oligo(dT)-cellulose and nuclear oestrogen receptors (NER4 and NER30) have been measured. The soluble oestrogen and progesterone receptors were measured at the Charing Cross Hospital in a 2000 x g supernatant fraction containing the cytosol (Fernandez et al., 1984). The total number of specimens amounted to 138, 94 (67.6%) postmenopausal and 44 (31.9%) premenopausal.

Comparison of Receptor Data Between the Two Series

Of all tumours analysed in the Charing Cross Hospital series of patients, 48.9% were positive for cytosol oestrogen receptor and 21.9% were positive for progesterone receptor. The overall distribution of incidence of receptor positive tumours in relation to menopausal status was similar in the Hammersmith Hospital and Charing Cross Hospital series' (Table 8), except for the cytosol progesterone receptor. The incidence of cytosol progesterone receptor-positive tumours was higher in the Hammersmith Hospital series, particularly for the postmenopausal group where the difference was highly significant (Table 8). The lower incidence of progesterone receptor-positive tumours in the Charing Cross Hospital series of patients, which is a reflection of a lower mean concentration of progesterone receptor, may be related to the use of a low speed cytosol preparation. However, the similarity of oestrogen receptor measurements between the two series would suggest a progesterone receptor effect. The difference in the incidence of unoccupied nuclear oestrogen receptor-positive tumours between postmenopausal patients in the two series only just attained statistical significance (Table 8). However, the general distribution of receptor

TABLE 8: COMPARISON OF THE INCIDENCE OF RECEPTOR-POSITIVE PARAMETERS BETWEEN THE CHARING CROSS (CX) AND THE HAMMERSMITH HOSPITAL (HH) SERIES OF PATIENTS.

	PREMENOPAUSAL				POSTMENOPAUSAL			
	CX		HH		CX		HH	
	(%)	(N)	(%)	(N)	(%)	(N)	(%)	(N)
CER	25.0	(11/44)	40.2	(47/117)	60.2	(56/93)	60.3	(105/174)
DT	26.5	(9/34)	42.3	(47/111)	60.0	(48/80)	60.6	(97/160)
CPR	25.0	(11/44)	64.1	(75/117)*	20.2	(19/94)	63.8	(111/174)**
NER30	30.8	(12/39)	19.6	(11/56)	55.3	(47.85)	35.5	(38/107)
NER4	22.5	(9/40)	19.6	(11/56)	58.5	(48/82)	29.9	(32/107)*

Significantly different from the corresponding incidence in the Charing Cross (CX) series of patients (Chi square analysis): *$p<0.05$, **$p<0.001$.

positive tumours was similar in both series of patients as was the mean concentrations of the receptor positive parameters.

Response to Hormonal Therapy

Follow-up data was not available for all the patients whose tumours had been analysed for receptor status. However, thirty two patients from the Charing Cross series were assessed after only six months from the start of endocrine therapy (both additive and ablative) and their clinical response correlated with the receptor status of the primary tumour. There are obvious limitations in considering response data after such a short period of time. However, the aim of the analysis was to obtain preliminary information of the relationship between activated oestrogen receptor and clinical response in comparison to the other relatively well accepted criteria, that is, cytosol oestrogen and progesterone receptors (Horwitz & McGuire, 1977; King et al., 1979) and nuclear oestrogen receptors (Garola & McGuire, 1977; Leake et al., 1981a). In this analysis the discriminant used to distinguish receptor positive tumours was $\geq$ 0.1 p.mol/g.wet weight. This did not affect the classification of activated oestrogen receptor positive tumours as any level of binding of receptor to oligo(dT)-cellulose constituted a positive assay. Clinical assessment was made following the criteria laid down by Hayward et al., (1977). Responders were those patients showing either partial or complete remission or no change. The incidence of objective response to endocrine therapy for these patients in relation to receptor status is presented in Table 9.

The incidence of cytosol and nuclear oestrogen receptors, cytosol progesterone receptors and activated oestrogen receptors were all higher

in patients responding to endocrine therapy compared to non-responders (Table 9a). The co-presence of cytosol oestrogen receptor and its ability to bind to oligo(dT)-cellulose was significantly higher in patients who responded (14/15) to endocrine therapy than those who failed (2/3) to respond (Table 9b). There was an apparent correlation between the co-presence of cytosol oestrogen and progesterone receptors and response to endocrine therapy (Table 9b), but this failed to achieve statistical significance.

The cytosol oestrogen receptor status appears to the most important predictive index of response to endocrine therapy in human breast cancer. However, this assessment also shows that breast tumours containing cytosol oestrogen receptors capable of binding to oligo(dT)-cellulose are likely to respond to endocrine therapy.

TABLE 9A: INCIDENCE OF OBJECTIVE RESPONSE IN BREAST CANCER PATIENTS RECIEVING ENDOCRINE THERAPY IN RELATION TO RECEPTOR STATUS.

RECEPTOR POSITIVE	RESPONDERS	NON-RESPONDERS
CER	16/18 (88.9%)	6/13 (46.2%)
NER4	16/18 (88.9%)	8/13 (61.5%)
NER30	16/18 (88.9%)	5/12 (41.7%)
CPR	13/18 (72.2%)	5/13 (38.5%)
DT	13/18 (72.2%)	4/11 (36.4%)

TABLE 9B: INCIDENCE OF COMBINED RECEPTOR STATUS IN RELATION TO OBJECTIVE RESPONSE TO ENDOCRINE THERAPY IN INDIVIDUAL PATIENTS

		RESPONDERS	NON-RESPONDERS
CER	DT		
+	+	14	2*
+	-	1	3
CER	CPR		
+	+	12	3
+	-	4	3

Significantly different from the corresponding distribution in patients responding to endocrine therapy (Chi square analysis): *$p<0.05$.

D: RELATIONSHIP OF STEROID RECEPTOR STATUS BETWEEN PRIMARY BREAST TUMOURS AND INVADED LYMPH NODES

The measurement of oestrogen receptors in breast cancer is useful for predicting response to hormonal manipulation in patients with metastatic disease (DeSombre et al., 1974; McGuire, 1975). However, when metastatic disease occurs, it may be in inaccessible locations, or the concentration of tumour cells may be insufficient to yield meaningful results. Therefore, in an attempt to understand the relationship between the receptor status in the primary and metastatic disease, the ability of the cytosol oestrogen receptor to bind to oligo(dT)-cellulose and nuclear oestrogen receptors have been measured in both the primary tumour and the metastatic nodal deposit(s) sampled simultaneously in a group of 35 patients from the Charing Cross Hospital. The cytosol oestrogen and progesterone receptors were measured at the Charing Cross Hospital. The concordance of the receptor status between the primary breast tumour and the invaded malignant lymph nodes has been evaluated.

Incidence and Receptor Levels in Primary Breast Tumours and Malignant Lymph Nodes

The incidence of cytosol progesterone receptors in tumours with involved lymph nodes was significantly lower than in tumours without nodal involvement (Table 10). The incidence of all receptor parameters in the invaded malignant lymph nodes was similar to that in the corresponding tumours (Table 10).

The mean concentration of oestrogen and progesterone receptors in primary tumours was not associated with nodal involvement, there being no significant difference in the concentration of receptors in tumours

TABLE 10: FREQUENCY OF OESTROGEN AND PROGESTERONE RECEPTORS IN PRIMARY TUMOURS AND INVADED LYMPH NODES.

	INCIDENCE OF RECEPTOR POSITIVES (%)		
	TUMOURS WITHOUT NODAL INVOLVEMENT	TUMOURS WITH NODAL INVOLVEMENT	
		(A)	(B)
CER	75.8	65.7	54.9
DT	34.8	43.8	60.0
CPR	50.5	30.3*	26.1
NER4	74.2	70.6	50.0
NER30	74.2	77.1	55.7

Receptor positive (A) tumour, and (B) corresponding lymph node. Significantly different from corresponding value in tumours without nodal involvement (Chi square analysis): *$p<0.01$.

with and without nodal involvement (Table 11). However, the mean concentration of cytosol oestrogen receptors and total nuclear oestrogen receptors was significantly lower in the lymph nodes than in the corresponding primary tumour (Table 12). This suggests that when tumours become more aggressive and begin to metastasise, there may be a trend for cells to lose receptors and the tissue to become receptor-negative reverting to autonomic growth.

Concordance of Receptor Status in Primary Tumours and Invaded Malignant Lymph Nodes

The consistency of receptor phenotypes in the primary tumours and the corresponding lymph nodes is shown in Table 13. Eighty two percent of the patients had the same cytosol oestrogen receptor status in the primary tumour and its corresponding malignant lymph nodes; 50.5% of the patients were positive for cytosol oestrogen receptor and 31.5% negative in both tissues (Table 13). The progesterone receptor status was identical in 81.8% of the primary tumours and the corresponding lymph nodes (Table 13). These results are similar to those reported by Klinga et al., (1982).

An alteration in cytosol oestrogen and progesterone receptor phenotype from positive in the primary tumour to negative in the corresponding invaded lymph node(s) occurred in less than 15% of the cases (Table 13). Conversely, a change in phenotype from negative in the primary tumour to positive in the lymph node(s) occurred less frequently(3-7% of the cases). Approximately 30-40% of the cases showed a change in nuclear oestrogen receptor phenotypes, mainly from positive to negative in agreement with observations of Leake et al., (1981a). Intrestingly, a significant proportion (34.4%) of samples that did not

TABLE 11: OESTROGEN AND PROGESTERONE RECEPTOR CONCENTRATIONS IN BREAST TUMOURS WITH AND WITHOUT NODAL INVOLVEMENT.

	TUMOURS WITHOUT NODAL INVOLVEMENT (p.mol/g.wet weight)	TUMOURS WITH NODAL INVOLVEMENT (p.mol/g.wet weight)
CER	3.21 ± 0.57 (66)	2.49 ± 0.63 (35)
DT	0.54 ± 0.17 (45)	0.49 ± 0.14 (30)
CPR	0.62 ± 0.15 (63)	0.87 ± 0.42 (35)
NER4	0.99 ± 0.19 (61)	0.86 ± 0.27 (34)
NER30	0.96 ± 0.15 (62)	0.77 ± 0.19 (34)

Receptor concentrations are Mean ± S.E.M. of the number of samples analysed given in parenthesis.

TABLE 12: OESTROGEN AND PROGESTERONE RECEPTOR CONCENTRATIONS IN PRIMARY BREAST TUMOURS AND CORRESPONDING INVADED LYMPH NODES.

	PRIMARY TUMOUR (p.mol/g.wet weight)	CORRESPONDING LYMPH NODE(S) (p.mol/g.wet weight)
CER	2.49 ± 0.63 (35)	1.61 ± 0.53 (56)*
DT	0.49 ± 0.14 (30)	0.37 ± 0.15 (23)
CPR	0.87 ± 0.42 (35)	0.26 ± 0.18 (48)*
NER4	0.86 ± 0.27 (34)	0.49 ± 0.13 (51)
NER30	0.77 ± 0.19 (34)	0.58 ± 0.16 (53)**

Significantly different from the corresponding value in the primary tumour (Wilcoxon's sign rank test): *$p<0.02$, **$p<0.003$. Figures in parenthesis are the numbers of samples analysed.

TABLE 13: DISTRIBUTION OF RECEPTOR PHENOTYPE IN PRIMARY TUMOUR AND INVADED MALIGNANT LYMPH NODES IN INDIVIDUAL PATIENTS.

		RECEPTOR PHENOTYPE IN TUMOUR / NODE (%)			
		+/+	-/-	+/-	-/+
CER	(35)	50.5	31.5	15.0	3.0
DT	(16)	26.0	21.9	17.7	34.4
CPR	(33)	19.2	62.9	11.1	7.1
NER4	(34)	41.7	21.1	28.9	8.3
NER30	(34)	49.5	16.7	27.6	6.2

In each patient with multiple nodal involvement, the receptor phenotype of each node was represented as a fraction of the total number of nodes involved in that patient.

contain activated oestrogen receptors in the primary tumour contained such receptors in the invaded lymph nodes.

Assessment of Clinical Response in Relation to Receptor Status

Thirty out of thirty-five patients were available for evaluation following mastectomy and their clinical response has been related to the receptor status of the primary tumour and the corresponding invaded malignant lymph node(s). Four patients died due to recurrent breast cancer with a median disease free interval of 8.5 months (range 3-14 months). Four patients survive with disease recurrence and 22 patients survive disease free, with a median disease free interval of 15 months (4-24 months) and 17 months (4-25 months) respectively.

In the primary tumours, the incidence of cytosol oestrogen receptor, its ability to bind to oligo(dT)-cellulose and cytosol progesterone receptor was significantly lower in patients with disease recurrence than those that remained disease free (Table 14). Similar observations for the oestrogen receptor status have been reported by several investigators (McGuire, 1980; Blamey et al, 1980; Howat et al, 1983). The incidence of cytosol oestrogen receptor in the invaded malignant lymph nodes of patients with early disease recurrence was also significantly lower than in the nodes of those patients who remained disease free (Table 15).

The concordance of cytosol oestrogen receptor positive status between the primary tumour and corresponding invaded malignant lymph node(s) was also significantly lower in patients with disease recurrence compared to those remaining disease free (Table 16). Conversely, the concordance of cytosol oestrogen receptor negative status between the two tissues was higher in patients with progressive disease (Table 16).

TABLE 14: INCIDENCE OF OESTROGEN AND PROGESTERONE RECEPTORS IN PRIMARY BREAST TUMOURS IN RELATION TO DISEASE RECURRENCE.

	No Recurrence		Early Disease Recurrence	
	% positive	n	% positive	n
CER	77.3	(17/22)*	37.5	(3/8)
DT	63.2	(12/19)**	14.3	(1/7)
CPR	50.0	(11/22)*	12.5	(1/8)
NER4	66.7	(14/21)	87.5	(7/8)
NER30	81.8	(17/21)	75.0	(6/8)

Significantly different from those patients with early disease recurrence (Chi square analysis): *$p<0.05$, **$p<0.01$.

TABLE 15: INCIDENCE OF OESTROGEN AND PROGESTERONE RECEPTORS IN MALIGNANT LYMPH NODES IN RELATION TO DISEASE RECURRENCE.

	No Recurrence		Early Disease Recurrence	
	% positive	n	% positive	n
CER	61.4	(13.5/22)*	16.6	(1.33/8)
DT	60.0	(6/10)	20.8	(1.66/8)
CPR	31.1	(6.84/22)	20.8	(1.66/8)
NER4	49.1	(9.33/19)	45.8	(3.66/8)
NER30	56.1	(10.66/19)	50.0	(4/8)

Significantly different from those patients with early disease recurrence (Chi square analysis): *$p<0.05$.

TABLE 16: CONCORDANCE OF OESTROGEN RECEPTOR PHENOTYPES BETWEEN THE PRIMARY TUMOUR AND THE CORRESPONDING INVADED LYMPH NODES IN RELATION TO DISEASE RECURRENCE.

	CYTOSOL OESTROGEN RECEPTOR PHENOTYPES IN TUMOUR / NODE(S)			
	+/+	-/-	+/-	-/+
NO RECURRENCE	12.5/22*	4/22	4.5/22	1/22
EARLY DISEASE RECURRENCE	1.33/8	5/8	1.66/8	0/8

Significantly different from early disease recurrence (Chi square analysis): *$p<0.03$.

TABLE 17: INCIDENCE OF COMBINED RECEPTOR STATUS IN PRIMARY BREAST TUMOURS IN RELATION TO DISEASE RECURRENCE.

	NO RECURRENCE	EARLY DISEASE RECURRENCE
CER/CPR	10/22	0/8*
CER/NER30	12/19	1/7
CER/NER4	13/21	3/8
CER/DT	13/21	3/8

Significantly different from patients with no recurrent disease (Chi square analysis): *$p<0.05$

The simultaneous occurrence of cytosol oestrogen and progesterone receptors was apparently associated with primary tumours from patients remaining disease free (figure 17). A similar trend was evident for the simultaneous occurrence in the primary tumour of cytosol oestrogen receptor with its ability to bind to oligo(dT)-cellulose but this was not statistically significant.

CHAPTER FOUR

RECEPTOR ACTIVATION AND MOLECULAR FORMS OF THE HUMAN BREAST CANCER OESTROGEN RECEPTOR

Activation of steroid-receptor complexes is a neccessary step in their regulatory function at the nuclear level. Despite the homologies of structure of the different steroid hormones and the broad similarities in their effector pathways and general metabolic effects, they show remarkably dissimilar characteristics of activation. The following studies were undertaken in order to gain an insight into the molecular properties and factors regulating the activation of human breast tumour cytosol oestrogen receptor.

A: Regulation of Activation of Breast Tumour Cytosol Oestrogen Receptor

In the following series of experiments, cytosol was prepared either from individual breast tumour specimens or from several tumour specimens pooled together and incubated with 5nM [^{3}H]-oestradiol. Nonspecific binding was determined in duplicate aliquots containing 1μM DES. Following incubation either for 1h or 18-24h at 4^{0}C and any further manipulations described in each experiment, the extent of oestrogen receptor activation was measured by its ability to bind to oligo(dT)-cellulose and related to the total specifically bound oestradiol in a duplicate aliquot of the cytosol.

Influence of Glycerol and Salt Concentrations on the Measurement of Cytosol Oestrogen Receptor and its Ability to Bind to Oligo(dT)-cellulose

Addition of glycerol and/or thioglycerol to the homogenisation buffers has been shown to markedly increase the stability of the progesterone receptor (Janne et al., 1975; Horwitz & McGuire, 1975; Pichon & Milgrom, 1977). Other investigators have demonstrated that the presence of physiological salt concentrations (0.1-0.2M KCl) is required to promote activation (Muller et al., 1981a) and transformation (Notides et al., 1975) of the rat uterine oestrogen receptor. The effect of glycerol and salt concentrations in cytosol preparations on activation of breast tumour oestrogen receptor has therefore been studied to define the optimal buffer conditions.

The presence of varying concentrations of glycerol and/or salt does not significantly affect the estimation of cytosol oestrogen receptor concentration (Table 18). However, there was a progressive loss of the ability of the breast tumour cytosol oestrogen receptor to bind to oligo(dT)-cellulose (i.e., activated oestrogen receptor) with increasing salt concentration (Table 19).

The presence of salt may be disrupting the ionic interactions between the steroid-receptor complex and oligo(dT)-cellulose matrix as binding of receptor to both DNA- and oligo(dT)- celluloses is disrupted by changes in ionic enviroment (Thrower et al., 1976). The studies of Traish et al., (1977) demonstrated that salt concentrations above 0.2M KCl interferes with nuclear binding of rat uterine oestrogen receptor. In contrast, Muller et al., (1983) have shown that 0.4M KCl promotes activation of the rat uterine oestrogen receptor as determined by DNA-cellulose binding. However, in that study the salt concentration of

TABLE 18: INFLUENCE OF SALT AND GLYCEROL ON THE MEASUREMENT OF CYTOSOL OESTROGEN RECEPTORS IN HUMAN BREAST TUMOUR SPECIMENS.

SALT (M)	CYTOSOL OESTROGEN RECEPTOR (p.mol/g. wet weight) GLYCEROL 0%	10%	30%
0.0	13.96	13.47	13.85
0.15	14.54	14.49	14.70
0.40	16.34	15.38	12.99

TABLE 19: INFLUENCE OF SALT AND GLYCEROL ON THE BINDING OF CYTOSOL OESTROGEN RECEPTOR TO OLIGO(dT)-CELLULOSE.

SALT (M)	OLIGO(dT)-CELLULOSE BINDING OF CER (%) GLYCEROL 0%	10%	30%
0.0	20.79	23.07	26.50
0.15	3.99	4.94	6.37
0.40	0	0	0

the oestrogen receptor preparations was diluted from 0.4M to 0.15M prior to assaying activation.

The proportion of activated oestrogen receptors measured in the presence of 30% glycerol was not significantly different from that measured in buffer without salt or glycerol. The presence of glycerol in the homogenisation buffer is required for stabilising the progesterone receptor and did not affect subsequent estimation of cytosol oestrogen receptor or its ability to bind to oligo(dT)-cellulose. Therefore, tumour cytosol prepared in buffer containing 30% glycerol was used for assay of both oestrogen and progestrone receptors.

Stability of the Activated Cytosol Oestrogen Receptor During Long-Term Incubation at 4°C

As shown in figure 18a, there was no significant loss of cytosol oestrogen receptor complexes in incubations maintained at 4°C for up to 90 hours. Similar findings have been reported for the rat uterine oestrogen receptor (Puca et al., 1971; Myatt, 1979). There was no significant increase in the proportion of activated oestrogen receptor complexes over the incubation period of 90 hours: $t = 0.6674$, NS. Similarly, there was no significant difference in the proportion of activated oestrogen receptors measured at 1h or 18h (figure 18b). Time dependent activation of oestrogen receptor has been demonstrated between 1 and 24 hours in animal systems (Muller et al., 1983; Myatt et al., 1984; White et al., 1984). However, Myatt (1979) observed a loss of activated oestrogen receptor during prolonged incubation (up to 70 hours) at 4°C, and suggested that this may be due to denaturation of the putative activating factor proposed to be involved in activation of the

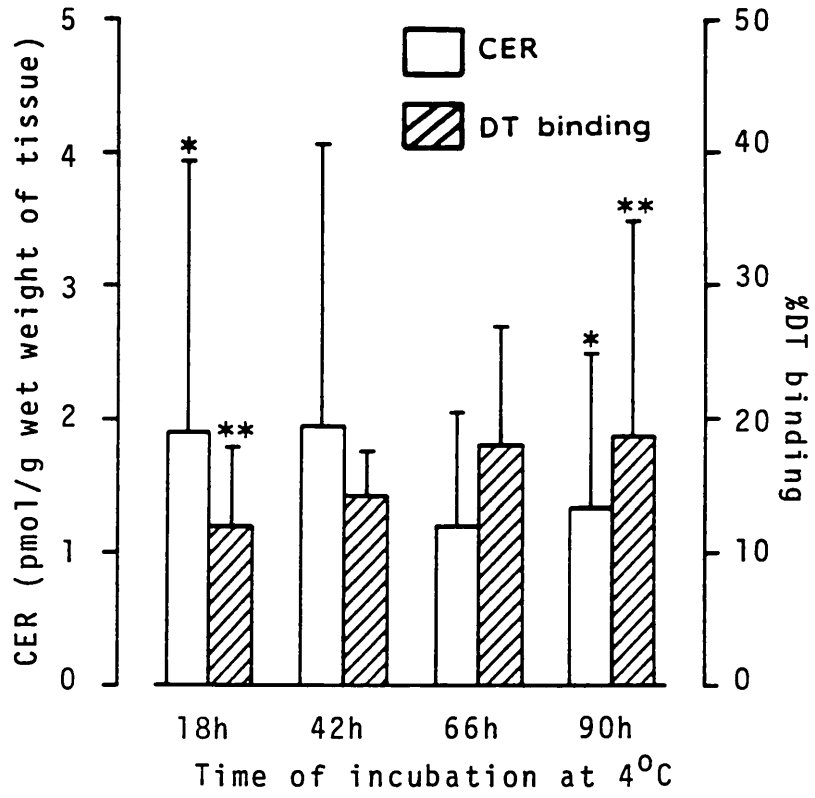


Fig. 18a. Stability of the activated oestrogen receptor to prolonged incubation at 4°C. Results are mean ± SD of five determinations. No significant difference either in the cytosol oestrogen receptor (*) concentration or the proportion of activated oestrogen receptors (**) between 18h and 90h incubation at 4°C.

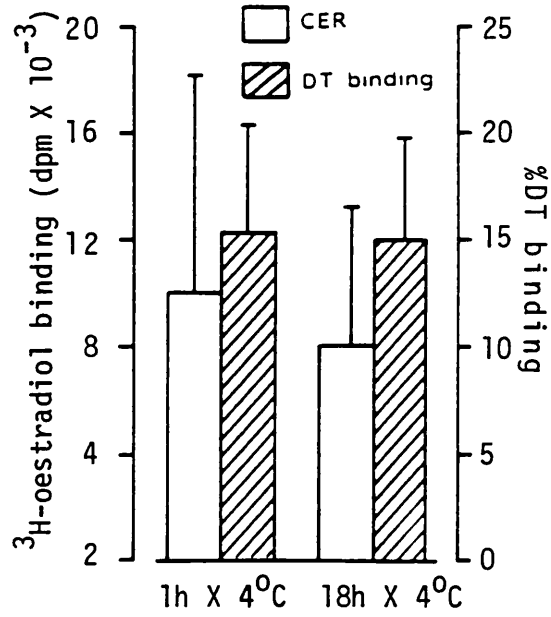


Fig. 18b. Stability of the activated oestrogen receptor between 1h and 18h incubation at 4°C. No significant difference either in the cytosol oestrogen receptor levels or the proportion of activated oestrogen receptors measured after 1h or 18h incubation at 4°C.

receptor. If such a putative activating factor is involved in activating the breast tumour oestrogen receptor, it may be more stable than that found in the rat uterus.

Effect of the Sulphydryl Reducing Agent, DTT, on Activation of Breast Tumour Cytosol Oestrogen Receptor

MacDonald and Leavitt (1982) demonstrated that reduced sulphydryl groups are required for the activation of the hamster uterine oestrogen receptor. Several other studies have suggested a role for sulphydryl groups in the binding of glucocorticoid (Young et al., 1975; Kalimi & Love, 1980), and progesterone (Kalimi & Banerjee, 1981) receptors to DNA. The effect of DTT on activation of breast tumour cytosol oestrogen receptor has therefore been investigated.

Incubation of breast tumour cytosol oestrogen receptor for 1h at 4^{o}C in the presence of the sulphydryl reducing agent, DTT, produced a concentration dependent increase in the proportion of cytosol oestrogen receptor bound to oligo(dT)-cellulose (figure 19). There was no significant difference in the level of cytosol oestrogen receptors measured in the presence of varying concentrations of DTT (figure 19). Maximal activation of oestrogen receptor, as measured by its ability to bind to oligo(dT)-cellulose, occurred in the presence of 10-20mM DTT (38-41%). In the absence of DTT, only 15-17% of the oestrogen receptor complexes are activated. The proportion of activated oestrogen receptor complexes measured in the absence of DTT was significantly lower than that measured in the presence of increasing concentrations of DTT above 1mM. However, there was no statistically significant difference in the proportion of activated oestrogen receptor complexes measured in the presence of 1mM and 20mM DTT, where the highest proportion of activated

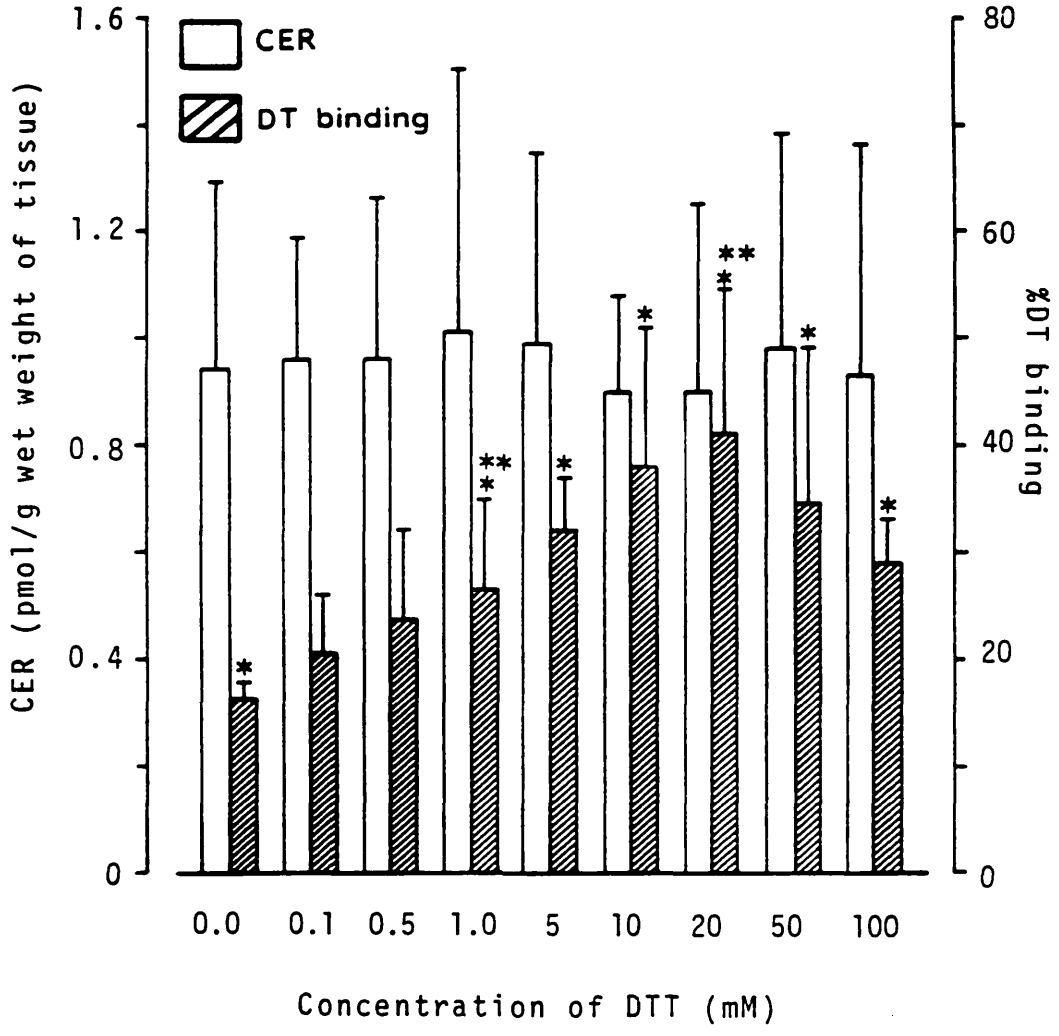


Fig. 19. Influence of DTT on activation of cytosol oestrogen receptors. Results are mean ± SD of three determinations; Students t-test, *p<0.05, **difference not significant.

complexes was observed:t = 1.625, NS. The presence of a sulphydryl reducing agent, therefore, promotes activation of breast tumour cytosol oestrogen receptor. This data suggests that the oligonucleotide binding domain of the breast tumour oestrogen receptor, or possibly the putative activating factor that interacts with the receptor and promotes activation requires reduced sulphydryl groups. Similar observations have been made by MacDonald and Leavitt (1982) for the activation of hamster uterine progesterone receptor.

Effect of Cytosol Dilution on Activation of Oestrogen Receptor

The influence of cytosol dilution on activation of oestrogen receptor was studied in three breast tumour samples. The protein concentration of the initial cytosol preparations ranged from 3.70 to 7.70 mg/ml of cytosol. Serial dilution of cytosol resulted in a linear decrease in the protein concentration (Figure 20a). There was a linear decrease in the cytosol receptor content with dilution (figure 20b), in parallel with the decrease in protein content. The amount of activated oestrogen receptor also showed a linear decrease, however, the proportion of activated oestrogen receptor showed an increase at the higher dilutions when the cytosol protein concentration was below 1mg/ml (figure 20c). Similar observations have been made for the rat uterine oestrogen receptor (Myatt et al., 1979a,b). Stancel et al., (1973) have demonstrated a protein concentration dependent aggregation of receptor. It is possible that dilution of cytosol with buffer, which results in a decrease in protein concentration, causes the breakdown of receptor aggregates and hence facilitates interaction of the oestrogen-receptor complex with oligo(dT)-cellulose.

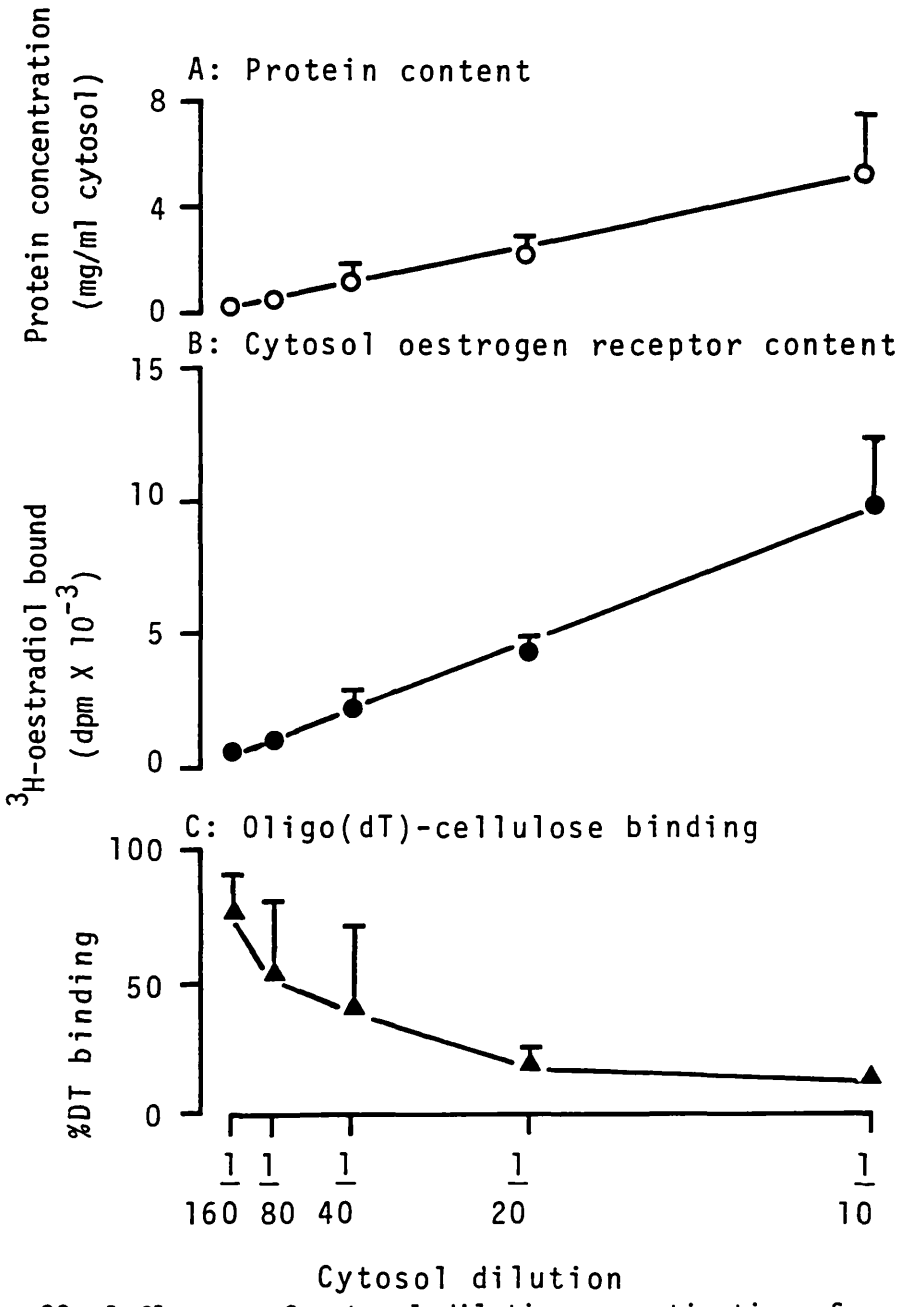


Fig. 20. Influence of cytosol dilution on activation of oestrogen receptor. Result are mean + SD of three separate determinations.

Routinely the assay for activated oestrogen receptors was not performed on diluted samples as levels measured at these dilutions were approaching the limits of detection. When all the data from both pre- and post- menopausal patients was analysed, there was no significant difference in the protein content of cytosols from pre- and post-menopausal tumours. Therefore, the differences observed in the capacity of oestrogen receptor binding to oligo(dT)-cellulose between samples cannot be attributed to an artefact of protein concentration.

Time-Course of Oestrogen Receptor Interaction with Oligo(dT)-cellulose

Aliquots of cytosol were incubated on oligo(dT)-cellulose columns for varying time periods from 0.5 to 8 hours before eluting the bound receptor complexes. In these experiments the cytosol preparations used had been equilibrated with 5nM (^{3}H)-oestradiol for either 1h or 18h at 4^{o}C. There was no significant difference in the concentration of cytosol oestrogen receptors measured at 1h or 18h after incubation with [^{3}H]-oestradiol at 4^{o}C. After an initial rapid increase in binding of oestrogen receptor to oligo(dT)-cellulose between 0.5 and 1.5 hours, an equilibrium was reached and no further increase in oligo(dT)-cellulose binding of oestrogen receptor was observed (Figure 21a). Similar results were obtained following both 1 hour and 18 hour (Figure 21a,b) prior-incubation with [^{3}H]-oestradiol at 4^{o}C. The limited proportion of oestrogen receptors binding to oligo(dT)-cellulose is not due to the availability of sites on the oligodeoxynucleotide matrix, which has infinite capacity (Thrower et al., 1976), but is attributed to the proportion of activated oestrogen receptor complexes present in the cytosol. Thrower et al., (1976) demonstrated that only about 30% of rat uterine cytosol oestrogen receptors bound to DNA- and oligo(dT)-celluloses.

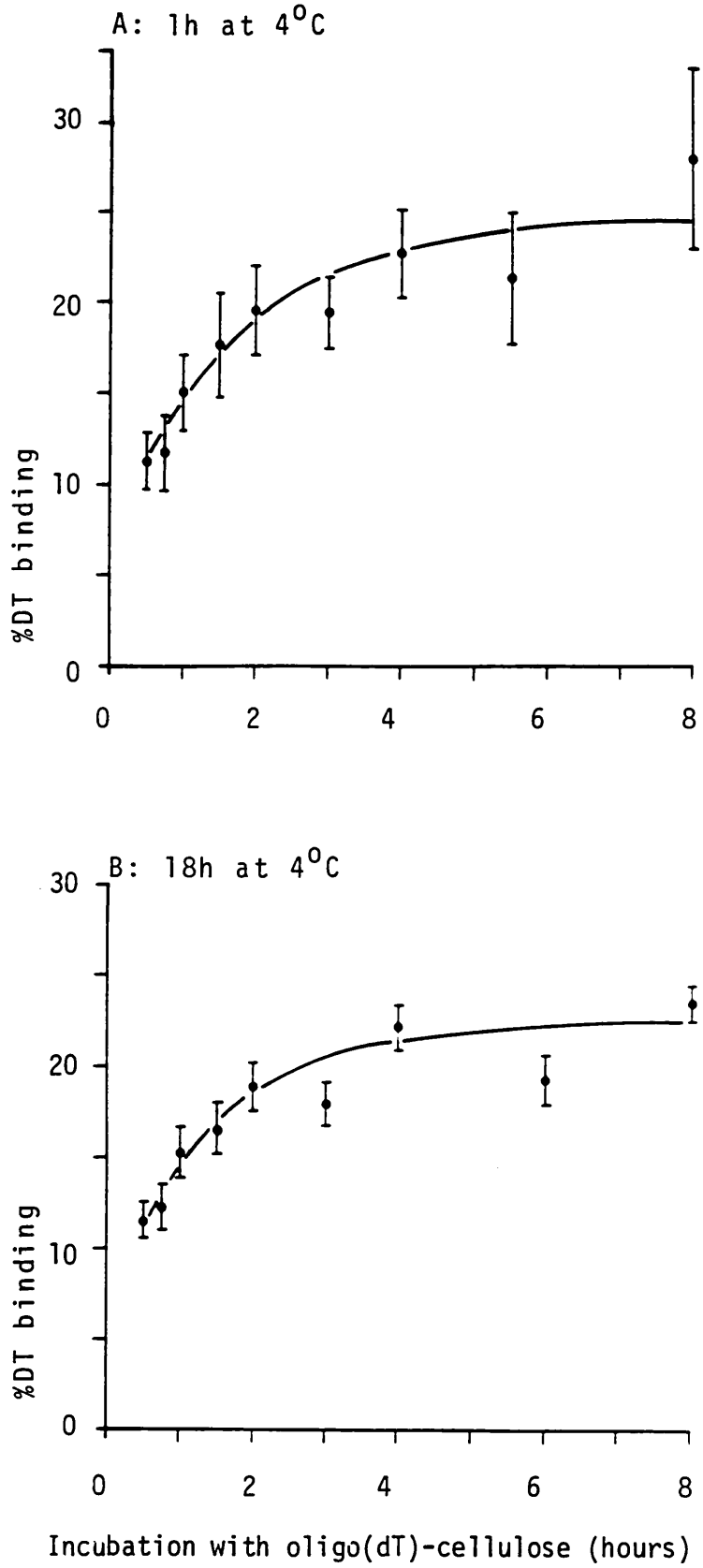


Fig. 21. Time course of cytosol oestrogen receptor interaction with oligo(dT)-cellulose following 1h and 18h incubation at 4°C. Results are mean ± SEM of five separate determinations.

Influence of Temperature on Activation of Oestrogen Receptor

Heat treatment promotes activation of rat uterine oestrogen receptor (Jensen et al., 1968, 1971; Buchi & Villee, 1976; Weichman & Notides, 1980). Breast tumour cytosol was therefore incubated at 4°C, 25°C, 30°C and 37°C for 10, 15, 20 or 30 minutes to establish if the promotion of oestrogen receptor binding to oligo(dT)-cellulose occurred and if so to establish the optimal conditions for receptor activation. Incubation of oestradiol-receptor complexes at elevated temperatures up to 30°C promoted activation as measured by its ability to bind to oligo(dT)-cellulose (Figure 22). The effect of heating cytosol at 37°C gave contrasting results in two separate experiments (Figure 22). One sample exhibited a decrease in the concentration of cytosol oestrogen receptor complexes at 37°C (Figure 22,I), possibly due to proteolytic degradation of receptor at higher temperature, whereas the receptor complexes were stable to heat treatment in another sample (figure 23,II). This probably reflects the heterogeneity of breast tumours which may contain variable amounts of proteolytic enzymes that are activated during heat treatment.

Activation of oestradiol-receptor complexes was maximal in samples heated at 25°C for 30 minutes compared with samples maintained at 4°C or heated to 37°C. These conditions (25°C for 30 min) were therefore employed in all subsequent experiments to study the effect of heat on oestrogen receptor activation.

Having established the optimal conditions for heat-induced activation of oestrogen receptor, this was studied in a series of 17 breast tumour samples. Heat treatment of cytosol preparations did not affect the estimation of oestradiol-receptor complexes but did promote receptor activation (figure 23). Aliquots of cytosol heated at 25°C for

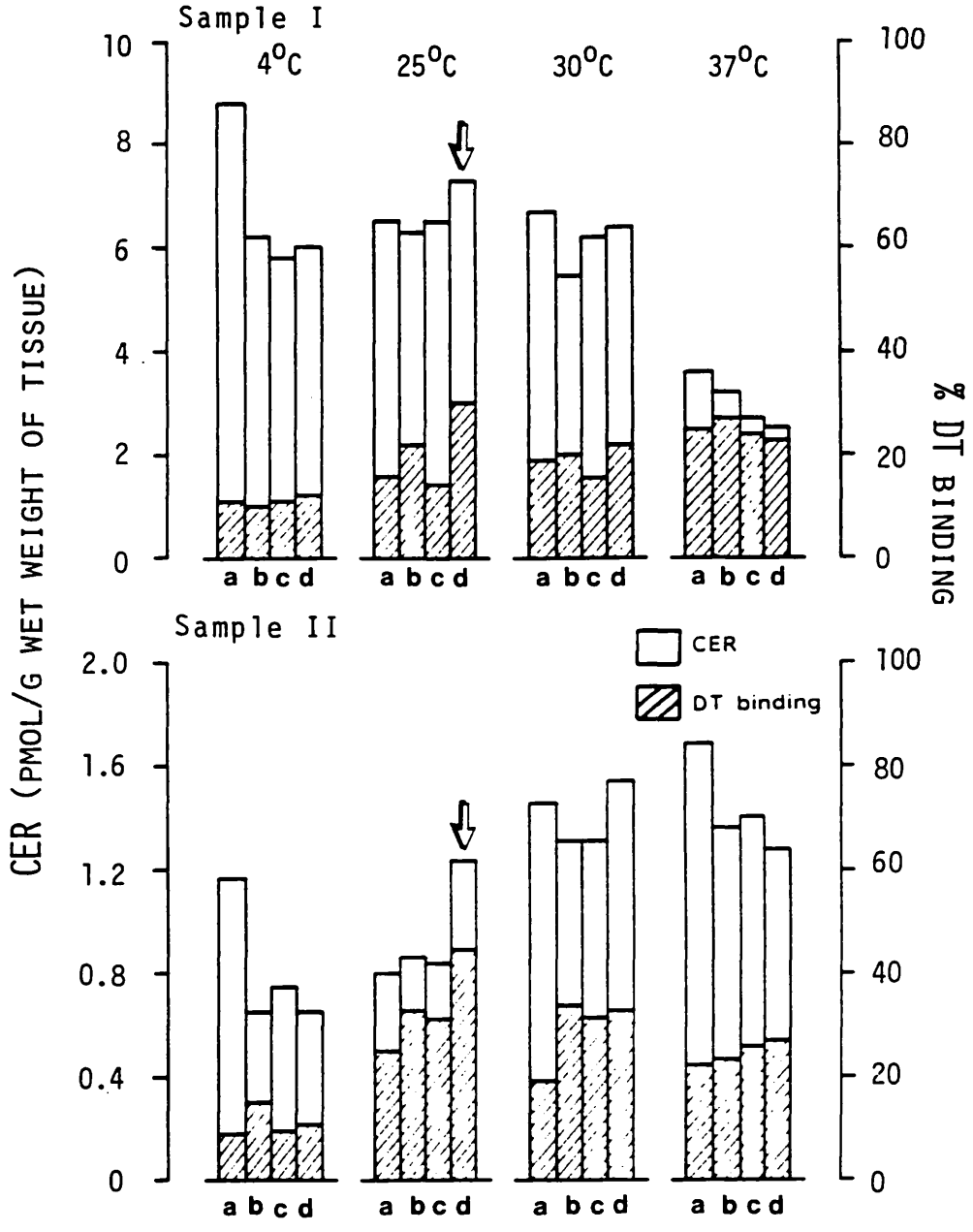


Fig. 22. Effect of elevated temperature on cytosol oestrogen receptor binding to oligo(dT)-cellulose in two separate experiments; a, 0 min; b, 10 min; c, 20 min; and d, 30 min. exposure to the various temperatures. Arrow indicates the conditions (25°C for 30 min) employed in subsequent experiments.

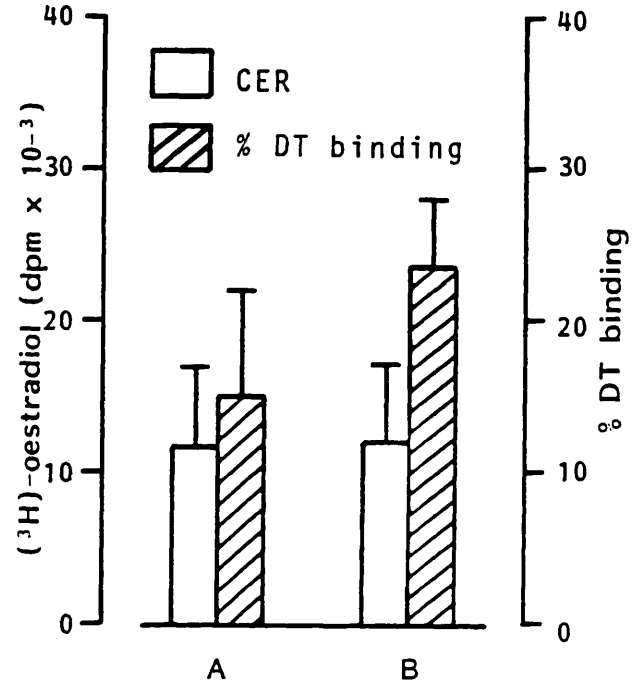


Fig. 23. Influence of heat treatment on activation of cytosol oestrogen receptors. A, 1h at 4°C; B, 1h at 4°C followed by incubation at 25°C for 30 min. Results are mean ± SD of 17 samples. Significantly different from corresponding heat treated samples (Student's t test), * $p<0.0275$.

30 minutes contained a significantly higher proportion of activated oestrogen receptor complexes than those maintained at 4°C (paired t test, $p < 0.025$).

Influence of Sodium Molybdate on Activation of Breast Tumour Cytosol Oestrogen Receptor

Miller et al., (1981) found that the presence of 10-20mM molybdate in the homogenisation buffer stabilised the receptor. It has been proposed that molybdate protects the receptor against specific proteolytic cleavage by stabilisation of phosphate groups. Molybdate has also been demonstrated to inhibit activation of rat uterine oestrogen (Mauck et al., 1982; Myatt et al., 1982a), glucocorticoid (Nielsen et al., 1977a,b; Shyamala & Leonard, 1980) and chick oviduct progesterone (Nishigori & Toft, 1980) receptors. The effect of sodium molybdate on oestrogen receptor activation has therefore been studied in a series of breast tumour cytosols.

There was no significant effect of molybdate on cytosol oestrogen receptor content (Table 20) [$t = 0.032$ ($n=11$), NS]. However, in the presence of molybdate there was a significant inhibition of oestrogen receptor activation as measured by oligo(dT)-cellulose binding (Table 20): $t = 6.73$ ($n=11$), $p << 0.001$. Total inhibition of activation was not observed; approximately 0-7% of the oestrogen receptor complexes were activated in the presence of molybdate. Myatt et al., (1982a) have reported similar findings for the rat uterine oestrogen receptor.

TABLE 20: INFLUENCE OF SODIUM MOLYBDATE ON ACTIVATION OF CYTOSOL OESTROGEN RECEPTOR.

CER -MOLYBDATE	%DT	CER +MOLYBDATE	%DT
1.26	10.4	1.19	0
4.85	19.1	4.91	6.8
0.20	31.0	0.23	4.7
4.62	22.2	4,58	5.6
0.37	18.3	0.41	3.1
2.31	12.9	2.12	2.8
1.21	15.4	1.23	7.2
0.60	29.7	0.62	3.2
1.20	8.5	1.31	0
7.36	24.5	7.10	4.1
1.35	22.9	1.30	4.0

Cytosol oestrogen receptor concentration expressed as p.mol/g. wet weight of tissue. The mean proportion of oestrogen receptors bound to oligo(dT)-cellulose in the absence of molybdate was significantly different from that in the presence of molybdate (Student's t test): $p<<0.001$.

Influence of ATP on Activation of Oestrogen Receptor

The effect of ATP was studied in 8 breast tumour samples. The capacity of the oestrogen receptor to bind to oligo(dT)-cellulose in these samples prior to addition of ATP ranged from 5.6% to 51.1% The effect of ATP was related to the initial proportion of activated oestrogen receptors present in the tumour sample. Tumours that contained $\geq$20% binding of oestrogen receptor to oligo(dT)-cellulose could not be further activated by ATP or heat treatment (figure 24a). There was no significant difference in the concentration of cytosol oestrogen receptors following ATP addition or heat treatment in these samples. Only those tumours that contained less than 20% activated oestrogen receptors at 4°C were activated by ATP or heat treatment. This effect was a consequence of changes in the ability to bind to oligo(dT)-cellulose and was not related to the absolute amounts of cytosol oestrogen receptor (Table 25).

When the subset of samples containing less than 20% activated complexes at 4°C were considered (Figure 24b), there was a significant increase in the proportion of activated receptor complexes in the presence of ATP ($p < 0.01$), but no effect on the concentration of cytosol oestrogen receptors. Similar findings have been reported for the effect of ribonucleotides on activation of oestrogen, glucocorticoid, androgen and progesterone receptors in MCF-7 cells (Chong & Lippman, 1982).

Heat treatment (25°C for 30 min) promoted activation of oestrogen receptor to the same extent both in the absence and presence of ATP without any significant effect on cytosol oestrogen receptor concentration.

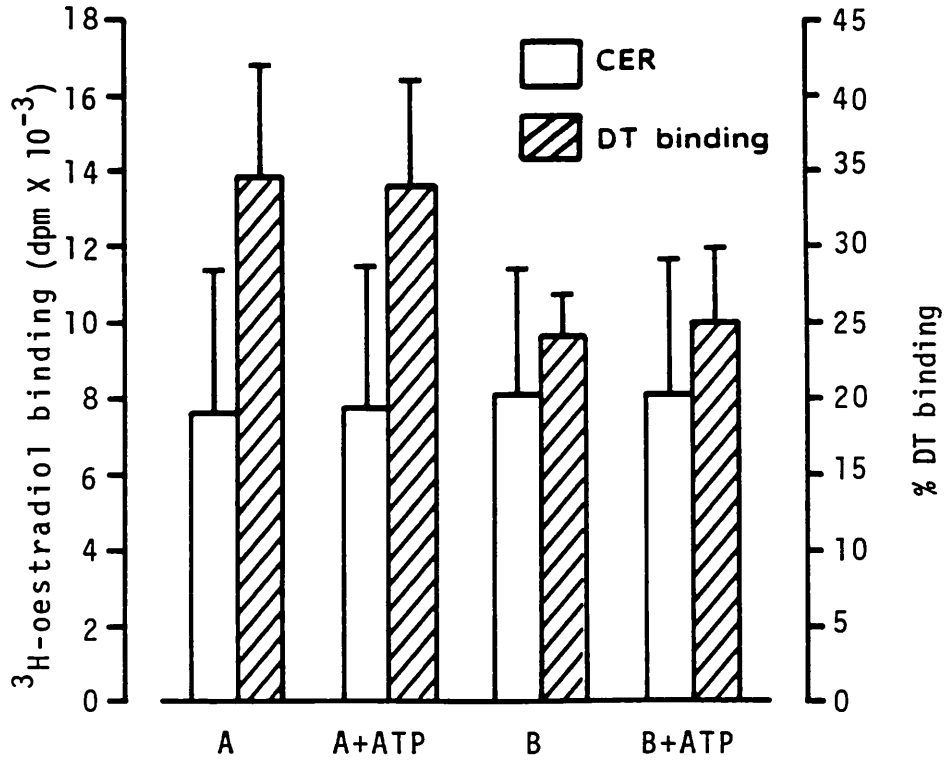


Fig. 24a. Influence of ATP on activation of cytosol oestrogen receptor. A, 1h at 4°C; B, 1h at 4°C followed by incubation at 25°C for 30 min. Results are mean ± SD of eight separate determinations.

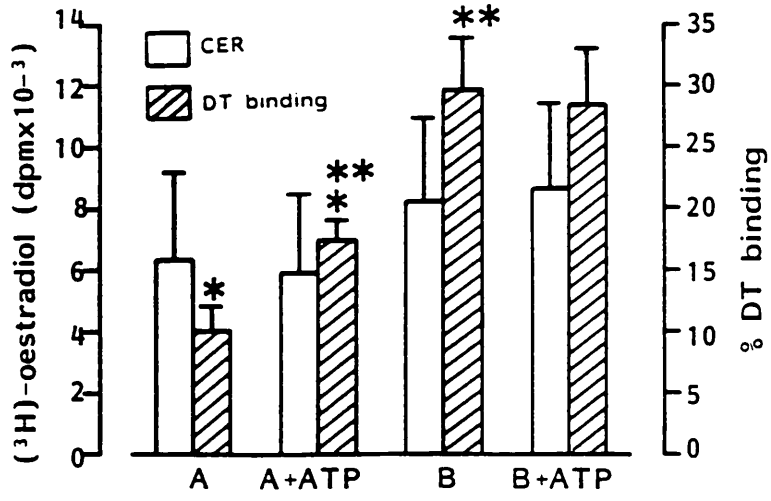


Fig. 24b. Influence of ATP on activation of cytosol oestrogen receptors in a subset of samples exhibiting less than 20% binding of oestrogen receptors to oligo(dT)-cellulose at 4°C. A, 1h at 4°C; B, 1h at 4°C followed by incubation at 25°C for 30 min. Results are mean ± SD of four separate samples; Student's t test, *$p<0.01$, ** $P<0.03$.

B: Relationship Between Activation and the Molecular Forms of the Breast Tumour Cytosol Oestrogen Receptor

Thrower et al., (1976) demonstrated that the activated cytosol oestrogen receptor from the rat uterus, as assessed by binding to oligo(dT)-cellulose, has a molecular weight of 120,000 daltons corresponding to the nuclear 5S receptor complex. Other studies have shown that activation of the rat uterine oestrogen receptor is accompanied by transformation of the 4S receptor to the 5S form (Jensen & DeSombre, 1973; Notides & Nielsen, 1974; Yamamoto, 1974; Notides et al., 1981). However, Park & Wittliff (1977) reported that heat-induced activation of the lactating rat mammary gland cytosol oestrogen receptor did not exhibit an alteration in sedimentation coefficient from 4S to the 5S form. In order, therefore, to establish if activation of human breast tumour cytosol oestrogen receptor is accompanied by transformation, the molecular forms of the activated receptor have been examined by high performance size exclusion gel chromatography (HPSEC). Size exclusion HPLC provides a more rapid method for analysing steroid receptor proteins than sucrose density gradient sedimentation (Pavlik et al., 1982). Resolution of receptor forms by sucrose density gradient analysis requires 13-18h of centrifugation; using HPSEC such resolution can be obtained in 40 minutes. Rapid separations minimise ligand dissociation, time dependent activation of receptor and proteolysis by endogenous proteases present in the cytosol preparations (Schneider & Dao, 1977; Tilzer et al., 1981; Miller et al., 1981), while allowing resolution of receptor forms that are qualitatively similar to those observed by sucrose density gradient analysis (Pavlik et al., 1982; Wiehle et al., 1984).

Characterisation of HPSEC columns

The distribution coefficients (Kav's) of reference molecular weight marker proteins were derived by monitoring their radioactivity or absorbance at 280 nm in 5 replicate determinations. The relationship defined by Ackers (1965) was used:

$$Kav = \frac{Ve-Vo}{Vt-Vo}$$

where Vo = the column void volume; Ve = peak elution volume of the sample; Vt = total volume of the column.

Using semi-logarithmic graph, Kav values for each protein standard were plotted on the linear scale against the corresponding molecular weight on the logarithmic scale. A straight line was obtained and the molecular weights of the receptor forms were derived by reference to this plot (Figure 25). Molecular Stokes radii of the receptor forms were derived from a plot of (Kav) against the molecular stokes radii of the standard proteins used for the calibration of the column (Figure 26) (Porath, 1963). Column performance was checked regularly using the reference molecular weight marker proteins.

The sedimentation coefficients were derived from the classical relationship:

$$R = S1/S2 = [MWt.1/MWt.2]$$

applied in sucrose density gradient centrifugation analysis (Martin & Ames, 1966), where S1, S2 and MWt.1, MWt.2 are the sedimentation coefficients and the molecular weights of the unknown and the standard reference proteins respectively.

The molecular weight of the receptor forms was derived from the plot of Kav against log(MWt) of the standard proteins and inserted in the above equation to derive the sedimentation coefficient.

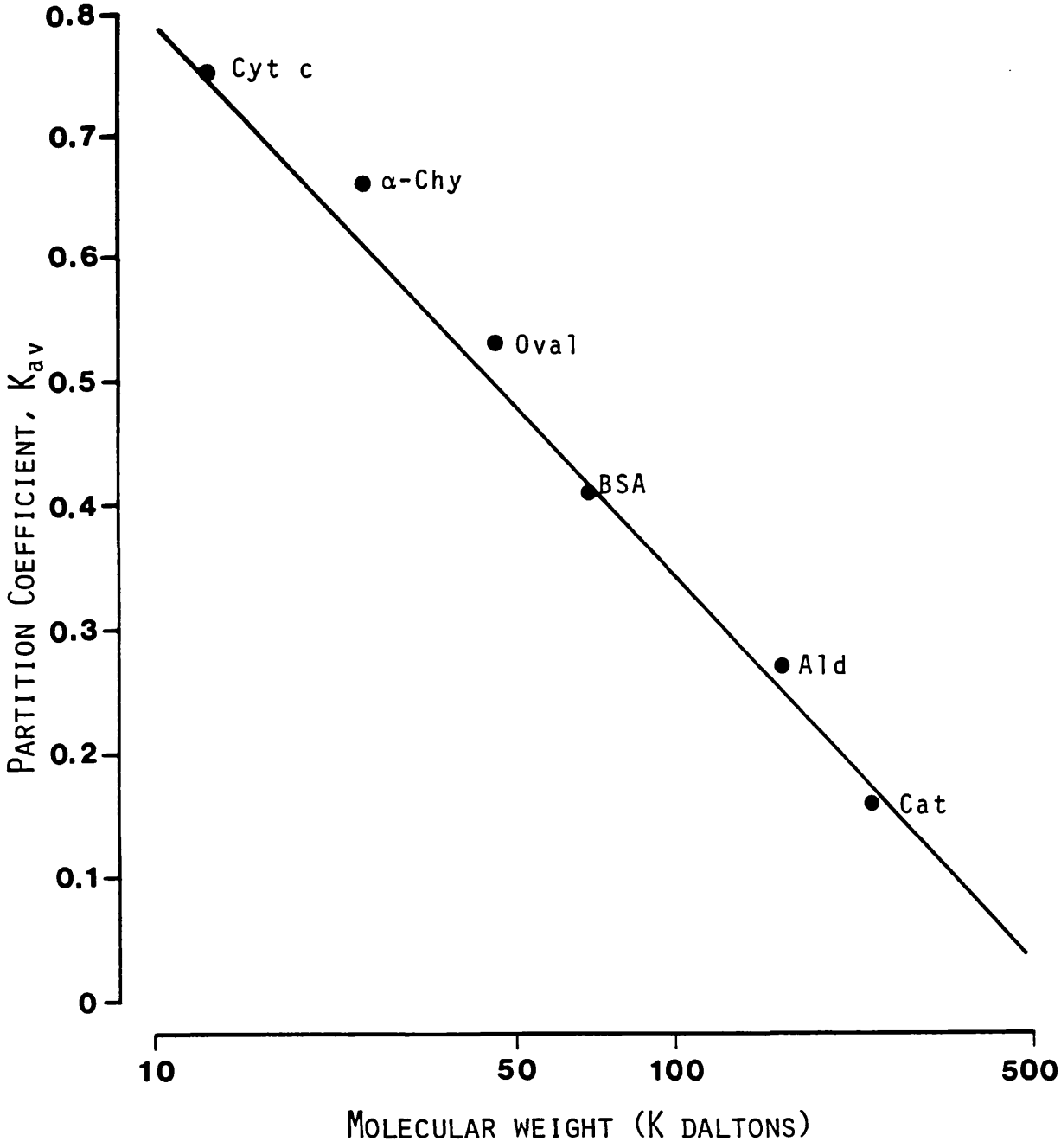


Fig. 25. Standard curve for the calibration of HPSEC columns. Standard reference proteins - Ca, catalase; Ald, aldolase; BSA, bovine serum albumin; Oval, ovalbumin; α-Chy, α-chymotrypsinogen; and Cyt c, cytochrome c - were separated on Spherogel-TSK 3000SW columns at a flow rate of 0.6 ml/min and the absorbance of the effluent monitored at 280nm. The distribution coefficient, Kav was calculated from Kav= (Ve - Vo)/(Vt - Vo), where Ve (elution volume) was the volume corresponding to to peak elution of the protein; Vo (void volume) was the volume corresponding to peak elution of ferritin or blue dextran 2000 (10.8 ml); and Vt (totoal volume) was 29.8 ml. The Kav of each protein standard was plotted against its molecular weight. The straight line is the best fit estimate determined by linear regression analysis. The molecular weights of the oestrogen binding forms were derived by interpolation of their respective Kav's.

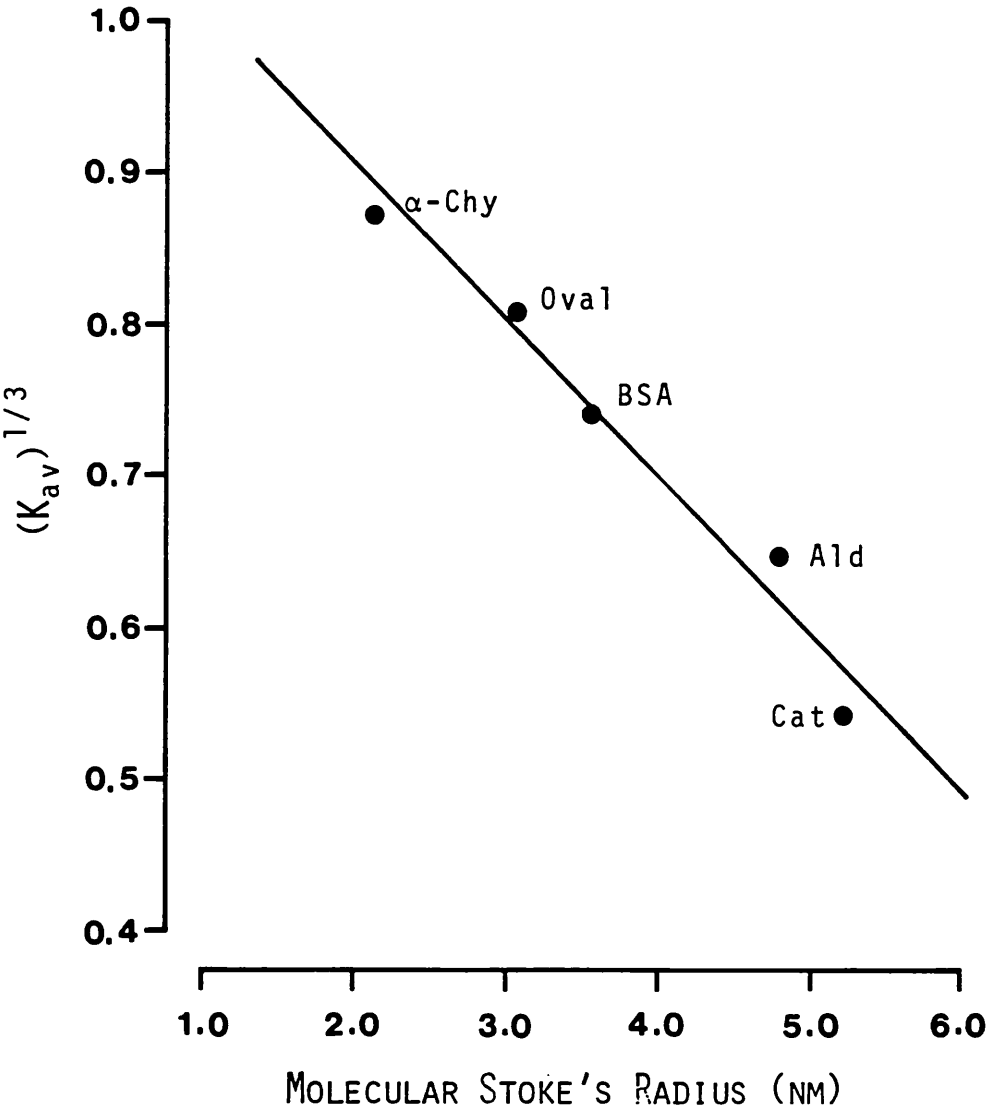


Fig. 26. Correlation of the partition coefficients, Kav's with molecular Stoke's radii. Stoke's radius for the standard proteins were from Page and Grodin (1969). The distribution coefficient, Kav was calculated as described in fig. 25 for each standard protein and plotted as a function of Stoke's radius. The straight line is the best fit estimate determined by linear regression analysis. The Stoke's radius of the oestrogen receptor forms were derived by interpolation of their respective Kav's.

Analysis of Breast Tumour Cytosol Oestrogen Receptor by HPSEC

Breast tumour cytosol was prepared and incubated with 5nM [^{3}H]-oestradiol for 1h at 4^{0}C and separated using HPSEC after treatment with DCC to remove unbound steroid. Radioactivity was determined in the fractionated samples obtained in the presence and absence of DES. Analysis of DES suppressible radioactivity allowed the determination of the distribution coefficient (Kav) from peak height analysis. The molecular weight and Stokes radius of the oestrogen receptor forms were derived by interpolation of the appropriate standard curves. Estimates of receptor recovery were always greater than 70% of input and frequently greater than 90%.

Resolution of Different Forms of the Oestrogen Receptor and their Relationship to Receptor Activation.

The human breast tumour cytosol oestrogen receptor could be resolved into two distinct components by HPSEC; a high-molecular-weight component with a molecular weight in the range 290-340K daltons and a low-molecular-weight component with the molecular weight in the range 60-66K daltons (Table 21; Figure 27). The high-molecular-weight oestrogen binding form had an estimated Stokes radius of 5.9-6.5 nm with the sedimentation coefficient in the range 11-13 S (Table 21). The smaller oestrogen binding form had an estimated Stokes radius of 3.2-3.7 nm with the sedimentation coefficient in the range 4.3-4.7 S (Table 21). These two components of the oestrogen receptor will be referred to as 300K and 60K respectively.

Only breast tumours previously shown to contain oestrogen receptors were analysed on HPSEC; they could be classified into two groups on the basis of the apparent molecular weights of the receptor

TABLE 21: MOLECULAR CHARACTERISTICS OF THE HUMAN BREAST TUMOUR CYTOSOL OESTROGEN RECEPTOR.

	COMPONENTS OF CYTOSOL OESTROGEN RECEPTOR	
	HIGH MOLECULAR WEIGHT	LOW MOLECULAR WEIGHT
APPARENT MOLECULAR WEIGHT (DALTONS)	290-340,000	60-66,000
ESTIMATED STOKE'S RADIUS (nm)	5.9 - 6.5	3.2 - 3.7
ESTIMATED SEDIMENTATION COEFFICIENT (SVEDBERGS)	11 - 13 S	4.3 - 4.7 S

TABLE 22: DISTRIBUTION OF OESTROGEN RECEPTOR FORMS IN A SERIES OF HUMAN BREAST TUMOURS.

	CYTOSOL OESTROGEN RECEPTOR	
	300K FORM ALONE PRESENT	300K AND 60K FORMS PRESENT
NUMBER OF SAMPLES ANALYSED	12	9
PROTEIN CONTENT - RANGE (mg protein/ml cytosol)	3.10 - 7.10	4.20 - 6.60
MEAN PROTEIN CONTENT - (MEAN ± S.D.)	5.19 ± 1.10	5.07 ± 0.87

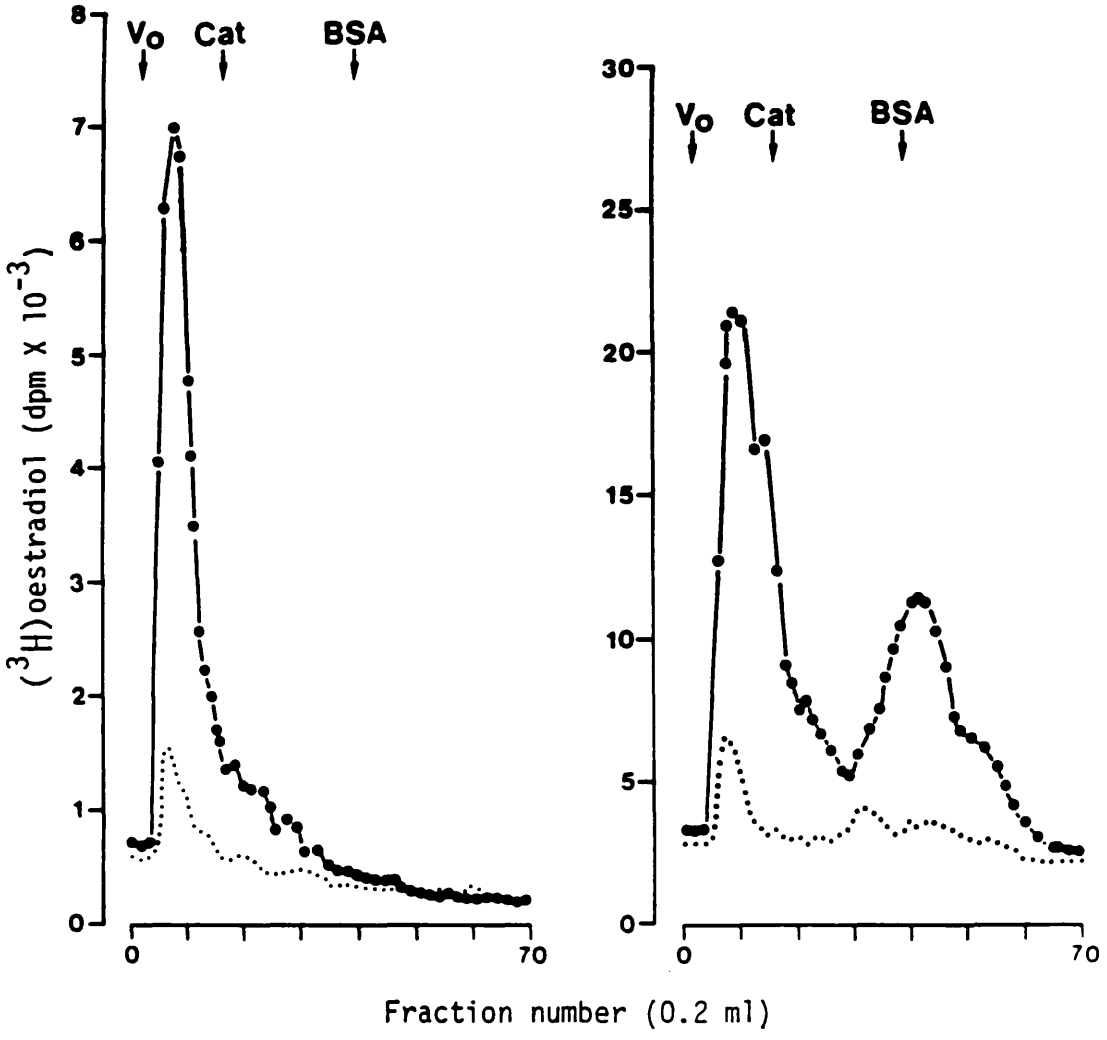


Fig. 27. Resolution of the molecular forms of the human breast tumour cytosol oestrogen receptor by HPSEC. Cytosols from different breast tumours (a,b) were incubated with 5nM ^{3}H-oestradiol in the presence (·····) and absence (●—●) of a 200-fold excess of DES. A 200µl aliquot was applied to the TSK-3000SW column and the receptors were eluted with PEG (phosphate buffer containing 1.5mM EDTA and 10% glycerol) buffer at a flow rate of 0.6 ml/min. Vo = void volume as determined by exclusion of ferritin or blue dextran 2000; Cat = catalase; BSA = bovine serum albumin.

forms. Of 21 breast tumours analysed, twelve samples contained only the high-molecular-weight (300K) form and nine samples contained both 300K and 60K components (Figure 27). There was no significant difference in the protein concentration of the samples analysed in each group (Table 22). None of the samples analysed exhibited the low-molecular-weight, 60K, form of the receptor alone. Wittliff et al., (1981) have made similar observations but, in addition, have reported the presence of the low-molecular-weight (2.9 nm) form alone in some human breast tumours.

In order to determine if the molecular form of the receptor was related to its ability to bind to oligo(dT)-cellulose, a comparison of HPSEC data and oligo(dT)-cellulose binding was made. This revealed that the proportion of activated oestrogen receptor complexes in tumours that contained only the 300K form of the receptor was significantly lower than in tumours that contained both 300K and 60K forms of the receptor (Table 23): $t = 4.6289$, $p < 0.001$. Thus the presence of 60K form of the receptor is apparently associated with an increase in the degree of receptor activation.

The presence of cytosol progesterone receptor, which is a determinant of a functional oestrogen response pathway (McGuire, 1977; see pp. 140-142), was also related to the presence of the 60K, activated oestrogen receptor (Table 24). The concentration of cytosol progesterone receptors was significantly higher in tumours containing both 300K and 60K forms of the oestrogen receptor compared to those exhibiting only the 300K aggregate.

The presence of the 60K form of the oestrogen receptor in human breast tumours is not only associated with receptor activation but is also correlated with the quantitative presence of cytosol progesterone receptor which is a functional end-point of response to oestradiol.

This suggests that the presence of the 60K activated oestrogen receptor is a functional determinant of the oestrogen receptor pathway.

TABLE 23: RELATIONSHIP BETWEEN THE MOLECULAR FORM(S) OF THE OESTROGEN RECEPTOR AND THE EXTENT OF RECEPTOR ACTIVATION.

300K FORM ONLY PRESENT %DT	300K AND 60K FORMS PRESENT %DT
7.2	11.8
11.2	16.3
14.3	19.1
4.8	17.6
10.3	18.6
10.1	18.0
9.3	15.4
8.4	9.0
6.9	18.6
13.1	
11.2	
8.2	

Activation of oestrogen receptor was assesed by its ability to bind to oligo(dT)-cellulose following incubation for 1 hour at 4°C. The proportion of activated oestrogen receptors was significantly higher in tumours that contained both the 300K and 60K forms of the oestrogen receptor (Student's t test): $p<0.001$.

TABLE 24: OESTROGEN AND PROGESTERONE RECEPTOR LEVELS RELATED TO THE MOLECULAR FORM(S) OF THE OESTROGEN RECEPTOR.

300K FORM ONLY PRESENT		300K AND 60K FORMS PRESENT	
CER	CPR	CER	CPR
10.02	1.59	0.37	2.73
2.19	1.58	1.33	5.46
5.48	0.48	2.49	1.68
0.38	0.09	2.08	0.70
1.26	2.47	13.67	4.45
0.79	3.38	0.57	3.78
1.00	0.08	1.35	5.35
7.34	5.65	11.18	3.61
1.27	0.28	1.26	2.47
12.31	0.66		
4.72	3.63		
1.65	0		

Oestrogen and progesterone receptor concentrations expressed as p.mol/g. wet weight of tissue. The mean cytosol oestrogen receptor concentrations were not different between the two groups; the mean concentration of cytosol progesterone receptor was significantly higher in tumours containing both the 300K and 60K forms of oestrogen receptor (Student's t test): $p<0.025$.

Effect of Heat Activation on the Molecular Form(s) of the Oestrogen Receptor

Activation of human breast tumour oestrogen receptor is promoted by heat activation (25°C for 30 min; see page 165). In order therefore to verify that an increase in oligo(dT)-cellulose binding was accompanied by a change in the molecular form of the receptor (see above), the HPSEC profiles of the heat-activated oestrogen receptor were determined. There was a change in the distribution of the forms of the oestrogen receptor from the high-molecular-weight (300K) region to the low-molecular-weight form, 60K, following heat treatment (Figures 28). This transformation of the 300K receptor to the 60K form of the human breast tumour cytosol oestrogen receptor is accompanied by a significant increase in the proportion of activated receptor as assessed by oligo(dT)-cellulose binding; $t = 9.2033$, $p < 0.001$ (Table 25).Heat treatment had no significant effect on the estimation of cytosol oestrogen receptor concentration.

The magnitude of the heat-induced increase in the proportion of receptor that was activated was apparently related to the molecular form(s) of the oestrogen receptor present in the sample. In those tumours that contained predominantly the 300K form at 4°C, the magnitude of the heat-induced increase in the proportion of activated oestrogen receptor complexes was significantly greater than in tumours containing both 300K and 60K forms of receptor (Table 25) (Wilcoxon's sign rank test; $p < 0.025$). This data indicates that activation of human breast tumour cytosol oestrogen receptor is associated with the transformation of the high-molecular-weight (300K) form of receptor to the low-molecular-weight (60K) form.

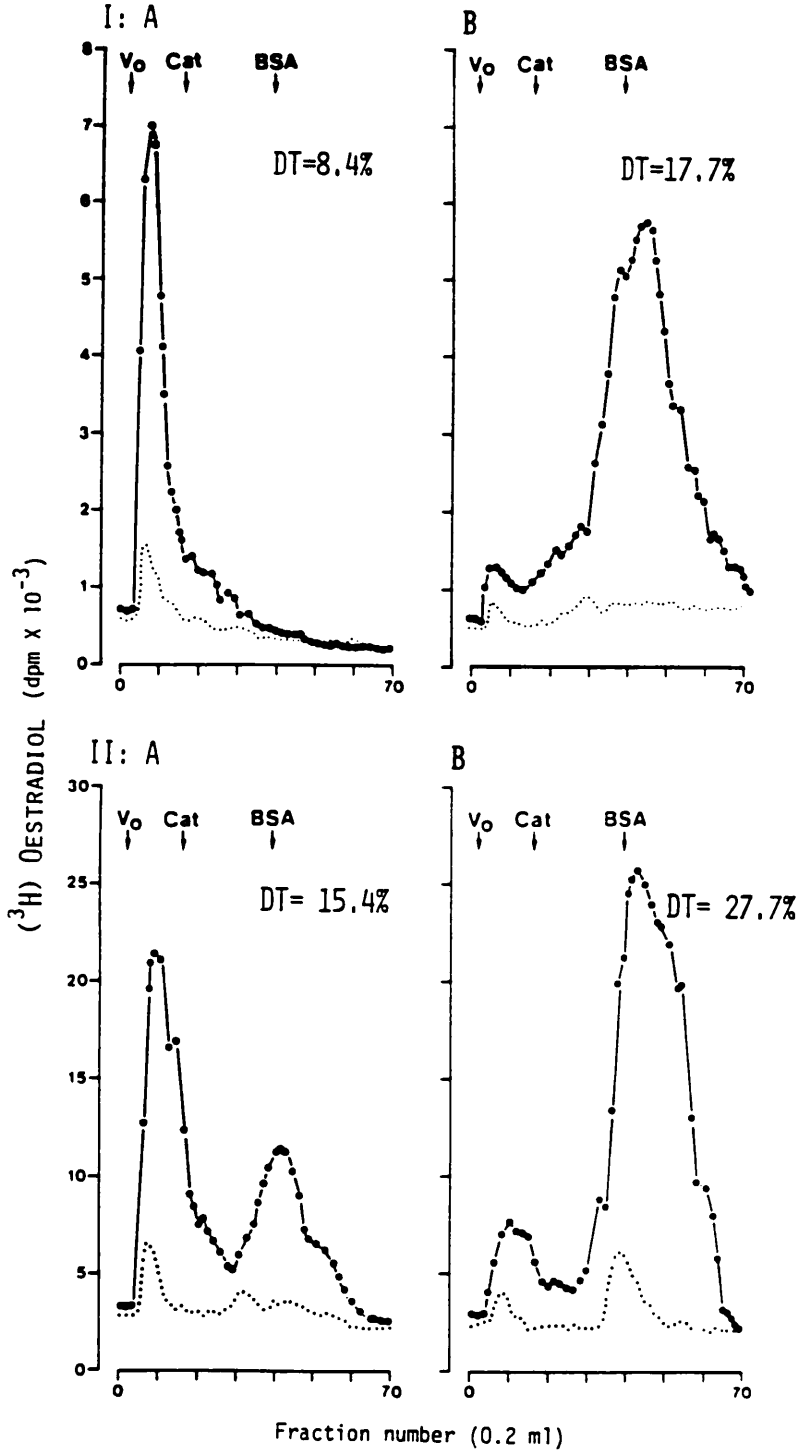


Fig. 28. Influence of heat treatment on the distribution of the molecular forms of the oestrogen receptor. Cytosols from two different breast tumour specimens (I, II) were incubated for 1h at 4°C (A) or heat treated at 25°C for 30 min following incubation for 1h at 4°C (B). Samples were chromatographed as detailed in legend to Fig. 27, and the proportion of activated receptors was measured in duplicate aliquots by oligo(dT)-cellulose chromatography. Identification of peaks as in figure 27.

TABLE 25: INFLUENCE OF HEAT TREATMENT ON ACTIVATION OF OESTROGEN RECEPTORS RELATED TO THE MOLECULAR FORM(S) OF THE OESTROGEN RECEPTOR.

300K FORM ONLY		300K AND 60K FORMS PRESENT	
%DT BINDING [a]	[b]	%DT BINDING [a]	[b]
7.2	21.5	11.8	22.6
11.2	26.4	16.3	22.6
14.3	27.8	19.1	33.3
4.8	26.0	17.6	24.2
10.3	18.4	18.6	28.3
10.1	25.0	18.0	33.0
9.3	32.1	15.4	27.7
8.4	21.5	9.0	22.0
6.9	16.1	18.6	29.7
13.1	24.6		
11.2	24.1		
8.2	21.0		

a; proportion of oestrogen receptors bound to oligo(dT)-cellulose after 1 hour incubation at 4°C, and *b;* after 1 hour at 4°C followed by 30 minutes at 25°C.

Influence of Potassium Chloride on the Molecular Form(s) of the Activated Oestrogen Receptor

Oestrogen receptor complexes prepared and eluted in low-ionic-strength buffer separated primarily as 300K form alone or 300K and 60K forms. When the oestrogen receptor complexes from breast tumour cytosols prepared in low-ionic-strength buffer were eluted in phosphate buffer containing 400 mM potassium chloride, the high-molecular-weight component was transformed to the low-molecular-weight, 60K, oestrogen binding form (Figure 29) in a manner similar to that reported for the lactating mammary gland of the rat (Gardner & Wittliff, 1973). Similar observations were made when the ionic-strength of the cytosols was adjusted to 400 mM salt by adding an appropriate amount of potassium chloride prior to analysis by HPSEC.

Heat activated oestrogen receptor complexes separated predominantly as 60K form in the absence or presence of 400 mM potasium chloride in the elution buffer (Figure 29).

Influence of Sodium Molybdate on the Apparent Molecular Weight of the Oestrogen Receptor

Sodium molybdate inhibits activation of the human breast tumour cytosol oestrogen receptor (see pp. 168). In the presence of 20 mM sodium molybdate virtually all of the specific oestrogen binding component eluted in the high-molecular-weight (300K) region (Figure 30). The apparent increase in the recovery of the 300K form of the oestrogen receptor may be attributed to the stabilising effect of sodium molybdate. Heat treatment of the molybdate-stabilised oestrogen receptor complexes did not result in a transformation of the high-molecular-weight (300K) form of the receptor to the 60K form

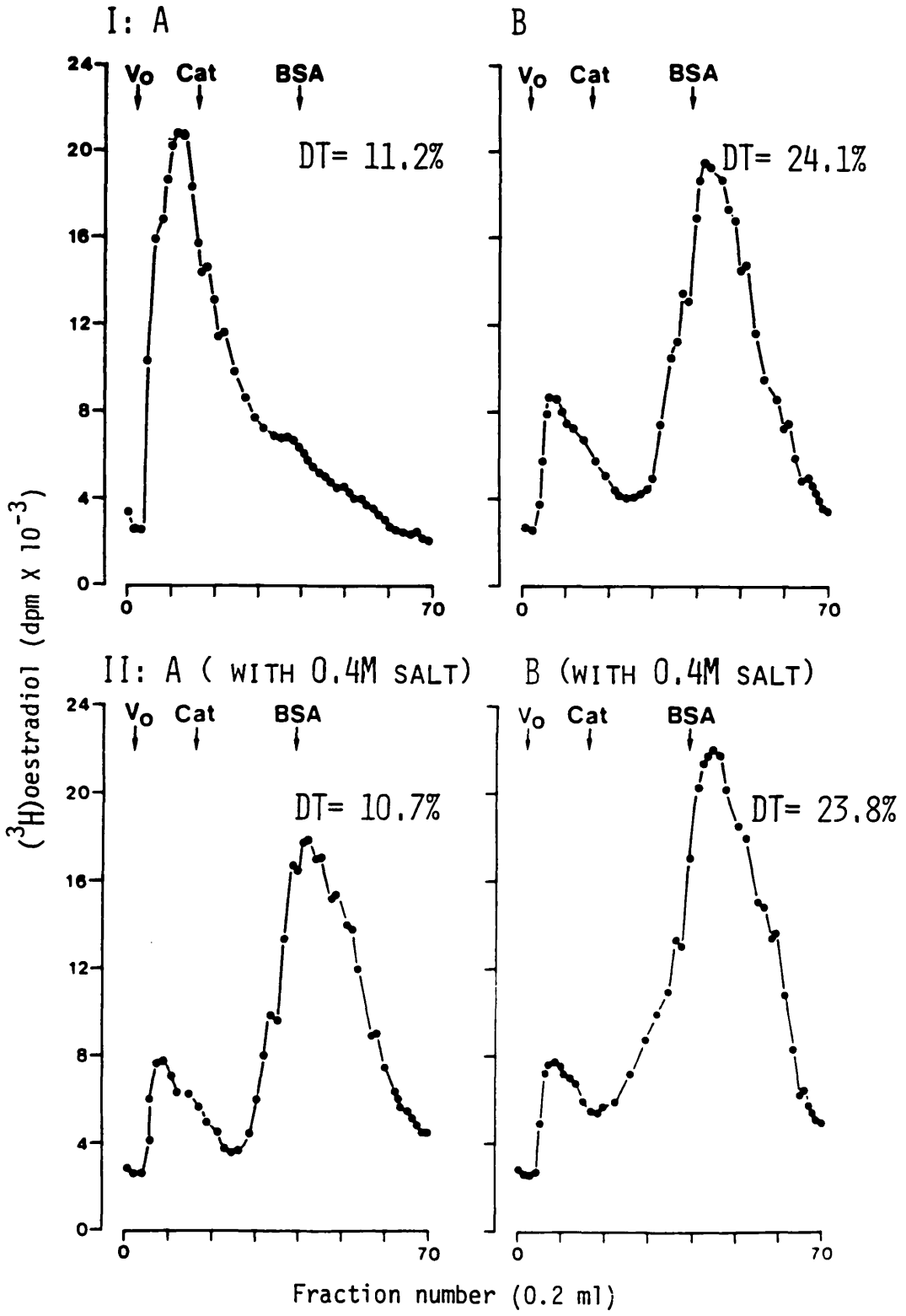


Fig. 29. Influence of potassium chloride (KCl) on the molecular forms of human breast tumour cytosol oestrogen receptor. Breast tumour cytosol was incubated as described in legend to figure 27 either in the absence (I) or presence of 0.4M KCl (II). Following incubation for 1h at 4°C (A), aliquots were heat activated at 25°C for 30min (B) before analysis of the molecular forms by HPSEC. The proportion of activated receptors was measured in duplicate aliquots of each sample by oligo(dT)-cellulose chromatography. Identification of peaks as in figure 27.

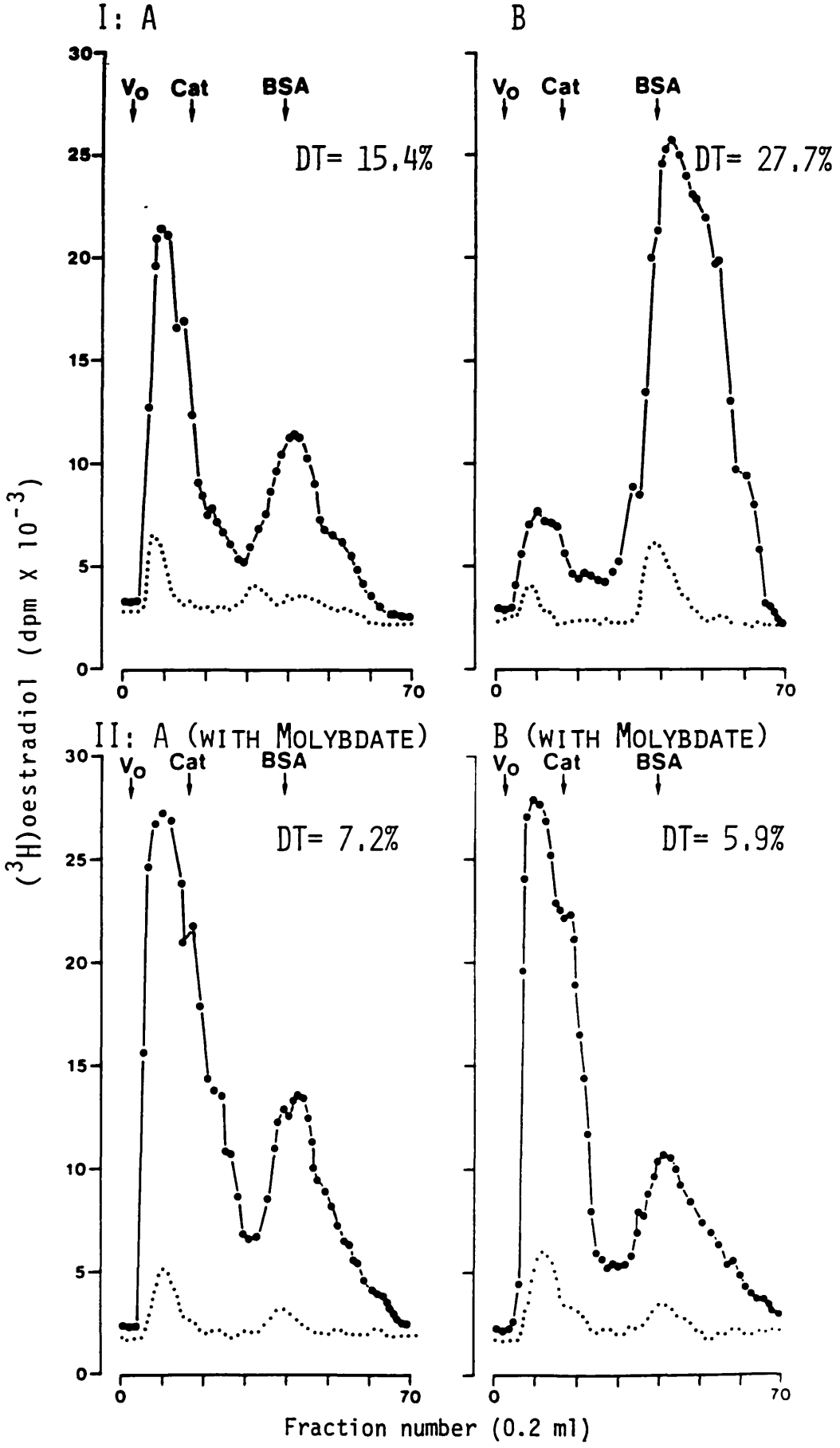


Fig. 30. Influence of sodium molybdate on the molecular forms of the human breast tumour cytosol oestrogen receptor.Breast tumour cytosol was incubated, as described in legend to figure 27, either in the absence (I) or presence of 10mM molybdate (II). See legend to figure 29 for details.

(Figure 30) and there was no increase in oligo(dT)-cellulose binding of the receptor. Therefore, sodium molybdate inhibits activation of human breast tumour cytosol oestrogen receptor and maintains the receptor in the high-molecular-weight (300K) form. In the absence of molybdate, heat activated oestrogen receptor complexes separated predominantly as the 60K form with an increase in the proportion of oestrogen receptor binding to oligo(dT)-cellulose (Figure 30). This data further confirms that activation of breast tumour cytosol oestrogen receptor is associated with a change in the molecular form of the receptor from the high-molecular-weight (300K) form to the 60K form.

Influence of ATP on the Molecular Form(s) of the Activated Oestrogen Receptor

The ribonucleotide triphosphate, ATP, has been reported to activate the oestrogen receptor from MCF-7 cells (Chong & Lippman, 1982) and also this study in human breast tumours (see pp. 170). The ATP induced activation of oestrogen receptor is less effective than that induced by heat treatment at 25°C for 30 minutes (see pp.165). In order to determine if such a differential effect on receptor activation could be distinguished in the molecular transformation of the receptor, the effect of ATP and heat activation were compared.

Heat treatment and the presence of ATP at 4 °C result in the transformation of the oestrogen receptor into the 60K form. However, a greater proportion of the receptor was found in the 60K region following heat treatment than in the presence of ATP at 4°C (Figure 31). This was reflected in the relative increase in oligo(dT)-cellulose binding; the increase following heat treatment (7.2% to 24.7%) being greater than that achieved by ATP at 4°C (7.2% to 13.7%). When cytosol was heated in

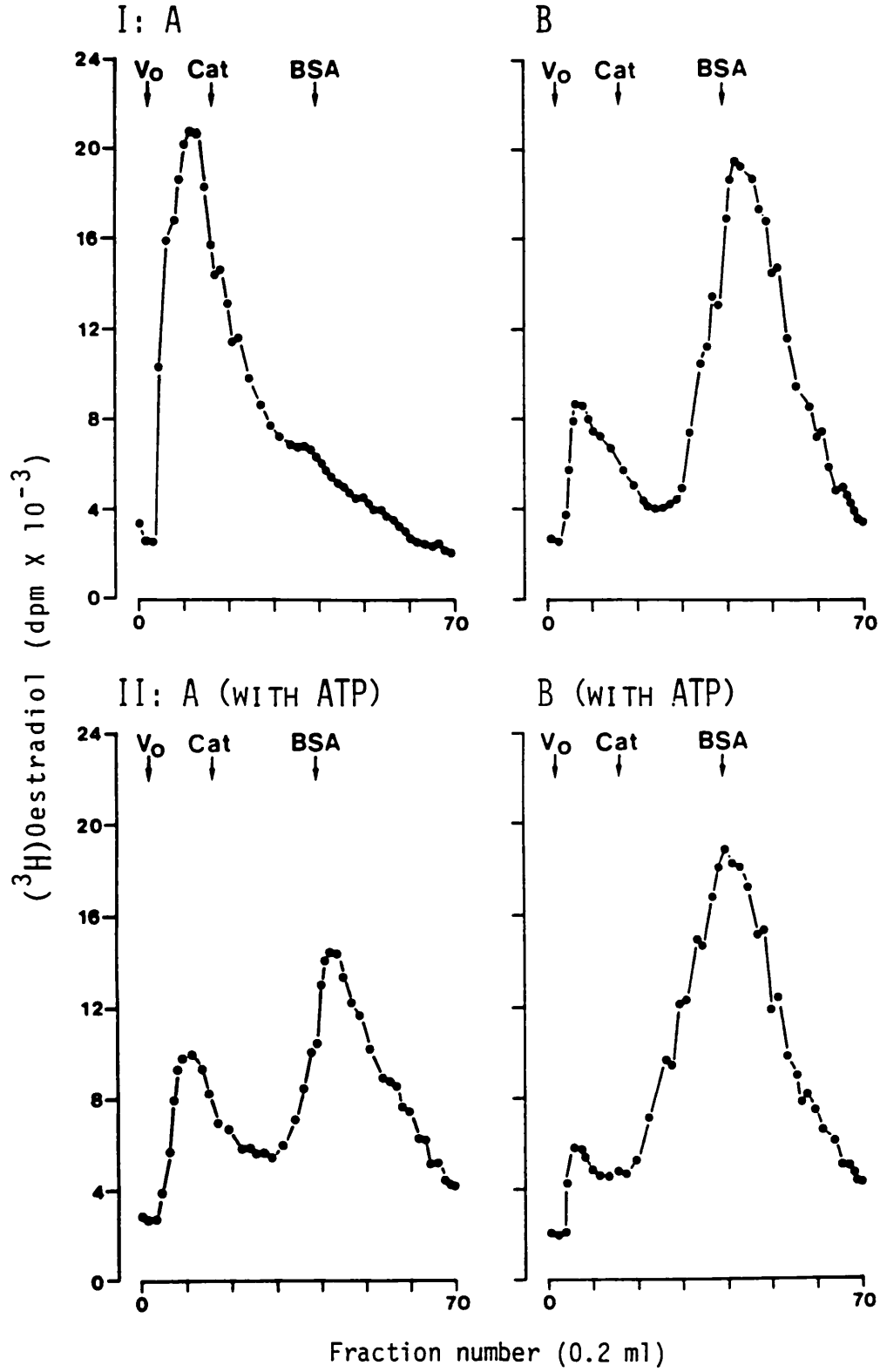


Fig. 31. Influence of ATP on the molecular forms of the human breast tumour cytosol oestrogen receptor. Breast tumour cytosol was incubated in the absence (I) and presence of ATP (II). See legend to figure 29 for details.

the presence of ATP (Figure 31, B(ATP)), the receptor was almost completely transformed to 60K with a concommitant activation (7.2% to 19.3%). The effect of ATP on the molecular form of the receptor was therefore similar to the effect of heat treatment.

All manipulations that activate the oestrogen receptor, for example heat treatment or the presence of ATP, caused the transformation of the receptor from the high-molecular-weight (300K) form of the receptor to the 60K form. However, agents such as sodium molybdate which inhibit activation maintained virtually all of the receptor in the high-molecular-weight form. It is therfore apparent that the activation of human breast tumour cytosol oestrogen receptor is associated with the transformation of the high-molecular-weight, 300K form of the receptor to the low-molecular-weight form with an apparent molecular weight in the range 60-66,000 daltons, Stokes radius, 3.2-3.7 nm and an estimated sedimentation coefficient in the range 4.3-4.7S.

CHAPTER FIVE

DISCUSSION

Numerous studies, including the present, have shown that approximately 50% of all biopsies of malignant breast tumours contain oestrogen receptors (Degenshein et al., 1979; Jensen et al., 1971, 1975; McCarthy & McCarthy, 1977; McGuire et al., 1975; Wittliff, 1974; Blamey et al., 1980). However, only about 50-60% of patients with tumours containing oestrogen receptors are responsive to either ablative or additive endocrine therapies. Several possible reasons have been proposed to explain this phenomenon including tumour heterogeneity and consequent non-uniformity of receptor status (Allegra et.al., 1980; Fidler & Hart,1981). There is evidence that some cells have various types of defects distal to the initial binding of steroid. Some cells may be able to bind oestrogen but are unable to translocate the receptor-complex into the nucleus. Such defective receptor proteins have been demonstrated in several experimental mouse mammary tumour systems (Shyamala, 1972; Vignon & Rochefort, 1976). Others may be defective at the nuclear level such that oestrogen receptor-complexes are translocated into the nucleus but induction of oestrogen specific responses is prevented possibly due to ineffective interaction with DNA (Nawata et al., 1981). Sato et al., (1981) have proposed a cytoplasmic factor in some oestrogen receptor-positive, endocrine resistant tumours which may inactivate the DNA-binding site of the receptor. It has been reported that specific nuclear acceptor sites for receptors are required for hormone action (Buller et al., 1975), and it is possible that absent or defective sites would lead to insensitivity to oestrogen receptor.

The presence of cytosol progesterone receptors in the cell indicates that the entire pathway of events involving oestrogen action is functional (McGuire, 1977). Therefore, tumours that contain oestrogen but not progesterone receptors then have one or several defects in the oestrogen receptor pathway, thus possibly explaining the decreased responsiveness to endocrine therapy in comparision to tumours that contain both receptors.

The molecular mechanism involved in generating the nuclear binding form of the oestrogen receptor are not clearly defined. However, using animal model systems it has emerged that this process, termed 'activation', is associated with an increased capability of the receptor-complex to bind to nuclei, DNA or oligo(dT)-cellulose *in vitro* (Thrower et al., 1976; Grody et al., 1982; Myatt et al., 1982b). This study has therefore investigated the ability of human breast tumour cytosol oestrogen receptors to bind to oligo(dT)-cellulose in an attempt to evaluate whether the activated receptor is a determinant of functionality of the receptor pathway and hence a potential indicator of the sensitivity of the tissue to endocrine manipulation. The relationship between the capability of the oestrogen receptor to bind to oligo(dT)-cellulose and the presence of nuclear oestrogen and cytosol progesterone receptors has been determined. The activation of human breast tumour cytosol oestrogen receptor has been partially characterised and related to the molecular form(s) of the receptor.

A: Steroid Receptor Status in Human Breast Cancer

Poulsen (1981) demonstrated that measurement of receptor levels was dependent on a critical protein concentration (1 to 3 mg protein per ml.) of cytosol. The protein content of all tumour samples assayed was in the range 0.33 to 13.00 mg protein/ml cytosol, with the majority of cytosols (87%) containing between 1.20 and 7.00 mg protein/ml of cytosol. It is therefore unlikely that receptor levels presented in this study are inappropriately decreased or false negative due to a low protein concentration in the assayed sample.

An oestrogen binding capacity of $\geq$ 10 fmol/mg cytosol protein has been used to discriminate between receptor-positive and receptor-negative breast tumours (Allegra, 1980b; Lippman, 1980; Lesser et al., 1981; Wittliff, 1984). This value corresponds to approximately 0.5 pmol/g wet weight of tissue (figures 15,16). Using the value of $\geq$ 0.5 pmol/g wet weight of tissue to distinguish receptor-positive from receptor-negative tumours for all receptor parameters resulted in the distribution of receptor phenotypes that was not significantly different from that generated using $\geq$ 10 fmol/mg protein as the discriminant (Table 2). Similar observations have been reported by Lippman (1980).

The higher incidence of cytosol progesterone receptors compared to cytosol oestrogen receptors in tumours from premenopausal patients (Table 2), and the higher concentration of cytosol oestrogen receptors in tumours from postmenopausal patients (Table 3) may be due to the lack of cyclic progesterone influence in these women, which in the premenopausal patients would repress cytosol oestrogen receptor synthesis (Saez et al., 1978). It has been reported that progesterone induces an 'oestrogen receptor regulatory factor', ReRF which preferentially metabolises the occupied nuclear oestrogen receptor

(Evans & Leavitt, 1980; Okulicz et al., 1981). This may possibly involve dephosphorylation of receptor (MacDonald et al., 1982). The presence of a phosphatase enzyme in the nuclei capable of inactivating nuclear oestrogen receptors has been demonstrated (Auriccio et al., 1980; 1981a,b,c). Inactivation of nuclear oestrogen receptor under the influence of progesterone may be part of the mechanism of progesterone regulation of cytosol oestrogen receptor replenishment. If this were the case in human breast cancer explaining the differences in cytosol oestrogen receptors between pre- and post- menopausal patients, then it would be expected that occupied nuclear oestrogen receptors will be selectively affected by progesterone action in premenopausal tumours. Infact the contrary was observed in that a greater proportion of premenopausal nuclear oestrogen receptor was occupied than in postmenopausal tumours. Alternatively, the lower concentration of cytosol oestrogen receptors in premenopausal tumours has been attributed to high levels of oestradiol occupying and hence masking the ligand binding sites (Hawkins et al., 1980). However, in vitro homogenisation procedures should dilute the endogenous concentration of oestradiol, and extended incubation for 18-24 hours in the presence of a saturating concentration of radiolabelled oestradiol should result in a complete exchange of any endogenously bound steroid with the [^{3}H]-oestradiol. Translocation of the cytosol receptor into the nucleus under the influence of high levels of circulating oestrogens may also explain the low levels of cytosol oestrogen receptors in tumours from premenopausal patients.

The significant increase in the mean concentration of available nuclear oestrogen (NER4) receptors in the postmenopausal group (Table 3) is in agreement with the report of Thorsen (1979). This may be due to the occupation of nuclear receptors by oestrone which has a lower

affinity for the oestrogen receptor than oestradiol (King & Mainwaring, 1974), and is the predominant plasma oestrogen in the postmenopausal woman (Siiteri & MacDonald, 1973). Thus, exchange of radiolabelled oestradiol with oestrone would occur readily at 4°C *in vitro*. However, such a simplistic interpretation of the occupancy of nuclear receptors based on plasma oestrogen may not be entirely valid as breast tissue can form oestrogens *in situ* from precursors in plasma (Adams & Li, 1975). King et al., (1980) have demonstrated in endometria of postmenopausal women that despite high levels of circulating oestrone in the plasma, oestradiol was the predominant and most active intranuclear form of oestrogen.

There was no significant difference in the incidence of cytosol progesterone receptor-positive tumours or in the concentration of progesterone receptors between the pre- and post- menopausal groups, in agreement with Koenders et al., (1980). It has been proposed from observations in human endometrium that the ratio of nuclear oestrogen receptors to cytosol progesterone receptors may be an index of end organ sensitivity reflecting the functional status of the receptor pathway (King & Whitehead, 1980). The ratios of both total nuclear oestrogen (NER30) and available nuclear oestrogen (NER4) receptors to cytosol progesterone receptors were higher in the postmenopausal group. A significantly lower ratio in the premenopausal tumours may be the result of a higher turnover rate of nuclear oestrogen receptors (Leake, 1981b). Given the importance of nuclear oestrogen receptors in the oestrogen response pathway, these observations suggest an impairment in the premenopausal tumours compared with postmenopausal tumours.

Assessment of the Functionality of the Oestrogen Receptor Pathway

The functionality of the oestrogen receptor pathway was assessed by the simultaneous occurrence of two or three receptor parameters, based on the rationale that each parameter represented a functional component of the cellular response pathway. In this way the importance of oligo(dT)-cellulose binding of cytosol oestrogen receptors to nuclear oestrogen receptors and cytosol progesterone receptors could be evaluated.

Simultaneous Occurrence of Two Receptor Parameters

The menopausal status of the patients was apparently a factor that influenced the simultaneous occurrence of the various receptor parameters. There was a significant association between the presence of cytosol oestrogen and progesterone receptors in both groups of patients (Table 5). However, the presence of cytosol oestrogen receptors was significantly associated with the presence of nuclear oestrogen (NER4 and NER30) receptors only in postmenopausal patients (Table 5). Both oestrogen and progesterone may regulate the concentration of their receptors at the cellular level in a functional receptor pathway (Janne et al., 1975). The presence of oestradiol would increase the sensitivity of the tissue by increasing oestrogen and progesterone receptor levels (Horwitz & McGuire, 1978a,b); progesterone, in turn, would inhibit replenishment of cytosol oestrogen receptor (Hsueh et al., 1975) by influencing processing of the nuclear oestrogen receptors (see above).

There was, therefore, an inconsistency between pre- and post-menopausal tumours with regard to functionality. On the basis of the copresence of cytosol oestrogen and progesterone receptors, the tumours

in each group were apparently similar yet the copresence of cytosol and nuclear oestrogen receptors (McGuire et al,., 1977a; Leake, 1981b) did not parallel this observation. In the normal endometrium throughout the menstrual cycle the changes in cytosol oestrogen and progesterone receptors are similar. However, changes in nuclear oestrogen receptors are distinct from cytosol oestrogen and progesterone receptors, with the difference between early and late proliferative phases being very pronounced (Pollow et al., 1983). Such changes in human breast cancer throughout the menstrual cycle (Kuttenen et al., 1981; Heise & Gorlich, 1982) could explain the observations. However, in tumours lacking cytosol oestrogen receptors, 39/70 premenopausal patients and 37/69 postmenopausal patients were positive for cytosol progesterone receptor suggesting that there may also be an oestrogen independent regulation of cytosol progesterone receptors (Horwitz & McGuire, 1977; Olea-Serrano et al., 1985).

The incidence of cytosol oestrogen receptors binding to oligo(dT)-cellulose was significantly higher in postmenopausal tumours (87/99) compared to premenopausal tumours (30/44). As discussed above, it is possible that high concentrations of progesterone in the luteal phase of the menstrual cycle in premenopausal women may influence the activation of cytosol oestrogen receptors in addition to affecting nuclear processing and replenishment of cytosol oestrogen receptors. Of all cytosol oestrogen receptor-positive tumours, the capacity of oestrogen receptor to bind to oligo(dT)-cellulose was significantly higher in postmenopausal patients (24.79 $\pm$ 16.2) compared to premenopausal patients (15.27 $\pm$ 20.1). The lower capacity of oestrogen receptor to bind to oligo(dT)-cellulose in premenopausal tumours may reflect a higher sensitivity to endogenous oestrogens such that a smaller number of cytosol oestrogen receptor complexes need to be

activated and translocated into the nucleus to evoke a response. This may explain the higher incidence of cytosol progesterone receptors compared to cytosol oestrogen receptors and may also explain the aggressiveness and thus poorer prognosis of breast tumours in premenopausal patients compared to postmenopausal patients (Stewart et al., 1983).

Simultaneous Occurrence of Three Receptor Parameters

In order to better define the functional state of the receptor pathway, the simultaneous occurrence of three receptor parameters (Barnes et al., 1979) was evaluated. The presence of cytosol oestrogen receptors capable of binding to oligo(dT)-cellulose was associated with the presence of cytosol progesterone receptors in both premenopausal and postmenopausal patients (Table 6); this being statistically significant in postmenopausal patients. Despite no significant differences in either the incidence or the concentration of cytosol progesterone receptors in tumours from pre- and post- menopausal patients (Tables 2,3 and 4), the distribution of activated oestrogen receptors in tumours containing both cytosol oestrogen and progesterone receptors was significantly different between the premenopausal (23/33) and postmenopausal (64/71) patients (Table 6). These results do not indicate an absolute relationship between the ability of cytosol oestrogen receptors to bind to oligo(dT)-cellulose and the presence of cytosol progesterone receptors which is a functional determinant of the oestrogen receptor pathway (Horwitz & McGuire, 1975). However, the data suggests that the regulation of cytosol progesterone receptors in premenopausal breast tumours may be different from that of postmenopausal patients.

B: Relationship between Receptor Status and Histological Grade of the Tumour

The histological grade of a tumour represents the extent of de-differentiation that has occurred during neoplastic transformation (Bloom & Richardson, 1957; Fisher et al., 1981). The relationship between the receptor parameters and tumour grade has been investigated to evaluate whether the receptor status reflects the degree of differentiation in human breast tumours. The presence of both cytosol oestrogen receptors and progesterone receptors were associated with the better-differentiated, grade II tumours compared with grade III tumours. A similar relationship between receptor status and histological grade has been suggested previously (Heuson, 1975; Maynard et al., 1978; Fisher et al., 1981; Stegner et al., 1983). Additionally, the ability of cytosol oestrogen receptor to bind to oligo(dT)-cellulose, that is, the activated oestrogen receptor was significantly associated with the better-differentiated tumours (Table 7). This suggests that the functional components of the cellular oestrogen response pathway are a biochemical reflection of breast tumour differentiation.

C: Receptor Status and Clinical Correlations in Human Breast Cancer

Accepting the obvious limitations of considering response data at an early stage of clinical follow-up (only 6 months), the preliminary data indicate that the measurement of activated oestrogen receptors may be a useful indicator of response to endocrine therapy in human breast cancer. Retrospective analysis of receptor data revealed that the cytosol oestrogen receptor status is an important indicator of response to endocrine therapy in human breast cancer (Table 9a). The incidence of other functional determinants of the oestrogen receptor pathway,

namely, nuclear oestrogen receptors (Leake et al., 1980b), and cytosol progesterone receptors (Horwitz & McGuire, 1977a; King et al., 1979) were also significantly higher in breast tumours from patients responding to endocrine therapy than those from non-responders (Table 9a). Additionally, a significantly higher proportion of breast tumours from patients responding (13/18) to endocrine therapy contained cytosol oestrogen receptors capable of oligo(dT)-cellulose binding compared to non-responders (4/11).

Analysis of the combined receptor status revealed a significantly higher proportion of breast tumours containing cytosol oestrogen receptors with the ability to bind to oligo(dT)-cellulose in patients responding to endocrine therapy (Table 9b). These observations suggest that the activated oestrogen receptor is a functional determinant of the cellular oestrogen response pathway in human breast cancer and hence a potential indicator of response to endocrine therapy.

Clinical response rate to endocrine therapy was also higher in patients with tumours containing both cytosol oestrogen and progesterone receptors. This is in accordance with observations by other investigators that patients with breast tumours containing both cytosol oestrogen and progesterone receptors have a better response rate to endocrine therapy than tumours containing cytosol oestrogen receptors alone (McGuire, 1980a).

D: Steroid Receptor Status in Primary Tumours and Invaded Malignant Lymph Nodes

The measurement of cytosol oestrogen and progesterone receptors (DeSombre et al., 1974; Leung et al., 1974; McGuire et al., 1977a) and activated oestrogen receptors are valuable indices in predicting

response to hormonal manipulation in breast cancer (Table 9; Myatt et al., 1982b; Fernandez et al., 1984). Breast tumours can metastasise to bone, lung (Samaan et al., 1981), liver, brain and skin (Leung et al., 1975; Lippman & Allegra, 1980), and also visceral and soft tissues (Stewart et al., 1980; Siebert & Lippman, 1982). Some of these metastatic lesions may be in inaccessible locations or the concentrations of tumour cells may be insufficient to yield meaningful results. There is disagreement as to whether the potential for nodal metastases is dependent on the receptor status of the primary tumour or not (Hahnel & Vivian, 1975; Heuson et al., 1975; McGuire, 1975; Leake et al., 1981a). In order to evaluate the relationship of the receptor status in the primary tumour and metastatic disease, the oestrogen and progesterone receptor status as well as the ability of the oestrogen receptor to bind to oligo(dT)-cellulose has been assessed in both primary tumours and lymph node metastases, and related to prognosis.

The overall concordance of cytosol oestrogen and progesterone receptors between the primary tumour and the corresponding malignant lymph nodes was similar to that reported by Klinga et al., (1982) for tumour and nodes, and Rosen et al., (1977) for multiple spatially separated tumour sites. There was a greater consistency of receptor positive phenotypes between the primary tumour and the invaded lymph nodes than for the negative phenotypes for each parameter (Table 13). The lower concordance of progesterone receptor-positive phenotypes suggests an impairment of the oestrogen response pathway during metastatic progression. This may reflect the lower incidence of progesterone receptor-positives in tumours with nodal involvement compared to those without (Table 13), and is consistent with the observations that patients with three or more nodes involved have a much

poorer prognosis than patients without nodal involvement (Stewart et al., 1983).

A change in receptor phenotype from positive in the primary tumour to negative in the corresponding nodal metastatic deposit occurred more frequently for nuclear oestrogen receptors suggesting a loss of receptor functionality in the course of the natural progression of the disease.

Receptor Status and Prognosis

In addition to the usefulness of the activated oestrogen receptor as a predictive index of response to endocrine therapy in primary breast tumours (see pp. 140-141; Myatt et al., 1982b; Fernandez et al., 1984), the presence of the activated oestrogen receptor may be valuable as a prognostic indicator in patients with nodal involvement. Preliminary clinical follow-up studies showed that the incidence of receptor components involved in the oestrogen response pathway was significantly lower in the primary tumours of patients with disease recurrence than those that remained disease free (Table 14). This is in agreement with reports of Blamey et al., (1980), McGuire, (1980b) and Howat et al., (1983). Similarly, the oestrogen receptor status of the corresponding malignant lymph nodes was also related to clinical recurrence (Table 15). The capability of the oestrogen receptor to bind to oligo(dT)-cellulose and the incidence of cytosol progesterone receptors was also lower in the primary tumours of patients with disease recurrence. In the malignant lymph nodes there was a similar trend, but this was not statistically significant.

The consistency of cytosol oestrogen receptor-positive status between the primary tumour and the corresponding invaded lymph node(s)

was significantly higher in patients remaining disease free (Table 16).

Such a consistency of receptor status was not apparent for either the activated oestrogen receptors, or nuclear oestrogen receptors or cytosol progesterone receptors. Thus, it appears that the oestrogen receptor status is not only important as a prognostic indicator in primary breast disease (McGuire et al., 1977a; Allegra et al., 1980b), but also reflects the prognosis in advanced metastatic breast cancer.

The copresence of cytosol oestrogen and progesterone receptors in primary tumours occurred less frequently in patients with early disease recurrence (Table 17). Therefore, those tumours which apparently lost functionality, that is, lack of cytosol progesterone receptors in oestrogen receptor-positive tumours, have a poorer prognosis than those tumours in which functionality of the oestrogen receptor pathway is demonstrated.

The cytosol oestrogen receptor status is an important prognostic indicator in primary breast cancer. Cytosol oestrogen receptor-positive tumours are less aggressive than those that lack the receptor (Nicholson et al., 1981). It has been suggested that a change in receptor status during the process of metastasising (mainly from oestrogen receptor positive to negative) possibly takes place mainly at the first step; namely, from primary to regional lymph nodes draining the tumour (Maass & Jonat, 1983). This reinforces the merit of assessing the oestrogen receptor status and the functionality of the oestrogen receptor pathway in the nodal metastatic deposit in conjunction with that of the primary tumour in providing prognostic information on the natural progression of the disease and facilitating selection of patients for particular modes of adjuvant therapy.

E: Activation of Cytosol Oestrogen Receptor

The molecular mechanism by which the steroid-receptor complex exerts its regulatory function in the nucleus is still unclear. After hormone binding, the steroid-receptor complex undergoes an activation process which results in an increased affinity for nuclear acceptor sites (Buchi & Villee, 1976) and accumulates in the cell nucleus, where it is believed to promote expression of the phenotypic effects (King & Mainwaring, 974; Schimke et al., 1975; Buller & O'Malley, 1976; Gorski & Gannon, 1976). This concept is based on *in vitro* observations that native receptors in crude cytosol are unable to bind to nuclei, DNA or oligonucleotides until heated or manipulated in other ways (Thrower et al., 1976; Park & Wittliff, 1977; Grody et al., 1982). Recent evidence has shown that the steroid-receptor complexes in three different systems recognise specific DNA sequences near the promoter regions of the genes under their control (Paywar et al., 1981; Compton et al., 1982; Mulvihill et al., 1982; Jost et al., 1984). This suggests a mode of action for steroid-receptor complexes similar to that observed for some procaryotic regulatory proteins, which recognise specific DNA sequences in or near the promoter regions of genes under their control and by binding to those regions modulate the activity of the adjacent promoter.

Activation of steroid-receptor complexes is a neccessary step before they can exert their regulatory function at the nuclear level. Various possible mechanisms have been proposed to be involved in the activation of steroid-receptor complexes including loss of an inhibitor of activation and/or interaction of a putative 'activating factor' among others (Grody et al., 1982). However, different steroid receptors show remarkably dissimilar characteristics of activation and therefore cannot be incorporated into a universal mechanistic model (Grody et al., 1982).

In an attempt to gain an insight into the molecular properties of the human breast tumour cytosol oestrogen receptor, the activation of the oestrogen-receptor complexes has been investigated and partially characterised in relation to the molecular form(s) of the receptor.

Factors Regulating the Activation of Human Breast Tumour Cytosol Oestrogen Receptor

This study has demonstrated the presence of activated oestrogen receptors in human breast tumours characterised by their association with oligo(dT)-cellulose. Breast tumour cytosol oestrogen-receptor complexes maintained at 0-4^{o}C exhibit a varying capacity of binding to oligo(dT)-cellulose from 1.3% to 86.3%. This variation may represent the extent of receptor activation *in vivo* in the individual tumours, or, alternatively, may reflect the molecular heterogeneity of oestrogen receptors (Kute et al., 1978), or heterogeneity of receptor status (Allegra et al., 1980b). Incubation of breast tumour cytosol oestrogen-receptor complexes at 0-4^{o}C did not show a time-dependent enhancement of oligo(dT)-cellulose binding between 1 and 18 hours contrary to the observations in the classical rat uterine oestrogen receptor system (Muller et al., 1983a; Myatt et al., 1984, 1985). These investigators explained the time-dependent activation in terms of the dissociation of an inhibitor from the oestrogen-receptor complex. The absence of such a time-dependent activation of oestrogen receptor in human breast tumours may suggest either a low concentration of such an inhibitor, or an inhibitor of lower affinity compared to that observed in the rat uterus. Perhaps this has some bearing on the biology of tumour cells in which loss of regulatory control of receptor function may have occurred during neoplastic transformation so that such an inhibitor of activation is less effective compared to normal cells.

Role of Sulphydryl groups in Oestrogen Receptor Activation

Addition of the sulphydryl reducing agent, DTT markedly increased the proportion of cytosol oestrogen-receptor complexes binding to oligo(dT)-cellulose at 0-4^{0}C without any effect on oestradiol binding (Figure 19). A requirement for reduced sulphydryl groups for the activation of progesterone and glucocorticoid receptors has been reported (MacDonald & Leavitt, 1982; Bodwell et al., 1984). Sulphydryl modifying agents such as iodoacetamide, N-ethyleneimide,methyl-methanethiosulfonate and 5,5'-dithiobis(2-nitrobenzoic acid) which oxidise sulphydryl groups of cysteine residues to either a disulphide or a thioester have been shown to inhibit activation of progesterone- and glucocorticoid- receptor complexes. The effects of these reagents was reversed at 0^{0}C by dithioerythritol and other sulphydryl reducing agents (MacDonald & Leavitt, 1982; Grody et al., 1982; Bodwell et al., 1984). This indicates the possible existence of a cysteine residue(s) in or near the DNA-binding domain of the receptor complex. It has been suggested that molybdate inhibition of steroid-receptor complexes may be due to its interaction with sulphydryl groups of the native receptors which results in the stabilisation of the protein (Kalimi & Banerjee, 1981; Brandt & Anderson, 1976).

Thus, it appears that reduced sulphydryl groups are essential for human breast cancer oestrogen receptor activation, and may be located in or near the oligonucleotide- or DNA- binding domain of the receptor which is distinct from the steroid binding site.

Stimulation of Activation by Dilution or Heat Treatment

Breast tumour oestrogen receptor binding to oligo(dT)-cellulose was sensitive to cytosol dilution and elevated temperature, reaching a maximum at 25°C (Figures 20,22). Similar observations have been reported for the classical rat uterine oestrogen receptor (Jensen et al., 1973; Myatt et al., 1982a), rat liver glucorticoid receptor (Higgins et al., 1973), chick oviduct progesterone receptor (Buller et al., 1975), and rat prostate androgen receptor (Mainwaring & Irving, 1973). Park and Wittliff (1977) have also demonstrated a temperature sensitive activation of oestrogen receptor complexes in the mammary gland of the rat. The temperature dependence of receptor activation suggests that a conformational change may be involved in this process. Such a change might have the effect of exposing additional positively charged residues on the surface of the molecule, increasing its affinity for DNA, phosphocellulose and other polyanions. However, heat treatment may cause dissociation and/or association of factors which may be involved in the regulation of oligonucleotide binding of the receptor complexes.

Promotion of oestrogen receptor activation by simple dilution may be explained by the dissociation of a putative cytosol inhibitory factor. Thus, with increasing dilution new equilibria are established between the bound and free inhibitor such that a greater proportion of the inhibitor is dissociated. The involvement of a low-molecular weight inhibitor of activation has been reported for the oestrogen receptor in the rat uterus (Shen et al., 1979; Myatt et al., 1979b) and mouse leydig cell tumours (Sato et al., 1979), glucorticoid receptor in rat liver (Bailly et al., 1977; Goidl et al., 1977), and androgen receptor in rat prostate (Shyr & Liao, 1978; Sato et al., 1980). Removal of this

inhibitor may facilitate, possibly via a steric modification or a conformational change in the receptor complex, the association of a putative 'activating factor' proposed by several investigators (Thrower et al., 1976; Myatt et al., 1979b; Thapman & Clark, 1981), or another non-steroid binding subunit described by Yamamoto (1974). The interaction of a second non-steroid binding subunit in the activation process of human breast tumour cytosol oestrogen receptor and generation of a 5S species seems unlikely as activation of cytosol oestrogen receptor was associated with the dissociation of the 8S receptor to the 4S form. A 5S form of the oestrogen receptor was not detected in any of the human breast cancer samples analysed (n=21).

Recently, DeRobertis et al., (1978) have described specific nuclear-binding proteins, termed karyophillc proteins which in their molecular structure contain a property or signal that enables them to accumulate selectively in the nucleus. It is possible that the putative 'activating factor' may be a karyophillic protein allowing nuclear translocation of the steroid-receptor complex after binding to it, since proteins that are not of nuclear origin have a restricted entry into the nucleus dependent on their size and shape (Paine et al., 1975; Bonner, 1975).

The presence of a low-molecular-weight inhibitor of activation may be serving a regulatory function in the receptor mediated action of steroids, such that, in the absence of ligand, activation of the receptor does not occur and therefore the receptor cannot be translocated into the nucleus. Native steroid receptor proteins are thought to be composed of a tetramer which dissociates into monomers after steroid binding (Sherman & Stevens, 1984). This monomeric subunit is capable of being activated by dimerisation with a second non-steroid

binding subunit or by interacting with a putative 'activating factor'. Receptors present in the cytosol may exhibit a variety of the above states and may account for the very wide range of binding capacities (1.3% to 86.3%) of oestrogen receptor to oligo(dT)-cellulose in human breast tumours. This is further discussed in relation to the molecular properties of the cytosol oestrogen receptor on pages 214-218.

Modulation of Oestrogen Receptor Activation by ATP and Molybdate

The enhancement in the binding of oestrogen-receptor complexes to oligo(dT)-cellulose by ATP may involve at least two processes, namely phosphorylation and/or ionic dissociation of receptor. Addition of ATP to cytosol containing oestrogen receptors resulted in an increase in the association of receptor-complexes with oligo(dT)-cellulose without any effect on receptor-ligand interactions (Figure 24). However, the presence of sodium molybdate inhibited, but did not completely block the activation of breast tumour cytosol oestrogen receptors, possibly due to the presence of receptors already activated *in vivo* (Table 20). Myatt et al., (1982a) have reported similar observations for the effect of molybdate on activation of rat uterine oestrogen receptors. Chong and Lippman (1982) have demonstrated that ATP promoted activation of oestrogen-, progesterone-, glucorticoid- and androgen- receptor complexes in the human breast cancer cell line, MCF-7. These investigators also showed that molybdate inhibited either ATP or temperature induced activation of these receptors. Similar observations have been made in our laboratory for the effect of ATP and molybdate on activation of oestrogen receptors in the rat uterus (Myatt et al., 1984).

The nucleotide triphosphate, ATP may act as a nucleotide to induce activation of the oligonucleotide binding domain of the receptor, or alternatively may participate in a phosphorylation/dephosphorylation reaction involved in receptor activation. Molybdate may act as a phosphoprotein phosphatase inhibitor (Paigen, 1958), suggesting that activation involves a dephosphorylation mechanism. Studies from our laboratory (Myatt et al., 1985), and those of Chong and Lippman (1982) have demonstrated that the non-metabolisable analogues of ATP and GTP, adenylylimidodiphosphate (AIDP) and guanylylimidodiphosphate (GIDP) also promoted activation indicating that phosphorylation of the receptor complex during activation was unlikely. Similarly, the observations that only molybdate but not other inhibitors of phosphatases, such as fluoride, p-nitrophenyl phosphate or arsenate, protects against heat inactivation of oestrogen receptor complexes from calf uterus argues against a phosphorylation/dephosphorylation mechanism of receptor activation (Muller et al., 1983a). Nishigori and Toft (1980) have come to similar conclusions on the effect of molybdate on activation of the chick oviduct progesterone receptor.

As discussed above, receptor activation has been proposed to involve the dissociation of a low-molecular-weight inhibitor of activation. The inhibitory action of molybdate on receptor activation may be by preventing the dissociation of such an inhibitor from the receptor-complex as suggested by Noma et al., (1980). ATP may interact either with the receptor itself or the inhibitor to facilitate dissociation of the putative low-molecular-weight inhibitor from the receptor-complex thereby allowing the association of the putative activating factor to promote activation. Studies from our laboratory (Myatt et al., 1985) and those of Holbrook et al., (1983a) suggest an ionic effect of ATP on activation of oestrogen and glucocorticoid

receptors respectively. Similarly, it is possible that molybdate inhibition of receptor activation also involves ionic interactions. Considering the solvation chemistry of molybdenum ions suggests it may influence receptor dynamics by affecting the transfer or storage of electrons (Moura & Xavier, 1978). Kalimi and Banerjee (1981) have presented findings which suggest that molybdate prevents activation of progesterone receptor complexes in the chick oviduct by interacting with sulphydryl groups of the native receptor. The binding of the molybdate ion to sulphydryl groups may form a molybdenum bridge between the receptor subunits resulting in the stabilisation of the protein (Brandt & Anderson, 1976). In the same system, Nishigori and Toft (1980) have demonstrated that molybdate inhibited activation by elevated temperature but not by the presence of high salt concentration which would disrupt the ionic interactions with molybdate. The ability of molybdate to donate and/or store electrons may prevent changes in electrostatic forces which normally occur in vitro to cause dissociation of receptor aggregates into subunits (Ogle, 1983). Such a mechanism would be consistent with the observations that molybdate stabilises receptor-complexes in the 300K (8S) form (see pp. 186), but once the receptor is dissociated into a 4-5S form, the presence of molybdate does not inhibit receptor activation (Thomas et al., 1983; Noma et al., 1980).

Pyridoxal phosphate has also been reported to inhibit activation of oestrogen receptor (Muller et al., 1980a; Traish et al., 1980), progesterone receptor (Nishigori & Toft, 1979), glucocorticoid receptor (Cake et al., 1978), and androgen receptor (Mulder et al., 1980). It reacts with free primary amino groups, such as the e-amino groups of lysine residues, by forming a Schiff base (Martial et al., 1975). This implies that lysine residues may be involved in the positively charged

oligonucleotide binding domain of the receptor complexes. Furthermore, Muller et al., (1983b) have demonstrated that the presence of 1,2-cyclohexanedione, which is a highly specific reagent for peptide arginine residues (Patthy & Smith, 1975a,b), also inhibited activation of oestrogen receptors. These observations, together with the effects of ATP and molybdate on receptor activation suggest that the nuclear binding domain of steroid-receptor complexes is positively charged and contains lysine and arginine residues. This is a common feature of enzymes and regulatory proteins which interact with negatively charged substrates, such as nucleic acids (Modak, 1976; Salvo et al., 1976).

In attempts to define the components of DNA recognised by steroid-receptor complexes, several studies, including those in our laboratory, have demonstrated using oligonucleotides covalently linked to cellulose that oestrogen-receptor complexes can discriminate among nucleotide moieties of DNA. The general order of preference is oligo(dG) > oligo(dT) > oligo(dC) > oligo(dA) (Gross et al., 1982; Kumar et al., 1983; Dickerman & Kumar, 1982; Hyder et al., 1984). Similarly, a strongly base composition dependent binding of rat liver glucocorticoid receptors to synthetic polynucleotides has been demonstrated (Romanov et al., 1983); the binding effectiveness being natural DNA's >poly(dA).poly(dT) $\geq$ poly d(A,T,G)7 $\geq$ poly(U) > poly(A).poly(U) > poly(A) $\equiv$ poly(C). The mouse kidney androgen receptor has also been reported to interact selectively with polyribonucleotides; it binds appreciably to synthetic poly(G) and moderately to poly(U), whereas there is negligible binding to poly(A) and poly(C) (Lin & Ohno, 1981). The nucleotide base preference of steroid-receptor complexes may be attributed to the pKa (ionic strength) of the functional group of peptidyl amino acids present in the oligonucleotide binding domain; the hyperreactivity of arginyl residues in the nuclear binding domain

(Muller et al., 1983a) has been attributed by Patthy and Thesz (1980) to the lower pKa of the guanidino group of peptidyl arginines. This would be consistent with a greater affinity of steroid receptor complexes for guanine containing oligo- or poly- nucleotides. This indicates that although electrostatic interactions between cationic amino acid residues of the receptor and the polyphosphate backbone of DNA are important, certain properties of the polynucleotide binding domain, such as the amino acid sequence, underlie specific base recognition.

F: Molecular Forms of the Activated Human Breast Tumour Cytosol Oestrogen Receptor

The molecular forms of the human breast tumour cytosol oestrogen receptors have been analysed by high-performance size exclusion gel chromatography (HPSEC). Rapid resolution of receptor forms was achieved within 40 minutes providing a critical advantage in minimising receptor modification compared to 16-18 hours of centrifugation required for sucrose density gradient analysis (Jensen et al., 1971a, 1973), during which significant ligand and/or subunit dissociation (Weichman & Notides, 1977; Pavlik & Rutledge, 1980), enzymatic processing and proteolysis (Schneider & Dao, 1977; Notides, 1978; Tilzer et al., 1981; Miller et al., 1981) can occur. The rapid separation time also affords an opportunity to characterise short-lived interactions between receptors and low-affinity ligands that appear to elicit physiological or pharmacological responses.

Human breast cancer cytosol oestrogen receptors were resolved into two distinct components by HPSEC which were qualitatively similar to those previously established by sucrose density gradient sedimentation analysis (Pavlik et al., 1982; Wiehle et al., 1984). The high-molecular-weight, 300K component of the receptor has an estimated Stoke's radius of 5.9-6.5 nm and a sedimentation coefficient in the range 11-13S (Table 21); the low-molecular-weight, 60K component has an estimated Stoke's radius of 3.2-3.7 nm and a sedimentation coefficient in the range 4.3-4.7S. These observations are consistent with the molecular forms described for a variety of steroid receptors (Jensen et al., 1973; Kalimi et al., 1975; Griffiths, 1975; Buller et al., 1975; Miller et al., 1981). Recently, Wiehle et al., (1984) have reported similar observations in human breast carcinoma using HPSEC.

Oestrogen receptors in the immature rat uterus undergo a change in their sedimentation behaviour from approximately 4S to 5S when warmed to 25-29°C and analysed on sucrose gradients containing KCl (Jensen et al., 1971b, 1973). In contrast with those of the rat uterus, oestrogen receptors from human breast tumours activated under identical conditions did not exhibit an alteration in molecular size of the 60K (~4S) receptor to the 5S form. However, heat activation resulted in the dissociation of the 300K receptor to the 60K form. This was accompanied by a concommitant increase in oligo(dT)-cellulose binding of the receptor complex. Activated oestrogen receptors from the rat mammary gland have been reported to exhibit similar characteristics, sedimenting as 4S species in sucrose gradients containing 400mM KCl (Park & Wittliff, 1977). Similarly, activation of chick oviduct progesterone receptor (Buller et al., 1975), and rat prostate androgen receptor (Mainwaring & Irving, 1973) is accompanied by the dissociation of the ~8S receptor to the 4S form. Thus, the transformation of 4S oestrogen receptor to give the 5S form which accompanies activation appears to be unique to the rat uterine oestrogen receptor system (Notides & Nielsen, 1975; Yamamoto & Alberts, 1975; Gorski & Gannon, 1976; Chan & O'Malley, 1976; Bailly et al., 1980; Jensen & DeSombre, 1973). However, it has been shown that activation (defined as an increase in the affinity of the receptor-complex for nuclear acceptor sites), and transformation (an increase in size from 4S to 5S form) of rat uterine oestrogen receptor are distinct processes, and that activation preceeds transformation (Buchi & Villee, 1976; Bailly et al., 1980; Muller et al., 1983a). Dissociation of the 8S receptor aggregate into the 4S form upon activation appears to be a common feature of steroid receptor function. Thus, activation consists of a dissociation of the 8S receptor aggregate into 4S subunits; each subunit may then undergo a conformational change

resulting in the exposure of the positively charged oligonucleotide binding domain. Alternatively, one can propose that each 4S subunit is already in the activated state, but the oligonucleotide binding domain is masked inside the 8S receptor aggregate; activation would then simply represent 8S to 4S dissociation (Muller et al., 1983a). Association of a putative 'activating factor' (Thrower et al., 1976; Myatt et al., 1979a,b; Thapman & Clark, 1981), which may be a karyophillic protein, with the 4S receptor may then simply facilitate nuclear accumulation of the receptor complex.

Activation of human breast tumour cytosol oestrogen receptor as assessed by an increased binding capacity for oligo(dT)-cellulose is accompanied by the dissociation of the 300K receptor aggregate to the 60K form. Addition of molybdate to breast tumour cytosol oestrogen receptor complexes inhibited the temperature dependent increase in oligo(dT)-cellulose binding. In the presence of molybdate, the receptor-complexes were maintained predominantly in the 300K form (Figure 30), probably by stabilising ionic interactions on the receptor aggregate (Nishigori & Toft, 1980). Other possibilities of the inhibitory action of molybdate on receptor activation, and thus preventing dissociation of the 300K receptor aggregate, may be by direct interaction with sulphydryl and/or phosphate moieties of the receptor complex (Leach et al., 1979; Sando et al., 1979b; Nishigori & Toft, 1980; Weathers et al., 1979), or by preventing the dissociation of a putative inhibitor of receptor activation as suggested by Noma et al., (1980)[discussed on pp. 209-211].

Activation of human breast tumour cytosol oestrogen receptors can be achieved by a variety of manipulations, including heat-treatment, dilution, exposure to high-ionic strength or addition of ATP, and

consists of a dissociation of the 300K ($\geq$8S) receptor aggregate to the 60K (4S) subunits (Figures 28-31). These subunits may then undergo a conformational change via interaction with a putative 'activating factor' resulting in the exposure of the positively charged nuclear binding domain. Studies with pyridoxal phosphate (Muller et al., 1983a; Traish et al., 1980), and 1,2-cyclohexanedione (Muller et al., 1983b) have indicated an involvement of functional lysine and arginine residues in this site. These peptidyl amino acids may be involved in the specific base recognition property of steroid-receptor complexes on the DNA.

The presence of the 8S form of the oestrogen receptor alone has been indicated to be an accurate predictive index of hormonally responsive breast cancers (Wittliff et al., 1976). These authors have suggested that unresponsive breast tumour cells contain only the 4S species of oestrogen receptors. Park & Wittliff (1980) have suggested that the 8S form of the oestrogen receptor binds more readily to DNA than the 4S species. However, the data from this study disagree with the above observations. In the studies of Park and Wittliff (1980), the molecular properties of human breast tumour cytosol oestrogen receptors were determined by prolonged sucrose density gradient sedimentation analysis during which not only time dependent aggregation of receptor occurs but significant ligand dissociation can occur (Weichman & Notides, 1977; Pavlik & Rutelidge, 1980). Activation of steroid receptors is generally defined as an increase in affinity for nuclear acceptor sites (Milgrom, 1981), a step neccessary for receptor function. In the present study the molecular forms of the breast tumour cytosol

oestrogen receptor were analysed by a rapid and sensitive method using HPSEC (see pp.214). The activation of the human breast tumour cytosol oestrogen receptor is accompanied by the dissociation of the 300K ($\geq$8S) receptor aggregate to the 60K (4S) form. Once the activated, 60K (4S) receptor complex is generated, the possibility of transformation to the 5S form during or post-translocation into the nucleus cannot be excluded (Figure 32). As discussed on page 200, the ability of the human breast tumour cytosol oestrogen receptor to bind to oligo(dT)-cellulose, that is, activated receptor, is a valuable predictive index of response to endocrine therapy. The concentration of cytosol progesterone receptors was higher in tumours that contained the 60K (4S) form of the oestrogen receptor than in those that did not. As discussed previously, cytosol progesterone receptor is an index of a functional oestrogen receptor pathway and is associated with a favourable response to endocrine therapy. The association between an increased concentration of cytosol progesterone receptors, an increased binding of oestrogen receptors to oligo(dT)-cellulose and the presence of the 60K oestrogen receptor would suggest that the lower molecular weight receptor species, rather than the 8S aggregate as suggested by Wittliff et al., (1976), is potentially a predictive index of hormonal sensitivity.

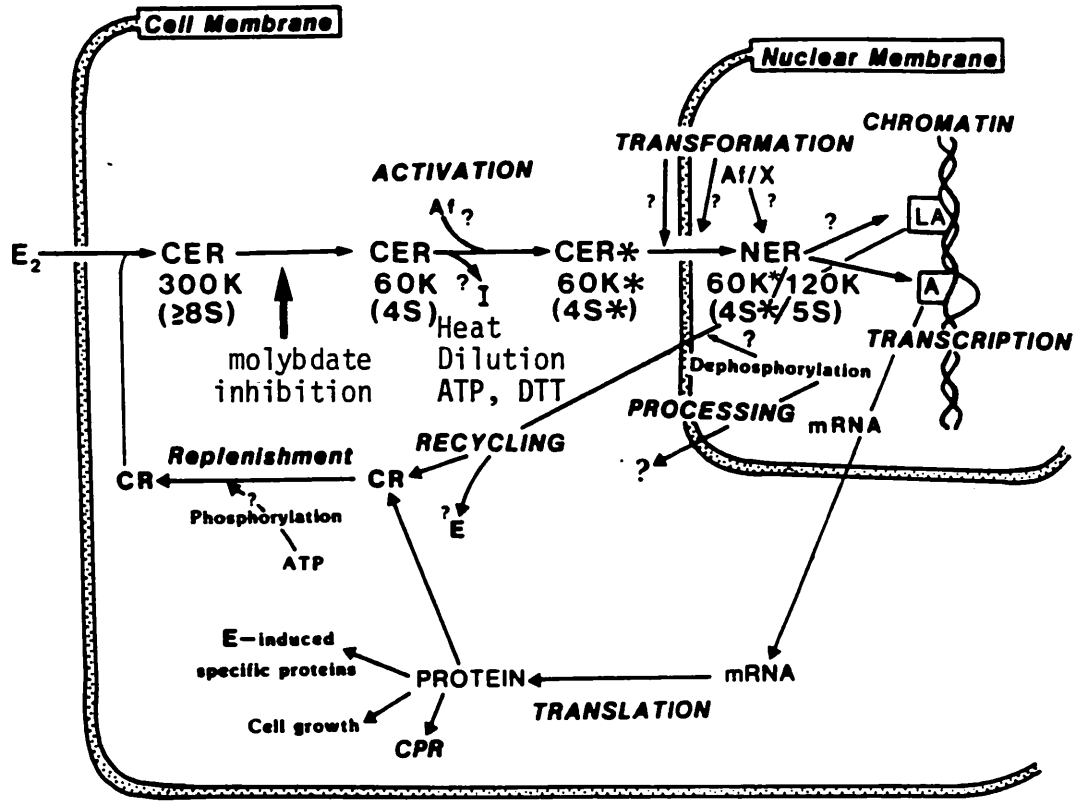


Figure 32. Possible mechanism of oestrogen action in human breast cancer. See legend to figure 7 for details. ? steps requiring further elucidation.

CHAPTER SIX

SUMMARY AND CONCLUSIONS

In normal target tissues, a functional oestrogen receptor pathway would consist of firstly a cytosol component; secondly a nuclear component derived from the cytosol component following translocation under the influence of oestrogen; and thirdly a cytosol progesterone receptor which is a specific end product of oestrogen action (Horwitz & McGuire, 1977; Koenders et al., 1977; Toft & O'Malley, 1972; Janne et al., 1975; Faber et al 1972; Fiel et al., 1972). The application of such a rationale in human breast cancer has demonstrated that patients who responded to endocrine therapy had tumours that were positive for two receptor parameters simultaneously. This is in accordance with observations by various investigators that patients whose tumours were positive for two or three receptor parameters simultaneously had a better response rate to endocrine therapy than those tumours containing cytosol oestrogen receptor only (McGuire et al., 1977a,b; Barnes et al., 1979; Hahnel & Vivian, 1980; Lippman, 1980; Leake et al., 1981a).

Little is known about the molecular mechanisms involved in generating the nuclear binding form of the oestrogen receptor. However, using model systems this process, termed 'activation', is associated with an increased capability to bind to DNA- or oligo(dT)-cellulose *in vitro* (Thrower et al., 1976; Myatt et al., 1982a; Grody et al., 1982). In the present study the capacity of human breast tumour cytosol oestrogen receptor to bind to oligo(dT)-cellulose has been investigated and an attempt has been made to establish if this measurement of receptor activation *in vitro* is related to the functionality of the receptor system in breast tumours *in vivo*.

Receptors in Breast Cancer

Histologically confirmed primary breast tumours have been analysed for receptor status from 117 premenopausal and 174 postmenopausal patients. Primary breast tumours from postmenopausal patients contained a significantly higher concentration of oestrogen receptors and a higher incidence of receptor-positive tumours than premenopausal patients, in agreement with other investigators (Wittliff et al., 1971; Hahnel & Vivian, 1975; Heusen et al., 1975; McGuire et al., 1975; Lippman & Allegra, 1980; Anderson et al., 1980; Hawkins et al., 1980; Myatt et al., 1982b; Fernandez et al., 1984). The lower concentration of cytosol oestrogen receptors in premenopausal tumours may be due to the influence of cyclic progesterone. This would repress cytosol oestrogen receptor synthesis (Saez et al., 1978), as well as replenishment possibly via induction of a ReRF (Evans & Leavitt, 1980; Okulicz et al., 1981) which regulates processing of nuclear oestrogen receptor probably by a dephosphorylation process (MacDonald et al., 1982).

The incidence of cytosol oestrogen receptor capable of binding to oligo(dT)-cellulose, i.e. activated receptor, as well as the distribution of activated receptors in tumours containing both cytosol oestrogen and progesterone receptors was significantly different between premenopausal and postmenopausal patients. The results show an apparent relationship between the presence of activated oestrogen receptor and the presence of cytosol progesterone receptor, which is a functional determinant of the oestrogen receptor pathway, suggesting that the activated oestrogen receptor is a functional component of the cellular oestrogen response pathway, but an absolute relationship was not evident. However, the data suggests that the regulation of cytosol progesterone receptors may be different depending on the endocrine status of the patients.

The activated oestrogen receptor and the presence of cytosol oestrogen and progesterone receptors were correlated with the better-differentiated tumours suggesting that these parameters are biochemical correlates of cellular differentiation.

Preliminary clinical follow-up data in a small number of patients suggest that the measurement of activated oestrogen receptors may be useful in human breast cancer. Retrospective analysis of receptor data showed that the incidence of activated oestrogen receptors, as well as those components thought to be functional determinants of the oestrogen receptor pathway, namely, nuclear oestrogen and cytosol progesterone receptors (Leake et al., 1981a, Horwitz & McGuire, 1977; King et al., 1979), were significantly higher in patients responding to endocrine therapy. A significantly higher proportion of patients who responded to endocrine therapy contained both cytosol oestrogen receptors and activated receptors. These observations further suggest that the activated oestrogen receptor is a functional determinant of the cellular oestrogen receptor pathway in human breast cancer and hence a potential indicator of response to endocrine therapy.

The presence of activated oestrogen receptor may also be valuable as a prognostic indicator in patients with nodal involvement. Short-term clinical follow-up studies revealed that primary tumours from patients with early disease recurrence were less frequently positive for cytosol oestrogen receptor, its ability to bind to oligo(dT)-cellulose or cytosol progesterone receptor than those remaining disease free. However, in the corresponding malignant lymph nodes of patients with early disease recurrence, only the cytosol oestrogen receptor status was significantly related to disease progression. The importance of the functionality of the oestrogen receptor pathway in the primary tumours

was reinforced by the observation that the co-presence of cytosol oestrogen receptors with either cytosol progesterone receptors or the activated oestrogen receptor was apparently associated with patients showing no evidence of disease recurrence.

Activation of Human Breast Tumour Cytosol Oestrogen Receptor and its Relationship to the Molecular Form(s) of the Receptor.

Activation of human breast tumour cytosol oestrogen receptor, as assessed by oligo(dT)-cellulose binding, is promoted by heat treatment and the presence of either DTT or ATP, but inhibited by sodium molybdate. Activation may therefore involve either a phosphorylation/ dephosphorylation process or a change in surface charge properties of the cytosol oestrogen receptor complex.

The human breast tumour cytosol oestrogen receptor exists as a high-molecular-weight, 300 K dalton form alone or together with a low-molecular-weight (60K) form in low ionic strength buffers in vitro. The proportion of oestrogen receptor capable of binding to oligo(dT)-cellulose, i.e. activated receptor was significantly higher in breast tumours containing both the 300K and 60K forms of the receptor.

In vitro manipulations of cytosol such as heat treatment and the addition of ATP which promoted activation (an increase in oligo(dT)-cellulose binding capacity) of oestrogen receptor was associated with an increase in the 60K form of the receptor and a corresponding decrease in the 300K form. This contrasts with reports that breast tumour 8S oestrogen receptor binds more readily to DNA than does the 4S species (Wittliff et al., 1981). However, these results are

consistent with the 300K species being a precursor for the 60K species which is or can be activated (Muller et al., 1983a).

The presence of sodium molybdate stabilised the 300K receptor aggregate during heat treatment consistent with its proposed mode of action in preventing activation (Shyamala & Leonard, 1980). In addition, molybdate also inhibited the binding of existing 60K oestrogen receptor to oligo(dT)-cellulose suggesting that further modification of receptor occurs during the activation step as suggested in other systems (Sakai & Gorski, 1984; Myatt et al., 1985).

Activation of breast tumour cytosol oestrogen receptor is accompanied by changes in the molecular forms of the receptor which are analogous to those observed in the rat prostate where the androgen receptor is transformed from 8S to 4.2S upon heat activation (Mainwaring & Irving, 1973), and the chick oviduct where the progesterone receptor is transformed from 6-8S to 4S (Buller et al., 1975). Transformation of the 60K receptor to the 120K (5S) species upon heat treatment was not observed in contrast to that reported for the rat and calf uterine oestrogen receptor (Jensen et al., 1971). Park and Wittliff (1977) have also demonstrated that heat-induced activation of the lactating rat mammary gland oestrogen receptor was not accompanied by a 4S to 5S transformation. However, the nature of the receptor eluted from the oligo(dT)-cellulose matrix was not investigated and therefore generation of a 120K species during interaction with the matrix cannot be excluded.

In conclusion, the present study has demonstrated in human breast cancer the presence of activated oestrogen receptors hitherto reported in animal model systems. The activated oestrogen receptor in human breast tumours is associated with the presence of nuclear oestrogen and cytosol progesterone receptors suggesting that it is a functional

determinant of the cellular oestrogen receptor pathway. This suggestion that the activated oestrogen receptor is associated with the functionality of the receptor pathway is further strengthened by the observations that it is not only an effective predictor of response to endocrine therapy but also a valuable prognostic indicator in patients with nodal involvement.

Accepting the limitations of *in vitro* assessments, activation of breast tumour cytosol oestrogen receptor probably involves disaggregation of the 300K receptor to the 60K form and the consequent exposure of the oligonucleotide binding domain. The 300K form of the receptor may or may not be an artefact of aggregation. The observations that DTT and ATP promote activation suggest that reduced sulphydryl groups and/or phosphorylation may be important in this process, however, ionic effects causing disaggregation of the 300K receptor cannot be discounted. However, generation of the nuclear binding form, as assessed by oligo(dT)-cellulose binding, is apparently facilitated by an increase in the 60K form of the receptor and a corresponding decrease in the 300K species. The increase in progesterone receptor content in the presence of 60K receptor suggests a biological association and further supports the need for qualitative assessment of oestrogen receptors in human breast cancer.

REFERENCES

Ackers, G. K. (1965)
Proc. Natl. Acad. Sci. (USA), 53; 342-45

Adams, J. B. and Li, K. (1975)
Br. J. Can. 31; 429-33

Ahmann, D. L. et al., (1977)
New Eng. J. Med. 297; 356-60

Alexieva-Figusch, J. et al., (1984)
Eur. J. Can. Clin. Oncol. 20; 33-40

Alitalo, K. et al., (1983)
Proc. Natl. Acad. Sci. (USA), 80; 1707-11

Allegra, J. C. et al., (1980a)
Cancer 45; 792-94

Allegra, J. C. et al., (1980b)
Eur. J. Can. 16; 323-31

American Joint Committee for Cancer Staging (AJCSS) and End-Results Results Reporting. Staging of Cancer of the Breast: manual for staging of cancer (1979); pp. 101-8
Whiting Press, Chicago.

Anderson, J. N. et al., (1972)
Biochem. J. 126; 561-567

Anderson, J. N. et al., (1973)
Endocrinology 92; 1488-95

Andre, J. and Rochefort, H. (1973)
FEBS Lett. 32; 330-34

Andre, J. and Rochefort, H. (1975)
FEBS Lett. 50; 319-23

Andre, J. et al., (1976)
Biochemistry 15; 2964-69

Ansfield, F. J. et al., (1976)
Cancer 38; 53-55

Auriccio, F. et al., (1980)
FEBS Letts. 117; 224-27

Auriccio, F. et al., (1981a)
Biochem. J. 194; 569-74

Auriccio, F. et al., (1981b)
Biochem. J. 198; 699-702

Auriccio F. et al., (1981c)
Biochem. Biophys. Res. Comm. 101; 1171-78

Auriccio, F, et al., (1984)
J. Steroid. Biochem. 20; 31-35

Bailly, A. et al., (1977)
J. Biol. Chem. 252; 858-63

Bailly, A. et al., (1980)
J. Biol. Chem. 255; 2729-34

Barbieri, R. L. and Ryan, K. J. (1981)
Am. J. Obstet. Gynaecol. 141 453-63

Barnes, D. M. et al., (1977)
Eur. J. Can. 13; 1133-43

Barnes, D. M. et al., (1979)
Br. J. Can. 40; 862-65

Barrack, E. R. and Coffey, D. S. (1980)
J. Biol. Chem. 255; 7265-75

Baudendistel, L. J. and Ruh, T. S. (1976)
Steroids 28; 223-37

Baulieu, E. E. et al., (1971)
Rec. Prog. Horm. Res. 27; 351-419

Baulieu, E. E. (1978)
Mol. Cell Endocrinology 12; 247-54

Baum, M. (Ed.) (1981)
In: Breast Cancer: The Facts.
Oxford University Press, New York.

Beatson, G. (1896)
Lancet ii; 104-7

Berenblum, I. (1941)
Cancer Research 1;807-14

Berenblum, I. (1978)
J. Nat. Can. Inst. 60; 723-26

Berenblum, I. and Shubik, P. (1947)
Br. J. Cancer 1; 379-91

Bishop, J. M. et al., (1980)
CHS Symp. Quant. Biol. 44; 99-30

Bishop, M. J. (1983)
Ann. Rev. Biochemistry 52; 301-54

Bittner, J. J. (1936)
Science 84; 162

Black, P. H. (1968)
Ann. Rev. Microbiology 22; 391-426

Blamey, R. W. et al., (1980)
Cancer 46; 2765-69

Bloom, H. J. and Boesen, E. (1974)
Br. Med. J. 2; 7-10

Bloom, N. D. and Richardson, W. W. (1957)
Br. J. Can. 11; 359-77

Bodwell, J. E. et al., (1984)
Biochemistry 23; 1392-98

Bonadonna, G. et al., (1977)
Cancer 39; 2904-15

Bonadonna, G. et al., (1978)
Ann. Rev. Med. 29; 149-75

Bonner, W. M. (1975)
J. Cell Biol. 64; 421-37

Borgna, J. L. et al., (1982)
Special FEBS Meeting, Athens Greece.
Abstract Book p.145.

Borthwick, N. M. and Smellie, R. M. S. (1975)
Biochem. J. 147; 91-101

Boutwell, R. K. (1974)
Crit. Rev. Toxicol. 2; 419-43

Boveri, T. (1914) 'Zur Frage der Entstehung Maligner Tumoren'
Gustav Fisher, Jena (English translation - Boveri, M. :
The origin of malignant tumours, 1929; Williams & Wilkins,
Baltimore).

Brambilla, C. et al., (1978)
Cancer Chemother. Pharmacol. 1; 35-39

Brandt, J. and Anderson, L. (1976)
Int. J. Peptide Protein Res. 8; 33-37

Brecher, P. I. et al., (1970)
Fed. Proc. 29; 249

Brem, D. and Folkman, J. (1975)
J. Exptl. Med. 141; 427-39

Buchi, K. and Villee, C. A. (1976)
J. Steroid Biochem. 7; 539-44

Bulbrook, R. J. (1966)
Proc. Canad. Can. Conf. 6; 36-49

Bulbrook, R. J. et al., (1980)
New Eng. J. Med. 303; 1246-48

Buller, R. E. and O'Malley, B. W. (1976)
Biochem. Pharmacol. 25; 1-12

Buller, R. E. et al., (1975)
J. Biol. Chem. 250; 809-18

Bullock, L. P. et al., (1978)
Endocrinology 103; 1768-82

Burkitt, D. (1963)
Int. J. Pathol. 2; 87-138

Burnet, M. (1978)
Adv. Cancer Res. 28; 1-29

Buttyan, R. et al., (1983)
J. Biol. Chem. 258; 14366-70

Buzdar, A. et al., (1982)
Proc. AACR, 557

Cake, M. H. et al., (1978)
J. Biol. Chem. 253; 4886-91

Carbone, P. P. and Davis, T. E. (1978)
Semin. Oncol. 5; 417

Carbone, P. P. et al., (1977)
Cancer 39 (Suppl.); 2916-22

Carlstedt-Duke, J. et al., (1983)
In: Gene Regulation by Steroid Hormones, Vol. 2
Eds. Roy, A. K. and Clark, J. H.
Springer, New York., pp.151-80

Carter, A. C. et al., (1977)
J. Am. Med. Assoc. 237; 2079-85

Carter, R. (1980)
Br. J. Cancer 41; 494

Chamness, G. C. et al., (1974)
Biochemistry 13; 327-31

Chamness, G. C. et al., (1980)
J. Histochem. Cytochem. 28; 792-8

Chan, L. and O'Malley, B. W. (1976)
New Eng. J. Med. 294; 1322-28

Charreau, E. H. and Baldi, A. (1977)
Mol. Cell Biochem. 16; 79-86

Chong, M. T. and Lippman, M. E. (1982)
J. Recept. Res. 2; 575-600

Cidlowski, J. A. and Muldoon, T. G. (1978)
Biol. Reprod. 18; 234-36

Ciejek, E. M. et al., (1973)
Nature 306; 604-9

Clark, J. H. and Markaverich, B. M. (1981)
J. Steroid. Biochem. 15; 49-54

Clark, J. H. and Peck. E. J. (976)
Nature 260; 635-37

Clark, J. H. and Peck, E. J. (1979)
Monographs in Endocrinology 14
Springer-Verlag, Berlin.

Clark, J. H. et al., (1972a)
Neuroendocrinology 77; 218-28

Clark, J. H. et al., (1972b)
Science 176; 528-30

Clark, J. H. et al., (1973a)
Steroids 22; 707-18

Clark, J. H. et al., (1973b)
Hormone Receptors and Reproduction Vol. 36
(Eds) O'Malley, B. W. and Means, A. R.
Plenum Press, New York pp.15-59

Clark, J. H. et al., (1977)
Ann. N. Y. Acad. Sci. 286; 161-79

Clark, J. H. et al., (1978)
J. Biol. Chem. 253; 7630-34

Clark, J. H. et al., (1983)
Endocrinology 113; 1167-68

Clifton, K. H. (1961)
Proc. Fourth Nat. Can. Conf., pp. 41-50
Lippincott, Philadelphia.

Clifton, K. and Sridharan, R. (1975)
In: Cancer, Vol. 3
Ed. Becker, F.
Plenum Press, New York., pp. 249-85

Coezy, E. et al., (1982)
Cancer Research. 42; 317-23

Cohen, E. et al., (1982)
Cancer 49; 1754-61

Collins, S. and Groudine, M. (1982)
Nature 298; 679-81

Compton, J. G. et al., (1983)
Proc. Natl. Acad. Sci. (USA), 80; 16-20

Cooper, M. R. et al., (1981)
Cancer 47; 2833-39

Corbin, A. (1982)
Yale J. Biol. Med. 55 27-47

Correa, P. and O'Connor, G. T. (1971)
Int. J. Cancer 8; 192-201

Cowie, A. T. et al., (1971)
In: Physiopathology of Lactation.
Physiology Society Monograph.
Edward Arnold, London.

de Boer, W. et al., (1978)
Nucleic Acid Res. 5; 87-103

de Klein, A. et al., (1982)
Nature 300; 765-67

de Vita, V. T. Jr. et al., (1975)
Cancer 35; 98-110

Dalla-Favera, R. D. et al., (1982)
Nature 299; 61-63

Davies, P. et al., (1980)
J. Endocrinology 87; 225-40

Davis, H. L. et al., (1981)
Proc. ASCO, 368

DeRobertis, E. M. et al., (1978)
Nature 272; 254-56

DeSombre, E. R. et al., (1974)
Can. Chemotherap. Rep. 58; 513-19

DeSombre, E. R. and Arbogast, L. Y. (1974)
Cancer Research. 34; 1971-76

Dean, D. C. et al., (1983)
Nature 305; 551-54

Degenshein, G. A. et al., (1979)
Current Prob. Surg. 16; 1-59

Dickerman, H. W. and Kumar, S. A. (1982)
Adv. Exp. Med. Biol. 138; 1-18

Djiane, J. and Durrand, P. (1977)
Nature 266; 641-43

Doerfler, W. (1968)
Proc. Natl. Acad. Sci. (USA), 60; 636-43

Doll, R. et al., (1970)
Acta. Un Int. Contra Cancrum 2; 341-57

Duax, W. L. and Weeks, C. M. (1980)
In: Estrogens in the Enviroment
Ed. McLachlan, J. A.
Elsevier/N. Holland, New York., pp.11-31.

Edwards, D. P. et al., (1979)
Biochem. Biophys. Acta. 560; 457-86

Edwards, D. P. et al., (1980a)
Cancer Research. 40; 1722-26

Edwards, D. P. et al., (1980b)
Biochem. Biophys. Res. Comm. 93; 804-12

Engelsman, E. et al., (1973)
Br. Med. J. 2; 750-52

Erdos, T. (1968)
Biochem. Biophys. Res. Comm. 32; 338

Erdos, T. et al., (1969)
FEBS Lett. 5; 161

Erdos, T. et al., (1970)
Anal. Biochem. 37; 244-52

Erikson, J. et al., (1983)
Proc. Natl. Acad. Sci. (USA), 80; 820-24

Eriksson, H. et al., (1978)
Biochem. Biophys. Res. Comm. 81; 1-7

Evans, E. et al., (1982)
Eur. J. Biochem. 128; 185-91

Evans, R. W. and Leavitt, W. W. (1980)
Proc. Natl. Acad. Sci. (USA), 77; 5856-60

Ewing, J. (1940),(Ed.)
In: Neoplastic Disease: A Treatise on Tumours (4th. Edition)
Saunders, Philadelphia.

Faber, L. E. et al., (1972)
J. Biol. Chem. 247; 5648-49

Fantone, J. et al., (1982)
In: Prostaglandins and Cancer.
Ed. Powles, T. J. et al.
Alan. R. Liss., New York., pp. 673

Fasal, E. and Paffenbarger, R. S. (1975)
J. Nat. Can. Inst. 55 767-73

Fazekas, A. G. and Mcfarlane, J. K. (1980)
J. Steroid Biochem. 13; 613-22

Feherty, P. et al., (1971)
Br. J. Can. 25; 697

Fernandez, M. D. et al., (1984)
Eur. J. Can. Clin. Oncol. 20; 41-46

Festa, R. S. et al., (1979)
Cancer 44; 1507-10

Fialkow, P. J. (1972)
Adv. Can. Res. 15; 191-23

Fidler, I. J. (1978)
Cancer Research 38; 2651-60

Fidler, I. J. and Hart, I. R. (1981)
Eur. J. Can. 17; 487-94

Fiel, P. D. et al., (1972)
Endocrinology 91; 738-46

Fisher, B. et al., (1977)
Cancer 39(Suppl.); 2883-903

Fisher, B. et al., (1981)
Cancer 48; 1273-80

Fisher, E. R. et al., (1976)
Am. J. Clin. Pathol. 65 21-30

Fisher, E. R. et al., (1981)
Breast Cancer Research Treat. 1; 37-41

Fishman, J. H. (1981)
Biochem. Biophys. Res. Commun. 103; 475-82

Fleischmann, G. and Beato, M. (1979)
Mol. Cell Endocrinology 16; 181-??

Folkman, J. and Greenspan, H. P. (1975)
Biochem. Biophys. Acta. 417; 211-36

Frantz, V. K. et al., (1951)
Cancer 4; 762-83

Friedwald, W. F. and Rous, P. (1944)
J. Exp. Med. 80; 101-26

Fulmer, A.W. and Fasman, G. D. (1979)
Biochemistry 18; 659-68

Furth, J. (1975)
In: Cancer: A Comprehensive Treatise, Vol. 1
Ed. Becker, F. F.
Plenum Press, New York., pp.75-120

Gambrell, R. D. (1984)
J. Obstet. Gynae. 4 (Supplement 2) S121-S127

Gardner, D. G. and Wittliff, J. L. (1973)
Biochemistry 12; 3090-96

Garola, R. E. and McGuire, W. L. (1977)
Cancer Research 37; 3333-37

Garola, R. E. and McGuire, W. L. (1978)
Cancer Research 38; 2216-20

Giannopolous, G. and Gorski, J. (1971)
J. Biol. Chem. 246; 2524-36

Ginsburg, M. et al., (1974)
Steroids 23; 773-92

Gjorgov, A. N. (1978)
Oncology 35 97-100

Gluksberg, H. et al., (1982)
Cancer 50; 423-34

Goidl, J. A. et al., (1977)
Biochemistry 16; 2125-30

Goldenberg, D. M. et al., (1974)
Nature 250; 649-51

Gorski, J. and Gannon, F. (1976)
Ann. Rev. Physiol. 38; 425-50

Gorski, J. et al., (1968)
Rec. Prog. Horm. Res. 24; 45-80

Gorski, J. (1964)
J. Biol. Chem. 239; 889-92

Greene, G. L. et al., (1980)
Proc. Natl. Acad. Sci. (USA), 177; 157-61

Griffiths, K. et al., (1975)
In: Normal and Abnormal Growth of the Prostate
Ed: Goland, M. et al.
Thomas Springfield, Ill., pp.417-30.

Grody, W. W. et al., (1982)
Endocrine Rev. 3; 141-63

Gross, L. (1978)
Cancer Research 38; 45-93

Gross, L. (1974)
J. Am. Med. Assoc. 230; 1029-32

Gross, S. C. et al., (1982)
J. Biol. Chem. 257; 4738-45

Gschwendt, M. and Kittstein, W. (1980)
Mol. Cell Endocrinology 20; 251-60

Gullino, P. M. (1978)
J. Nat. Can. Inst. 61; 639-43

Gullino, A. and Pasqualini, J. R. (1982)
Cancer Research 42; 1913-21

Hahnel, R. et al., (1979)
Cancer 44; 671-75

Hahnel, R. and Vivian, A. B. (1975)
In: Estrogen Receptors in Human Breast Cancer
Eds. McGuire, W. L., Carbone, P. P. and Vollmer, E. P.
Raven Press, New York., pp. 205-35

Harris, J. and Gorski, J. (1978)
Endocrin. 103; 240-45

Haselbacher, G. K. and Eisenfeld, A. J. (1976)
Biochem. Pharmacol. 25; 2571-81

Hawkins, R. A. et al., (1980)
Br. J. Surg. 67; 153-69

Hayward, J. L. et al., (1977)
Eur. J. Can. 13; 89-94

Heath, C. W. Jr. et al., (1975)
In: Persons at High Risk Cancer.
Ed. Fraumeni, J. F.
Academic Press, New York., pp. 241-64

Heise, E. and Gorlich, M. (1982)
Oncology 39; 34-44

Henderson, B. E. et al., (1974)
J. Nat. Can. Inst. 53; 609-14

Henningson et al., (1980)
Rec. Results in Cancer Res. 71; 122-24

Henrikson, K. P. et al., (1981)
Endocrinology 109; 1196-202

Heppner, D. C. et al., (1978)
Cancer Research 38; 3758-63

Heubner, R. J. and Todaro, G. J. (1969)
Proc. Natl. Acad. Sci. (USA), 64; 1087-94

Heuson, J. C. (1975)
In: Estrogen Receptors in Human Breast Cancer.
Eds. McGuire, W. L., Carbone, P. P. and Vollmer, E. P.
Raven Press, New York., pp.57-70

Heuson, J. C. (1976)
Can. Treat. Rep. 60; 1463-66

Higgins, S. J.. and Gehring, U. (1978)
Adv. Can. Res. 28; 313-97

Higgins, S. J. et al., (1973)
J. Biol. Chem. 248; 5866-79

Holbrook, N. J. et al., (1983a)
J. Biol. Chem. 258; 14885-94

Holbrook, N. J. et al., (1983b)
J. Biol. Chem. 258; 6477-85

Honn, K. V. (1982)
In: Prostaglandins and Cancer.
Eds. Powles, T. J. et al.
Alan. R. Liss., New York., pp. 733

Honn, K. V. et al., (1983)
Biochem. Pharmacol. 32; 1-11

Horwitz, K. B. and McGuire, W. L. (1975)
Steroids 25; 497-505

Horwitz, K. B. and McGuire, W. L. (1977)
J. Biol. Chem. 253; 2223-28

Horwitz, K. B. and McGuire, W. L. (1978a)
J. Biol. Chem. 253; 2223-28

Horwitz, K. B. and McGuire, W. L. (1978b)
J. Biol. Chem. 8185-91

Horwitz, K. B. and McGuire, W. L. (1980)
J. Biol. Chem. 255; 9699-705

Howat, J. M. et al., (1983)
Br. J. Can. 47; 629-40

Hsueh, A. J. et al., (1975)
Nature 254; 337-39

Hubay, C. A. et al., (1980)
Cancer 46; 2805-8

Huggins, C. et al., (1959)
J. Exp. Med. 109; 25-42

Huggins, C. (1967)
Science 156; 1050-54

Hughes, M. R. et al., (1981)
Biochemistry 20; 2481-91

Hyder, S. et al., (1984)
Biochem. Soc. Trans. 12; 322-23

IARC Monographs (1979)
Sex Hormones, Springer Verlag, Berlin.

Ikeda, M. (1982)
Biochem. Biophys. Acta. 718; 66-73

Illmensee, K. and Mintz, B. (1976)
Proc. Natl. Acad. Sci. (USA), 73; 549-53

Izuo, M. et al., (1981)
Breast Cancer Res. Treat. 1; 125-30

Jackson, V. and Chalkey, R. (1974a)
J. Biol. Chem. 249; 1615-26

Jackson, V. and Chalkey, R. (1971b)
J. Biol. Chem. 249; 1627-36

Janne, O. et al., (1975)
J. Steroid Biochem. 6; 501-9

Jensen, E. V. and Jacobson, H. I. (1962)
Rec. Prog. Horm. Res. 18; 387-414

Jensen, E. V. and DeSombre, E. R. (1972)
Ann. Rev. Biochem. 41; 203-30

Jensen, E. V. and DeSombre, E. R (1973)
Science 182; 126-34

Jensen, E. V. et al., (1967)
Science 158; 385-87

Jensen, E. V. et al., (1968)
Proc. Natl. Acad. Sci. (USA), 59; 632-38

Jensen, E. V. et al., (1971a)
Natl. Can. Inst. Monogr. 34; 55-79

Jensen, E. V. et al., (1971b)
Biochem. Soc. Sym. 32; 133

Jensen, E. V. et al., (1973)
Rec. Results in Cancer Research. 42; 55-62

Jensen, E. V. et al., (1974)
Vitamins and Hormones 32; 89-127

Jensen, E. V. et al., (1975)
In: Estrogen Receptors in Human Breast Cancer. Vol. 1: Current Approaches to Therapy. Ed. McGuire, W. L.
Plenum Press, New York., pp. 217-62

Jick, H. et al., (1980)
Am. J. Epidemiol. 112 577-85

John, J. K. and Moudgil, V. K. (1979)
Biochem. Biophys. Res. Commun. 90; 1242-48

Johnson, M. and Everitt, R. (Eds.) (1980)
Essential Reproduction,
Blackwell Scientific Publications, Oxford, pp. 293-342.

Jordan, V. C. and Allen, K. E. (1980)
Eur. J. Can. 16; 239-51

Jordan, V. C. et al., (1980)
In: Pharmacological Modulation of Steroid Action.
Eds. Gennazzani, F., DiCarlo, F. and Mainwaring, W. I. P.
Raven Press, New York., pp. 81-98

Jost, J. P. et al., (1984)
Proc. Natl. Acad. Sci. (USA), 81; 429-33

Juret, P. (1976)
Eur. J. Cancer 12 701-4

Kalimi, M. and Love, K. (1980)
J. Biol. Chem. 255; 4687-90

Kalimi, M. and Banerjee, A. (1981)
J. Steroid Biochem. 14; 593-97

Kalimi, M. et al., (1975)
J. Biol. Chem. 250; 1080-86

Kallos, J. and Hollander, V. P. (1978)
Nature 272; 177-79

Kallos, J. et al., (1981)
Proc. Natl. Acad. Sci. (USA), 78; 2874-78

Kaschka-Dierich, C. et al., (1976)
Nature 260; 302-6

Kassis, J. A. and Gorski, J. (1981)
J. Biol. Chem. 256; 7378-82

Kassis, J. A. and Gorski, J. (1983)
Mol. Cell. Biochem. 52; 27-36

Katzenellenbogen, B. S. et al., (1978)
J. Biol. Chem. 253; 697-707

Katzenellenbogen, B. S. et al., (1979)
Rec. Prog. Horm. Res. 35; 259-300

Katzenellenbogen, B. S. et al., (1981)
J. Steroid Biochem. 15; 219-29

Kauffman, S. (1971)
J. Theoret. Biol. 31; 429-51

Kay, C. (1981)
Br. Med. J. 282 2089-92

Kelner, K. L. and Peck, E. J. Jr. (1981)
J. Recept. Res. 2; 47-62

Kennedy, B. J. (1974)
Semin. Oncol. 1; 119-30

Kiang, D. T. and Kennedy, B. J. (1977)
Ann. Intern. Med. 87; 687-90

King, R. J. B. and Gordon, J. (1972)
Nature New Biol. 240; 185-87

King, R. J. B. and Mainwaring, W. I. P. (Eds.) (1974)
Steroid Cell Interactions.
University Press, Baltimore, MD.

King, R. J. B. and Whitehead, M. I. (1980)
In: Perspectives in Steroid Receptor Research.
Ed. Bresciani, F.
Raven Press, New York., pp. 259-71

King, R. J. B. et al., (1971)
Advances in Biosciences 7; 21-44

King, R. J. B. et al., (1979)
In: Steroid Receptor Assay in Human Breast Tumours: Methodological and Clinical Aspects. Ed. King, R. J. B.
Alpha-Omega-Alpha, Cardiff., pp. 55-72

King, R. J. B. et al., (1980)
J. Steroid Biochem. 13; 377-82

King, W. J. and Greene, G. L. (1984)
Nature 307; 745-47

Kirschner, M. A. (1977)
Cancer 39 2716-26

Kledzik, G. S. et al., (1976)
Cancer Research 36 3265-68

Klein, G. (1972)
Proc. Natl. Acad. Sci. (USA), 69; 1056-64

Klein, G. (1979)
Proc. Natl. Acad. Sci. (USA), 76; 2442-46

Klein, G. (1981)
Nature 294; 313-18

Kleinerman, J. and Liotta, L. (1977)
In: Cancer Invasion and Metastasis: Biologic Mechanisms and Therapy.
Ed. Day, S.B. Raven Press, New York., pp. 135-43

Knight, W. A. et al., (1977)
Cancer Research 37; 4669-73

Klinga, K. et al., (1982)
Oncology 39; 337-39

Knudson, A. G. et al., (1976)
New Eng. J. Med. 295; 1120-23

Knudson, A. G. (1973)
Adv. Can. Res.. 17; 317-52

Koenders, A. J. M. et al., (1977)
In: Progesterone Receptors in Normal and Neoplastic Tissues.
Eds. McGuire, W. L., Raynaud, J. P. and Baulieu, E. E.
Raven Press, New York., pp. 71-84

Koenders, A. J. M. et al., (1980)
In: Perspectives in Steroid Receptor Research.
Ed. Bresciani, F.
Raven Press, New York., pp. 247-57

Korenman, S. G. (1969)
Steroids 13; 163-77

Kraemar, P. M. et al., (1974)
CSH Symp. Quant. Biol. 38; 133

Kumar, S. A. et al., (1980)
Proc. Natl. Acad. Sci. (USA), 77; 3341-45

Kumar, S. A. et al., (1983)
J. Cellular Biochem. 21; 19-27

Kute, T. E. et al., (1978)
Cancer Research 38; 4307-13

Kuttenn, F. et al., (1981)
J. Clin. Endo. Metab. 52; 1225-29

Lacassagne, A. (1948)
Schweiz. Med. Wschr. 78; 705-08

Lacour, F. et al., (1982)
Rec. Results Cancer Research. 80; 200-6

Lacour, J. et al., (1984)
Br. Med. J. 288; 589-92

Lathrop, A. and Loeb, L. (1913)
Proc. Soc. Exp. Biol. Med. 11; 38-40

Lea, O. A et al., (1979)
Endocrinology 105; 1350-60

Leach, K. L. et al., (1979)
J. Biol. Chem. 254; 11884-90

Leake, R. E. et al., (1981a)
Br. J. Cancer 43; 59-66

Leake, R. E. et al., (1981b)
Br. J. Cancer 43; 67-71

Leclerq, G. et al., (1973)
Eur. J. Can. 9; 665-73

Legha, S. S. et al., (1978)
Annals. Int. Med. 88; 69-76

Legha, S. S. et al., (1981)
Oncology 39; 140-45

Lerner, L. J. (1964)
Rec. Prog. Horm. Res. 20; 435-90

Lesser, M. L. et al., (1981)
Cancer 48; 299-309

Leung, B. S. et al., (1974)
Surg. Gynaecol. Obstet. 139; 525-28

Leung, B. S. et al., (1975)
In: Estrogen Receptors in Human Breast Cancer.
Eds. McGuire, W. L., Carbone, P. P. and Vollmer, E. P.
Raven Press, New York., pp. 107-29.

LiVolsi, V. A. (1978)
New. Eng. J. Med. 299 381-85

Liao, S. et al., (1973)
Nature New Biol. 241; 211-13

Lin, S. and Ohno, S. (1981)
Eur. J. Biochem. 124; 283-87

Linkie, D. M. (1977)
Endocrinology 101; 1862-70

Linkie, D. M. and Siiteri, P. K. (1978)
J. Steroid. Biochem. 9; 1071-78

Lippman, M. E. (1980)
In: Perspectives in Steroid Receptor Research.
Ed. Bresciani, F.
Raven Press, New York., pp. 217-37

Lippman, M. E. and Allegra, J. C. (1980)
In: Recent Results in Cancer Research, Vol. 71
Eds. Henningsen, B., Linder, F. and Steichele, C.
Springer-Verlag, New York., pp. 155-61

Lippman, M. E. et al., (1976)
Cancer Research. 36; 4595-601

Lipsett, M. (1979)
Cancer 43; 1967-81

Lohmar, P. H. and Toft, D. O. (1975)
Biochem. Biophys. Res. Commun. 67; 8-15

Lowry, O. H. et al., (1951)
J. Biol. Chem. 193; 265-75

Lupulescu, A. et al., (1978)
J. Cell Biol. 79; 187a, abstract 1205

Maass, H. and Jonat, W. (1983)
J. Steroid Biochem. 19; 833-37

MacDonald, R. G. and Leavitt, W. W. (1982)
J. Biol. Chem. 257; 311-15

MacDonald, R. G. et al., (1982)
Biochem. Biophys. Res. Comm. 104; 570-76

MacDonald, R. G. et al., (1983)
Mol. Cell Endocrinology 32; 301

MacMahon, B. et al., (1970)
Bulletin of the World Health Organisation 43; 209-21

Mainwaring, W. I. P. and Irving, R. (1973)
Biochem. J. 134; 113-27

Mainwaring, W. I. P. et al., (1976)
Biochem. J. 156; 129-41

Mark, J. (1969)
Eur. J. Cancer 5; 307-15

Mark, J. (1974)
In: Chromosomes and Cancer
Ed. German, J.
John Wiley & Sons, New York., pp. 497-517.

Mark, J. (1977)
Rec. Adv. Can. Res.. 24; 165-222

Markaverich, B. M. et al., (1981)
J. Steroid. Biochem. 14; 125-32

Markaverich, B. M. et al., (1983)
J. Biol. Chem. 258; 11663-71

Martial, J. et al., (1975)
Biochem. 14; 4907-11

Martin, R. G. and Ames, B. N. (1961)
J. Biol. Chem. 236; 1372-79

Martin, P.M. and Sheridan, P. J. (1980)
Experientia 36; 620-22

Martin, P. M. and Sheridan, P. J. (1982)
J. Steroid. Biochem. 16; 215-19

Massol, N. et al., (1978)
Nucleic Acid Res. 5; 723-38

Mauck, L. A. et al., (1982)
Biochem. 21; 1788-93

Maynard, P. V. et al., (1978)
Br. J. Can. 38; 745-48

McCarthy, K. S. Sr. and McCarthy, K. S. Jr.(1977)
Am. J. Pathol. 86; 705-44

McGuire, W. L. (1973)
J. Clin. Invest. 52; 73-77

McGuire, W. L. (1975)
Ann. Rev. Med. 26; 353-63

McGuire, W. L. (1977)
In: Breast Cancer. Advances in Research and Treatment. Vol. I:
Current Approaches to Therapy. Ed. McGuire, W. L.
Plenum Press, New York., pp.217-62.

McGuire, W. L. (1980a)
In: Perspectives in Steroid Receptor Research.
Ed. Bresciani, F.
Raven Press, New York., pp. 339-46

McGuire, W. L. (1980b)
In: Breast Cancer Experimental and Clinical Aspects.
Eds. Mouridsen, H. T. and Palshof, T.
Pergamon Press, New York., pp. 39-43

McGuire, W. L. and DeLaGarza, M. (1973)
J. Clin. Endo. Metab. 36; 548-52

McGuire, W. L. and Horwitz, K. B. (1977)
Annals. New York Acad. Sci. 286; 90-100

McGuire, W. L. and Julian, J. A. (1971)
Cancer Research 31; 1440-45

McGuire, W. L. et al., (1975)
In: Estrogen Receptors in Human Breast Cancer
Eds. McGuire, W. L., Carbone, P. P. and Vollmer, E. P.
Raven Press, New York., pp.1-8

McGuire, W. L. et al., (1977a)
Cancer 39; 2934-47

McGuire, W. L. et al., (1977b)
Cancer Research 37; 637-39

McGuire, W. L. et al., (1978)
In: Receptors and Hormone Action, Vol. II
Eds. O'Malley, B. W. and Birnbaumer, L.
Academic Press, New York., pp.401-41

Meakin, J. W. et al., (1979)
Canad. Med. Assoc. J. 120;1221-29

Meiss, R. et al., (1982)
Andrologia 14 317-21

Mester, J. and Baulieu, E. E. (1975)
Biochem J. 146; 617-23

Mester, J. et al., (1970)
Biochem. J. 120; 831-36

Meyer, J. S. et al., (1977)
Cancer 40; 2290-98

Migiliaccio, A. et al., (1982)
Biochem. Biophys. Res. Comm. 109; 1002-10

Migiliaccio, A. et al., (1984)
Proc. Natl. Acad. Sci. (USA), 81; 5921

Milgrom, E. (1981)
In: Biochemical Actions of Hormones.
Ed. Litwack, G.
Academic Press, New York., pp. 465-92.

Milgrom, E. et al., (1973)
Biochem. Biophys. Acta. 320; 267-83

Miller, A. B. (1977)
Cancer 39; 2704-8

Miller, L. K. et al., (1981)
Endocrinology 108; 1369-78

Miller, E. C. (1978)
Cancer Research 38; 1479-96

Miller, J. B. and Toft, D. O. (1978)
Biochem 17; 173-77

Mintz, B. and Illmensee, K (1975)
Proc. Natl. Acad. Sci. (USA), 72; 3585-89

Mobbs, B. G. and Johnson, I. E. (1976)
Canad. Med. Assoc. J. 114; 216-19

Modak, M. J. (1976)
Biochemistry 15; 3620-26

Mohla, S. et al., (1972)
Biochem. Biophys. Res. comm. 46; 661-67

Moncharmont, B. et al., (1982)
J. Steroid Biochem. 16; 361-68

Moon, H. et al., (1950)
Cancer Research 10; 293-308

Moore, F. D. et al., (1974)
Surgery 76; 376-90

Mortimer, B. and Lipsett, M.D. (1979)
Cancer 43; 1967-81

Moura, J. J. G. and Xavier, A. V. (1978)
In: New Trends in Bio-Organic Chemistry.
Eds. Williams, R. J. P. and DaSilva, J. R. R.
Academic Press, New York., pp. 79-119.

Mouridsen, H. T. et al., (1978)
Cancer Treat. Rev. 5; 131-41

Mueller, C. B. and Jeffries, W. (1975)
Ann. Surg. 182; 334-41

Mueller, G. C. et al., (1972)
Rec. Prog. Horm. Res. 28; 1-49

Muhlbock, O. (1972)
J. Nat. Can. Inst. 48; 1213-16

Mulder, E. et al., (1980)
Steroids 36; 633-46

Muldoon, T. G. (1978)
J. Steroid. Biochem. 9; 485-94

Muldoon, T. G. (1971)
Biochemistry 19; 3780-84

Muller, R. E. et al., (1980)
J. Biol. Chem. 255; 4062-67

Muller, R. E. et al., (1983a)
J. Biol. Chem. 258; 9227-36

Muller, R. E. et al., (1983b)
J. Biol. Chem. 258; 11582-89

Mullins, D. E. and Rohrlich, S. T. (1983)
Biochem. Biophys. Acta. 695; 177-214

Mulvihill, E. R. et al., (1982)
Cell 24; 621-32

Mulvihill, J. J. et al., (1977)
In: Genetics of Human Cancer, pp. 519
Eds. Mulvihill, J. et al.
Raven Press, New York.

Mulvihill, E. R. and Palmiter, R. D. (1977)
J. Biol. Chem. 252; 2060-68

Musliner, T. A. et al., (1970)
Biochemistry 9; 4448-53

Myatt, L. (1979a)
PhD Thesis, University of London.

Myatt, L. et al., (1979b)
Biochem. Soc. Trans. 7; 15-16

Myatt, L. et al., (1982a)
Biochem. J. 202; 203-9

Myatt, L. et al., (1982b)
Br. J. Can. 45; 964-67

Myatt, L. et al., (1984)
Biochem. Soc. Trans. 12; 321-22

Myatt, L. et al., (1985)
Biochem. Biophys. Act. in press.

NIH Consensus Development Conference, (1980)
Cancer 46; 2759-63

Nawata, H. et al., (1981)
J. Biol. Chem. 256; 6895-902

Neel, B. G. et al., (1982)
Proc. Natl. Acad. Sci. (USA), 79; 7842-46

Nenci, I. (1976)
J. Steroid. Biochem. 7; 505-10

Nenci, I. (1978)
Cancer Res. 38; 4204-11

Nenci, I. et al., (1980)
J. Histochem. Cytochem. 28; 1081-88

Neuwald, P. D. et al., (1980)
J. Nat. Can. Inst. 64; 447-59

Newsome, H. H. et al., (1977)
Cancer 39; 542-46

Nicholson, G. L. (1978)
BioScience 28; 441-47

Nicholson, R. et al., (1981)
J. Steroid Biochem. 15; 193-99

Nicholson, R. I. and Golder, M. P. (1975)
Eur. J. Can. 11; 571-79

Nielson, C. J. et al., (1977a)
J. Biol. Chem. 252; 7568-78

Nielson, C. J. et al., (1977b)
Cancer Research. 37; 3420-26

Nishigori, H. and Toft, D. O. (1979)
J. Biol. Chem. 254; 9155-61

Nishigori, H. and Toft, D. (1980)
Biochemistry 19; 77-83

Nissen-Meyer, R. (1975)
In: Hormones and Breast Cancer.
Eds. Namer, M. and Lalanne, C. M.
INSERM, Paris., pp. 151-58

Noma, K. et al., (1980)
Endocrinology 107; 1205-11

Noteboom, W. D. and Gorski, J. (1965)
Arch. Biochem. 111; 559-68

Notides, A. C. (1978)
Recept. Horm. Action 2; 33-61

Notides, A. C. and Gorski, J. (1966)
Proc. Natl. Acad. Sci. (USA), 56; 230-35

Notides, A. C. and Nielsen, S. (1974)
J. Biol. Chem. 249; 1866-73

Notides, A. C. and Nielsen, S. (1975)
J. Steroid Biochem. 6; 483-86

Notides, A. C. et al., (1973)
Endocrinology 93; 210-16

Notides, A. C. et al., (1975)
J. Biol. Chem. 250; 3945-50

Notides, A. C. et al., (1981)
Proc. Natl. Acad. Sci. (USA), 78; 4926-30

O'Malley, B. W. and Means, A. R. (1974)
Science 183 610-20

O'Malley, B. W. et al., (1971)
J. Biol. Chem. 246; 1117-22

O'Malley, B. W. et al., (1972)
Nature 235; 141-44

Ogle, T. F. (1983)
J. Biol. Chem. 258; 4982-88

Okulicz, W. C. et al., (1981)
Endocrinology 109; 2273-75

Olea-Serrano, P. et al., (1985)
IABCR Proceedings, Abstract 2-25

Ory, H. et al., (1981)
New Eng. J. Med. 294 419-22

Oskarsson, M. et al., (1980)
Science 207; 1222-24

Page, M. and Godin, C. (1969)
Biochem. Biophys. Acta. 19; 329-31

Paigen, K. (1958)
J. Biol. Chem. 233; 388-94

Paine, P. L. and Feldherr, C. M. (1973)
Exp. Cell Res. 74; 81-98

Paine, P. L. et al., (1975)
Nature 254; 109-14

Pall, M. L. (1981)
Proc. Natl. Acad. Sci. (USA), 78; 2465-68

Panko, W. B. and McLeod, R. M. (1978)
Cancer Research 38; 1948-51

Panko, W. B. et al., (1981)
J. Steroid. Biochem. 14; 1311-16

Panko, W. B. et al., (1982)
Cancer 49; 2148-52

Parada, L. F. et al., (1982)
Nature 297; 474-78

Parchman, L. G. and Litwack, G. (1977)
Arch. Biochem. Biophys. 183; 374-82

Park, D. C. and Wittliff, J. L. (1977)
Biochem. Biophys. Res. Commun. 78; 251-58

Park, D. C. and Wittliff, J. L. (1980)
Tumour Diagnostik 5; 261-65

Patthy, L. and Smith, E. L. (1975a)
J. Biol. Chem. 250; 557-64

Patthy, L. and Smith, E. L. (1975b)
J. Biol. Chem. 250; 565-69

Patthy, L. and Thesz, J. (1980)
Eur. J. Biochem. 105; 387-93

Pavlik, E. J. and Coulson, P. B. (1976)
J. Steroid. Biochem. 7; 357-68

Pavlik, E. J. and Rutledge (1980)
J. Steroid Biochem. 13; 1433-41

Pavlik, E. J. et al., (1982)
Biochem. 21; 139-45

Paywar, F. et al., (1981)
Proc. Natl. Acad. Sci. (USA), 78; 6628-32

Peck, E. J. et al., (1973)
Biochem. 12; 4596-603

Penney, G. C. and Hawkins, R. A. (1982)
Br. J. Can. 45; 237-46

Petrakis, N. L. (1977)
Cancer 39 2709-15

Pfahl, M. (1982)
Cell 31; 475-82

Pichon, M. F. and Milgrom, E. (1977)
Cancer Research 37; 464-71

Pietras, R. J. and Szego, C. (1977)
Nature 265; 69-72

Pike, M. C. et al., (1983)
Lancet ii 926-30

Pitot, H. C. (Ed.) (1981)
Fundamentals of Oncology
Marcel Dekker, New York.

Plapinger, L. et al., (1973)
Endocrinology 93; 1119-28

Pollow, K. et al., (1983)
In: Steroids and Endometrial Carcinoma.
Eds. Jasonni, V. M. et al.
Raven Press, New York, pp.37-60

Pooley, A. S. and King, R. J. B. (1969)
Biochem. J. 115; 48P

Porath, J. (1963)
Pure Appl. Chem. 6; 233-43

Poste, G. (1977)
In: Cancer Invasion and Metastasis: Biologic Mechanisms
and Therapy.
Ed. Day, S.B. Raven Press, New York., pp. 163-74

Poulsen, H. S. (1981)
Eur. J. Can. 17; 495-501

Pratt, W. B. et al., (1979)
In: Steroid Hormone Receptor Systems.
Eds. Leavitt, W. W. and Clark. J. H.
Plenum Press, New York., pp. 343-56

Puca, G. A. and Bresciani, F. (1968)
Nature 218; 967-69

Puca, G. A. and Bresciani, F (1969a)
Nature 223; 745-47

Puca, G. A. and Bresciani, F. (1969b)
Endocrinology 85; 1-10

Puca, G. A. et al., (1971)
Biochem. 10; 3769-79

Puca, G, et al., (1974)
Proc. Natl. Acad. Sci. (USA), 71; 979-83

Puca, G. et al., (1975)
J. Biol. Chem. 250; 6452-59

Ramcharan, S. et al., (1981) In: The Walnut Creek Drug Study: A prospective study of the side effects of oral contraceptives, Vol. III. Department of Health and Human Services (National Institutes of Health), Bethesda.

Rao, B. R. (1982)
Mol. Cell Endocrinology 21; 97-108

Ratajczak, T. et al., (1982)
FEBS Lett. 149; 80-84

Rawls, W. F. et al., (1969)
Am. J. Epidemiol. 89; 547-54

Raynaud, J. P. et al., (1977)
In: Research in Steroids, Vol. VII
Eds. Vermulen, A., Jungblut, P. and Klupper, A.
Elsevier, North Holland., pp.281-90.

Regato, J. A. (1977)
Semin. Oncol. 4; 33-38

Ritzen, E. M. et al., (1974)
J. Biol. Chem. 249; 6597-604

Roblin, R. et al., (1975)
Adv. Can. Res.. 22; 203-60

Rochefort, H. and Baulieu, E. E. (1973)
Biochimie 54; 1303-17

Rochefort, H. et al., (1983)
J. Steroid Biochem. 19; 69-74

Rochefort, H. and Borgna, J. L. (1981)
Nature 292; 257-59

Romanov, G. A. et al., (1983)
Biochem. Int. 6; 339-48

Rosen, P. P. et al., (1977)
Cancer 39; 2194-200

Rosen, S. W. et al., (1980)
J. Clin. Endo. Metab. 50; 834-41

Rossi, A. et al., (1981)
Br. Med. J. 282; 1427-31

Rousseau, G. C. et al., (1975)
J. Mol. Biol. 79; 539-44

Rowley, J. D. and Potter, D. (1976)
Blood 47; 705-21

Rowley, J. D. (1973)
Nature 243; 290-93

Rowley, J. D. (1977)
Proc. Natl. Acad. Sci. (USA), 74; 5729-33

Rubin, H. (1980)
J. Nat. Cancer Inst. 64; 995-1000

Ruh, T. S. and Baudendistel, L. J. (1977)
Endocrin. 100; 420-26

Ruh, T. S. and Baudendistel, L. J. (1978)
Endocrin. 102; 1838-46

Ruh, T. S. and Ruh, M. F. (1974)
Steroids 24; 209-24

Sachs, L. (1980)
Proc. Natl. Acad. Sci. (USA), 77; 6152-56

Saez, S. et al., (1978)
Cancer Research 38; 3468-73

Sajdel, E. M. and Jacob, S. T. (1971)
Biochem. Biophys. Res. Comm. 45 707-15

Sakai, D. and Gorski, J. (1984)
Biochem. 23; 3541-47

Sala-Trepat, J. M. et al., (1977)
Nucleic Acid Res. 4; 649-62

Salsbury, A. J. (1975)
Can. Treat. Rev. 2; 55-72

Salvo, R. A. et al., (1976)
Biochemistry 15; 493-97

Samaan, N. et al., (1981)
Cancer 47; 554-60

Sanborn, B. et al., (1971)
Biochemistry 10; 4955-62

Sandberg, A. A. (1973)
Proc. Nat. Cancer. Conf. 7; 333-43

Sando, J. J. et al., (1979a)
J. Biol. Chem. 254; 4772-78

Sando, J. J. et al., (1979b)
J. Biol. Chem. 254; 4779-89

Santen, R. J. et al., (1980)
J. Clin. Endo. Metab. 51 473-77

Santen, R. J. et al., (1977)
J. Clin. Endo. Metab. 45 469-79

Sar, M. and Stumpf, W. E. (1976)
J. Steroid. Biochem. 7; 391-94

Sarff, M. and Gorski, J. (1971)
Biochemistry 10; 2557-63

Sato, B. et al., (1978)
Endocrinology 102; 545-55

Sato, B. et al., (1979)
Endocrinology 104; 1474-79

Sato, B. et al., (1980)
Endocrinology 106; 1142-48

Sato, B. et al., (1981)
J. Steroid Biochem. 14; 295-303

Scatchard, G (1949)
Ann. N. Y. Acad. Sci. 51; 660-72

Schimke, R. T. et al., (1975)
Rec. Prog. Horm. Res. 31; 175-208

Schneider, S. L. and Dao, T. L. (1977)
Cancer Research 37; 382-87

Schwab, M. et al., (1983)
Nature 303; 497-501

Segaloff, A. and Maxfield, W. (1971)
Cancer Research 31; 166-68

Shen, G. et al., (1979)
Biochem. J. 182; 241-43

Sherman, M. R. and Stevens, J. (1984)
Ann. Rev. Physiol. 46; 83-105

Sherman, M. R. et al., (1974)
J. Biol. Chem. 249; 5351-63

Sherman, M. R. et al., (1978)
Fed. Proc. 37; 167-73

Sherman, M. R. et al., (1980)
Endocrinology 106; 1715-27

Sherman, M. R. et al., (1981)
In: Physiopathology of Endocrine Diseases and Mechanisms of Hormone Action. Eds. Soto, R. J., DeNicola, A. and Blaquier, J. Alan. R. Liss., New York., pp.321-37

Sheridan, P. J. et al., (1979)
Nature 282; 579-82

Shyamala, G. and Gorski, J. (1969)
J. Biol. Chem. 244; 1097-103

Shyamala, G. and Leonard, L. (1980)
J. Biol. Chem. 255; 6028-31

Shyamala, G. and Nandi, S. (1972)
Endocrinology 91; 861-67

Shyamala, G. (1971)
Nature New Biol. 231; 246-48

Shyamala, G. (1972)
Biochem. Biophys. Res. Comm. 46; 1623-30

Shyamala, G. (1975)
Biochem. Biophys. Res. Commun. 64; 408-15

Shyr, C-I. and Liao, S. (1978)
Proc. Natl. Acad. Sci. (USA), 75; 5969-73

Siebert, K. and Lippman, M. E. (1982)
In: Clinics in Oncology, Vol. 1(3)
Ed. Baum, M.
W. B. Saunders Co. Ltd., London., pp. 735-94.

Siiteri, P. et al., (1980)
In: Hormones and Cancer.
Eds. Iacobelli, S., King, R., Lindner, H., and Lipmann, M.
Raven Press, New York., pp.499-505

Siiteri, P. K. and MacDonald, P. C. (1973)
In: Handbook Of Physiology. Section 7, Vol. II
Williams & Wilkins, Baltimore, MD., pp.615-29

Siiteri, P. K. (1982)
Cancer Research 42(Suppl.); 32695-735

Simons, M. J. et al., (1976)
J. Nat. Can. Inst. 57; 977-80

Singhagowinta, A. et al., (1975)
In: Estrogen Receptors in Human Breast Cancer.
Eds. McGuire, W. L., Carbone, P. P. and Vollmer, E. P.
Raven Press, New York., pp. 131-49

Sparks, F. C. et al., (1977)
In: Adjuvant Therapy of Cancer
Ed. Salmon, S. E. and Jones, S. E.
North Holland Publ., Amsterdam, pp. 109-14

Spelsberg, T. C. and Toft, D. O. (1976)
In: Receptors and the Mechanisms of Action of Steroid Hormones, Part I. Ed. Pasqualini, J.
Marcel Dekker, New York., pp. 262-309

Spelsberg, T. C. et al., (1971)
J. Biol. Chem. 246; 4188-97

Spelsberg, T. C. et al., (1972)
J. Biol. Chem. 247; 1368-74

Spelsberg, T. C. (1976a)
Biochem. J. 156; 391-98

Spelsberg, T. C. et al., (1976b)
Nature 262; 65-67

Spelsberg, T. C. et al., (1976c)
J. Steroid. Biochem. 7; 1091-101

Spindler, B. J. et al., (1975)
J. Biol. Chem. 250; 4113-19

Stancel, G. M. et al., (1973)
Biochem. 12; 2130-41

Starthling, W. H. and O'Malley, B. W. (1976)
Eur. J. Biochem. 66; 435-41

Steggles, A. W. and King, R. J. B. (1970)
Biochem. J. 118; 695-701

Steggles, A. W. and King, R. J. B. (1971)
Proc. Natl. Acad. Sci. (USA), 68; 1479-82

Stegner, H. E. et al., (1983)
J. Steroid Biochem. 19; 297-303

Stein, G. et al., (1975)
Biochemistry 14; 1859-66

Stewart, J. F. et al., (1980)
Eur. J. Can. 17; 449-53

Stewart, J. F. et al., (1983)
Eur. J Can. Clin. Oncol. 19; 1381-87

Stoll, B. A. (1969)
Hormonal Management Of Breast Cancer.
Pitman, London.

Stoll, B. A. (Ed.) (1981)
Hormonal Management of Endocrine-Related Cancers.
Lloyd-Luke Medical Books Ltd., London.

Strauli, P. and Weiss, L (1977)
Eur. J. Cancer 13; 1-12

Straus, D. S. (1981)
J. Nat. Cancer Inst. 67; 233-41

Stumpf, W. E. (1969)
Endocrinology 85; 31-37

Sugarbaker, E. V. (1981)
Cancer Biol. Rev. 2 235-78

Sutherland, R. L. and Jordan, V. C. (1981)
Non-Steroidal Antioestrogens.
Academic Press, New York.

Sutherland, R. L. et al., (1980)
Nature 288; 273-75

Sutherland, R. L. et al., (1983a)
Eur. J. Can. Clin. Oncol. 19; 307-18

Sutherland, R. L. et al., (1983b)
Eur. J. Can. Clin. Oncol. 19; 615-21

Svedberg, T. and Pederson, K. O. (1940)
In: The Ultracentrifuge.
Oxford University Press, London. p.478.

Takizava, S. et al., (1970)
Cancer Research 30; 206-11

Talwar, G. P. et al., (1964)
Proc. Natl. Acad. Sci. (USA), 52; 1059-66

Tarin, D (1976)
In: Fundamental Aspects of Metastasis.
Ed. Weiss, L.
N. Holland, Amsterdam., pp. 151-87

Taylor, S. G. and Gelber, R. (1982)
Cancer Treat. Rep. 66; 1594-95

Taylor, S. G. (1962)
Surg. Gynnecol. Obstet. 115; 443-48

Temin, H. M. (1971)
J. Nat. Can. Inst. 46; 3-7

Temin, H. M. (1974)
Cancer Research 34; 2835-41

Temin, H. M. (1976)
Science 192; 1075-80

Terayama, H. et al., (1976)
In: Control Mechanisms in Cancer.
Eds. Criss, W. E. et al.
Raven Press, New York., pp. 83-97.

Terenius, L. (1967)
Mol. Pharmacol. 3; 423-28

Thanki, K. H. et al., (1978)
J. Biol. Chem. 253; 7744-50

Thapman, T. N. and Clark, J. H. (1981)
Nature 290; 152-54

Thomas, T. et al., (1983)
Biochem. Biophys. Res. Comm. 115; 685-91

Thorsen, T. (1979)
J. Steroid Biochem. 10; 661-68

Thrower, S. et al., (1976)
Biochem. J. 160; 271-80

Thrower, S. et al., (1981)
Biochem. J. 194; 667-71

Tilzer, L. L. et al., (1981)
Cancer Research 41; 1058-63

Toft, D. O. (1972)
J. Steroid Biochem. 3; 515-22

Toft, D. O. and Gorski, J. (1966)
Proc. Natl. Acad. Sci. (USA), 55; 1574-81

Toft, D. O. and O'Malley, B. W. (1972)
Endocrinology 90; 1041-45

Tooze, J. (1973)
In: The Molecular Biology of Tumour Viruses,
Cold Springs Harbour Laboratory,
Cold Springs Harbour, New York.

Traish, A. et al., (1977)
J. Biol. Chem. 252; 6823-30

Traish, A. et al., (1980)
J. Biol. Chem. 255; 4068-72

Trinkaus, J. P. (1976)
In: The Cell Surface in Animal Embryogenesis and Development
Eds. Poste, G. and Nicholson, G. L.
N. Holland, Amsterdam., pp. 225-29.

Tsai, M. J. et al., (1975)
J. Biol. Chem. 250; 5165-74

Tseng, L. and Gurpide, E. (1976)
J. Steroid. Biochem. 7; 817-22

Union Internationale Contre Le Cancer, UICC (1978)
TNM Classification of Malignant Tumours,
Geneva.

Uriel, J. (1976)
Cancer Research 36; 4269-75

van der Riet-Fox, M. F. et al., (1980)
Cancer 44; 2108-19

Varmus, H. E. et al., (1973)
Proc. Natl. Acad. Sci. (USA), 70; 3067-71

Vedeckis, W. V. et al., (1980)
Biochemistry 19; 335-49

Vedeckis, W. V. et al., (1983)
Biochemistry 22; 1975-83

Vessey, M. P. et al., (1981)
Br. Med. J. 282 2093-97

Vessey, M. P. et al., (1982)
Br. J. Cancer 45 327-33

Vessey, M. P. et al., (1983)
Br. J. Cancer 47 455-62

Viadana, E. et al., (1973)
Br. J. Cancer 27; 336-40

Vignon, F. and Rochefort, H. (1976)
10th. Meeting on Mammary Cancer, KOBE, p.42

Wallgren, A. et al., (1981)
In: Adjuvant Therapy of Cancer, III
Eds. Salmon, S. E. and Jones, S. E.
Grune & Stratton. pp. 345-50

Walters, M. R. et al., (1980)
J. Biol. Chem. 255; 6799-805

Warren, B. A. and Vales, O. (1972)
Br. J. Exptl. Pathol. 53; 301-11

Watson, C. S. and Clark, J. H. (1980)
J. Recept. Res. 1; 91-111

Weathers, B. J. et al., (1979)
J. Am. Chem. Soc. 101; 917-924

Webster, D. J. T. et l., (1978)
Am. J. Surg. 136; 337-38

Weichman, B. M. and Notides, A. C. (1977)
J. Biol. Chem. 252; 8856-62

Weichman, B. M. and Notides, A. C. (1979)
Biochem. 18; 220-25

Weichman, B. M. and Notides, A. C. (1980)
Endocrinology 106; 434-39

Weiss, L. and Poste, G. (1976)
In: Scientific Foundations in Oncology
Ed. Symington, T.
Heinemann, London., pp. 25-35.

Welshons, W. V. et al., (1984)
Nature 307; 747-49

White, J. O. and Lim, L. (1980a)
Biochem. J. 172; 37-47

White, J. O. and Lim, L. (1980b)
Biochem. J. 190; 833-37

White, J. O. et al., (1981)
Biochem. J. 196; 557-65

White, J. O. et al., (1982)
Biochem. J. 208; 199-204

White, J. O. et al., (1984)
Biochem. Soc. Trans. 12; 323-24

Wiehle, R. D. et al., (1984)
J. Chromatography 307; 39-51

Williams, D. and Gorski, J. (1971)
Biochem. Biophys. Res. Comm. 45; 258-64

Williams, D. and Gorski, J. (1972)
Proc. Natl. Acad. Sci. (USA), 69; 3464-68

Willis, R.A., (1960) (Ed.)
In: Pathology of Tumours.
Butterworths Medical Publications, London.

Wira, C. R. and Baulieu, E. E. (1971)
C. R. Acad. Sci. (Paris) 273; 218-21

Wittliff, J. L. (1974)
Semin. Oncol. 1; 109-18

Wittlif, J. L. (1984)
Cancer 53; 630-43

Wittliff, J. L. and Savlov, E. D. (1978)
Int. J. Radiat. Oncol. Biol. Phys. 4; 463-67

Wittliff, J. L. et al., (1971)
Cancer Research 32; 1983-92

Wittliff, J. L. et al., (1976)
In: Recent Results In Cancer Research.
Eds. St. Arneault, G., Ban, P. and Israel, L.
Springer-Verlag, New York., pp. 59-77

Wittliff, J. L. et al., (1978)
In: Hormones, Receptors and Breast Cancer.
Ed. McGuire, W. L.
Raven Press, New York., pp.325-59

Wittliff, J. L. et al., (1981)
In: Physiopathology of Endocrine Diseases and Mechanisms of Hormone Action. Eds. Soto, R. J., DeNicola, A. F. and Blaqvier, J. A. Alan. R. Liss., New York., pp. 375-96

Wolman, S. R. and Horland, A. A. (1975)
In: Cancer: A Comprehensive Treatise, Vol. 3
Ed. Becker, F. F.
Plenum Press, New York, pp.155-98

Yamamoto, K. R. (1974)
J. Biol. Chem. 249; 7068-75

Yamamoto, K. R. and Alberts, B. M. (1972)
Proc. Natl. Acad. Sci. (USA), 69; 2105-9

Yamamoto, K. R. and Alberts, B. M. (1974)
J. Biol. Chem. 249; 7068-86

Yamamoto, K. R. and Alberts, B. M. (1975)
Cell 4; 301-10

Yogeeswaran, G. and Salk, P. L. (1981)
Science 212; 1514-16

Young, H. A. et al., (975)
Proc. Natl. Acad. Sci. (USA), 72;3060-64

Zanker, K. S. et al., (1981)
J. Can. Res. Clin. Oncol. 100; 135-48

Zava, D. T. and McGuire, W. L. (1977)
J. Biol. Chem. 252; 3703-8

Zech, I. et al., (1976)
Int. J. Cancer 17; 47-56