THE ROLE OF STEROIDS IN NEOPLASTIC TISSUE GROWTH

by

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This thesis is dedicated to my parents and

my future husband, Mike.

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ABBREVIATIONS

The standard abbreviations, as recommended in revised "Instructions to Authors" (Biochem. J. (1978) 169, 1-27), are used throughout this thesis, with the following additions:-

DES	diethylstilboestrol
DTT	dithiothreitol
POPOP	1,4-di-{2-(5-phenyloxazolyl)}-benzene
PPO	2,5-diphenyloxazole
SHBG	sex hormone binding globulin
HEPES	N-2-hydroxy-piperazine-N'-2-ethane sulphonic acid
LH	luteinizing hormone
FSH	follicle stimulating hormone

NAMING OF STEROID COMPOUNDS

Listed below are the systematic names of some compounds which will be referred to in the text by their trivial names:-

TRIVIAL NAME	SYSTEMATIC NAME
aldosterone	18,11 hemiacetal of 11β, 21-dihydroxy-3,20-dioxo-4 pregnen-18-al
androstenedione	4-androstene-3,17-dione
cholesterol	cholest-5-en-3β-ol
hexoestrol	meso-3,4-bis(p-hydroxyphenyl)-n-hexane
hydrocortisone	11β, 17α, 21-trihydroxy-4-pregnene-3,20-dione
oestradiol-17α	oestra-1,3,5(10)-triene-3,17α-diol
oestradiol-17β	oestra-1,3,5(10)-triene-3,17β-diol
oestriol	oestra-1,3,5(10)-triene-3,16α, 17β-triol
oestrone	3-hydroxy-oestra-1,3,5(10)-triene-17-one
pregnenolone	3β-hydroxypregn-5-en-20-one
progesterone	pregn-4-ene-3,20-dione
testosterone	17β-hydroxy-4-androsten-3-one

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SUMMARY

The evaluation of the level of oestrogen receptor protein in the soluble fraction of mammary tumour tissue has been shown to provide an index of response by breast cancer patients to hormonally-based therapeutic regimes. This method, although giving a better indication than physical or clinical features, is not completely reliable, since some tumours do not respond favourably to endocrine manipulation despite containing receptor. It has been of interest, thus, to expand the study of receptor availability as an index of hormonal stimulation by examining the integrity of the hormonal mechanism of action as a whole.

In the present study, nuclear as well as cytoplasmic receptor levels have been considered on the basis that entry of receptor into the nucleus and subsequent binding to acceptor sites on chromatin are essential pre-requisites of long-term hormonal stimulation. The presence of receptor within the nucleus should thus indicate that the cell's translocation mechanism had survived malignant transformation, and give further evidence of a hormonal component in tissue growth. A defect in translocation would be a major point of blockage in the endocrine system, although further steps may also be sensitive to damage.

Using this approach, oestrogen receptor levels have been measured in 1000 mammary tumour biopsies representing both primary and advanced lesions from women of all ages. Using the criterion that only samples containing receptor at both cellular levels are truly hormone-dependent, 33% of tumours were identified as potentially responsive to hormone therapy.

Two abnormal situations were discovered where receptor was present in one cellular fraction only. These represented only a small percentage of cases, and appeared to indicate tumour autonomy. Good, but not perfect, continuity was noted in receptor status estimated in primary and secondary or early and late secondary biopsies from the same patient.

Receptor content was examined in relation to several variables. Absolute level of both cytosol and nuclear receptor was seen to increase with patient age, but this effect seemed to be menopausally - rather than age - related. Menopause also appeared to influence slightly the distribution of receptors within the cell. Most dramatically, the situation where receptors were present in the nucleus alone was detected in only postmenopausal tissue. No correlation between tissue receptor status and nodal involvement, histological grade, lactalbumin content, plasma steroid levels or plasma receptor content was noted.

Follow-up data were collected concerning a number of patients who had received hormonal manipulation as the sole form of treatment at some time subsequent to assessment of receptor status in a tumour biopsy. In 70% of cases where hormonal-dependence was indicated by receptor presence in both soluble and pellet fractions, the patient experienced benefit for at least six months. Only 10% of those patients whose biopsies had contained no detectable receptor responded to any form of hormone therapy, and this was presumably by some indirect pathway. In cases where receptor presence was limited to one fraction, only a small percentage responded. Overall, in 85% of cases the response to hormonal therapy was correctly predicted from receptor status of the biopsy.

Further follow-up data revealed that the presence of receptor at both cellular levels indicated a much improved prognosis over complete absence. The disease free interval in cases where receptor had been detected in only one fraction was similar for either fraction and tended towards the pattern displayed by receptor-negative tumours. A comparison was made between the influence of receptor and nodal status on prognosis. Receptor-positive lesions with no nodal infiltration indicated the best prognosis, receptor-negative biopsies displaying nodal involvement giving the worst. In cases of no receptor, but some nodal involvement, or no nodal infiltration, but detectable receptor levels, very similar prognosis was noted initially, with nodal status becoming predominant with time.

In the course of designing this assay system, the instability at low temperatures of the hormone/receptor complex from both fractions became apparent. Dissociation of the hormone was accelerated at higher temperatures, but was appreciable at $4^{\circ}C$ over a period of 18 hours. This shows that exchange of added for endogenous steroid can occur at low as well as elevated temperatures, and that this assay system measures both filled and unfilled sites. Thus, it is not possible to distinguish between filled and empty sites by this method.

The abnormality of receptors found only in the soluble fraction has been investigated. Preliminary results showed that these receptors could not be induced to translocate into nuclei of a hormone-responsive tissue. This suggests that the defect lies with the receptor rather than cellular functioning in these cases.

Receptors were studied in other human tissue types; notably colonic carcinoma.

Specific binding was not detected, despite the fact that tentative evidence of hormonal response does exist.

An in vitro approach to the study of hormonal dependence was attempted. The isolation procedures and subsequent growth requirements for epithelial cells from mammary tumour biopsies were studied. The most successful method involved the use of a confluent monolayer of non-dividing cells (feeder layer) onto which collagenase-digested tissue was innoculated. In the majority of cases, this enhanced the development of epithelial colonies, while inhibiting stromal cell attachment and growth.

Preliminary studies were carried out on the effects of hormones using this system. Initial results suggested that growth on a feeder layer might be a marker of malignancy. However, subsequent findings that "normal" mammary epithelial colonies developed on feeder layer challenged the validity of this marker, and emphasised the requirement for further methods of characterizing cells cultured in this manner.

PART I

The Interaction of Steroid Hormones with Normal and Neoplastic Tissue

1. INTRODUCTION

1.1 CONTROL MECHANISMS

The elucidation of the control mechanisms which regulate the human body has proved much more difficult than the study of those found in a unicellular bacterium. This is due mainly to the fact that the more complex system is controlled by a compilation of extracellular as well as intracellular interactions. Despite this fact, it has been possible to find two general control systems regulating the human body - the central nervous system and the endocrine system. In the case of the nervous system, effects on even the most distant parts of the body are achieved instantaneously by a series of electrical impulses emanating from the brain. The endocrine system acts more slowly, however, utilising, instead of electrical, chemical messages in the form of hormones which are synthesised in and released from one organ to act on another organ(s). It may be many hours before the external effects of hormonal stimulation are evident.

The organs which synthesise hormones are known as endocrine glands. From these, the hormone is released into the bloodstream and carried on a transport protein(s) to other parts of the body. Although a hormone may come into contact with a number of organs throughout the body, it will only affect specific ones which are known as target organs for that hormone. These are parts of the body which respond to a given hormone in some physiological manner, whether it be by synthesis of some special substance or by an overall growth and division of cells. Target organs may be

sensitive to just one hormone or a combination of many and, in the same way, one hormone may have one target organ or a selection of them throughout the body.

Although it was in 1849 that Berthold produced the first demonstration of the ability of one gland to effect the functioning of the whole body (Malkinson, 1975), it was not until 1905 that the term "hormone" was first introduced by Starling. He used the word in reference to secretin, the existence of which he had demonstrated three years previously (Bayliss & Starling, 1902). The word "hormone" he derived from a Greek word meaning "I excite" or "arouse". At the time of its introduction, there was much controversy over the exact literal meaning of the Greek word, but, although attempts were made to introduce a different term, the use of "hormone" was adopted (Wright, 1978). In 1914, Starling further defined a hormone as "any substance normally produced in the cells of some part of the body, and carried by the bloodstream to distant parts, which it affects for the good of the organism as a whole". This definition has been challenged by Huxley in 1935 and more recently by Robison et al.,(1971) as being too generalised, but can still be used to describe hormones as a whole.

Since the pioneering work with hormones by Berthold, much investigation has been carried out into the exact location of the endocrine glands and the nature and effects of the hormones produced by each one. Different methods of classifying hormones have emerged from these studies, using the type of response, structure or mechanism of action as the criteria for classification. Robison et al.,(1971) considered the type of response evoked by the hormone, and found two classes - rapidly-acting ones, such as glucagon or luteinizing

hormone, and slow-acting onces, including insulin and all steroid hormones. Insulin can, of course, also invoke some rapid responses. If, instead, structure is taken into account, three classes emerge - peptides, steroids and amino acid derivatives. The last type of classification, by mechanism of action, yields two groups. One group, containing steroid and thyroid hormones, exerts its actions by actually entering the target cell. The other group, including peptide hormones and catecholamines, was initially thought to act exclusively via a second messenger within the cell, the hormones never actually entering the cells themselves. However, there is now evidence that some of these hormones do enter the cell at a later stage, perhaps along with their cell surface receptors, but the reason for their entry is not yet understood (Kolata, 1978) although it may simply be to give controlled degradation of the hormone.

It is clear that there exists a wide variety of properties which must be considered when undertaking a study of hormones. Since the subject of this thesis relates directly to steroid hormones, the different aspects of these will be considered in more detail.

1.2 STEROID HORMONES

1.2.1 STRUCTURE AND NOMENCLATURE

1.2.1.1 General Structure

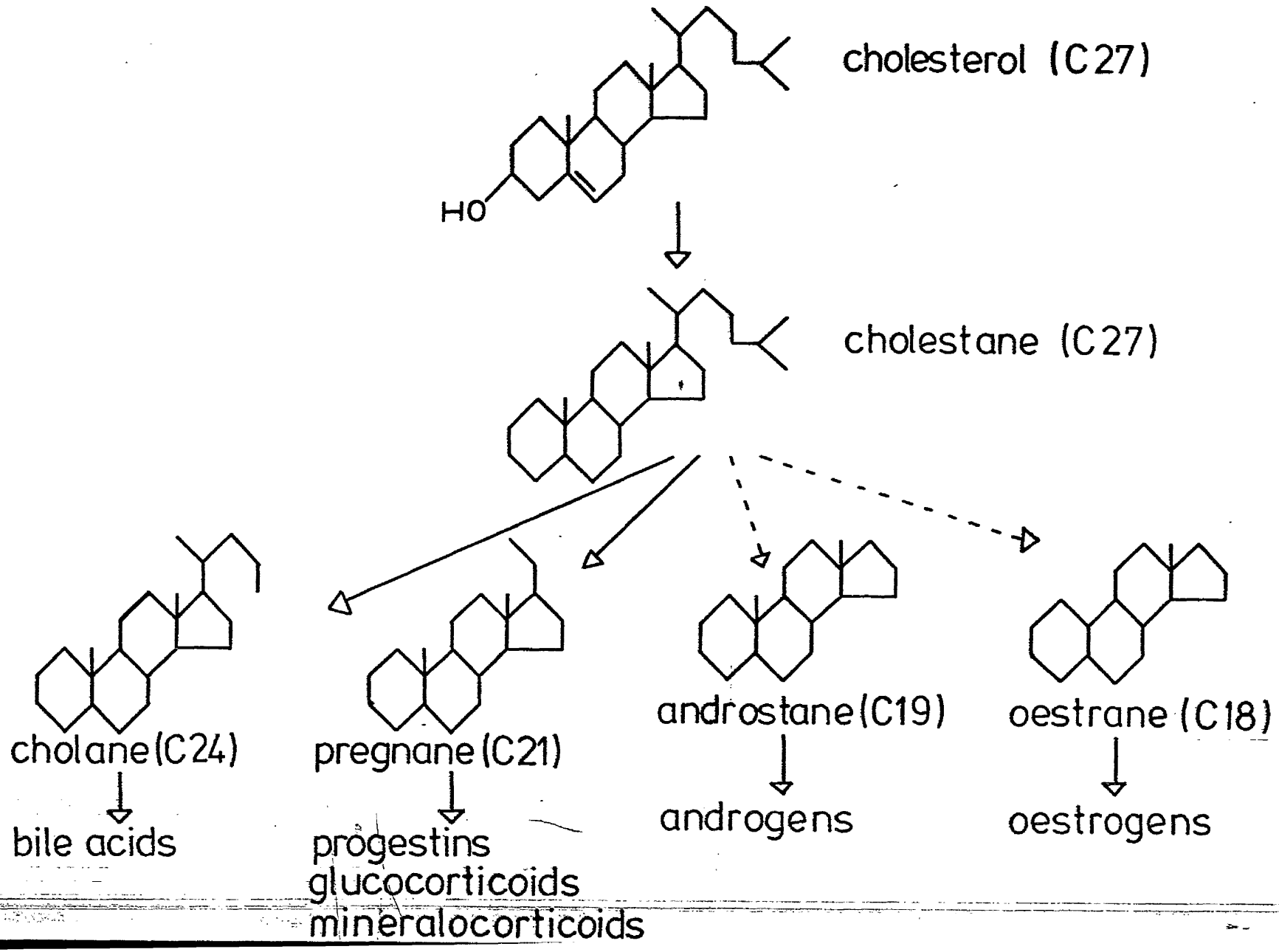
In contrast to peptide hormones, whose structures include a wide range of numbers of amino acids (from three in the case of thyrotropin releasing hormone to almost two hundred in the case of growth hormone and even to dimers such as LH and FSH, which contain in excess of two hundred amino

acids), and thus vary tremendously in size, steroid hormones are derived all from a single parent compound, cholesterol. Cholesterol is synthesised mainly in the liver and intestine, which together account for about 60% of the daily yield. It is synthesised also in glands which produce steroid hormones, such as the adrenal cortex, ovaries and testis. Other tissues, such as brain,do contribute to the body's pool of cholesterol, but only to a very limited extent (Ramsey & Nicholas, 1972). Some cholesterol is taken into the body in the diet, and the overall level at any one time is a function of the level of absorption in the diet, the amount of de novo synthesis and the excretion of either cholesterol itself or any one of its metabolic products. In order to be converted to steroids, cholesterol is modified in a series of dehydrogenation reactions to give hormones of different carbon atom content. The skeletons formed contain 21 carbon atoms (progestins, glucocorticoids, mineralocorticoids), 19 carbon atoms (androgens) or 18 carbon atoms (oestrogens) {see Figure 1}. All have a common ring structure of three six-membered rings and one five-membered ring. In addition, there may be a number of different substituents at different positions on this skeleton. Steroids found in nature may have such substituents as double bonds, hydroxyl groups or ketone groups, either alone or in combination. The aldehyde group is another possible substituent, but this is not so common, the only notable example being that of aldosterone. An unsaturated A ring is common in biologically active steroids.

1.2.1.2 Nomenclature

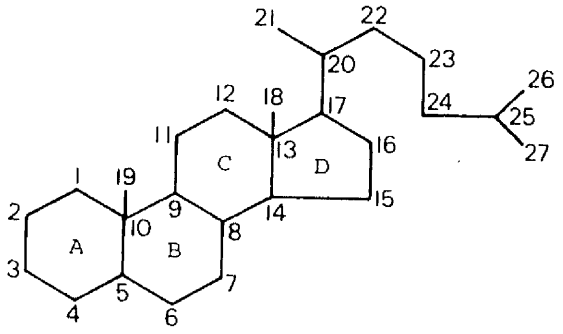
Early studies to isolate steroid hormones from different tissues of the body and metabolites from urine proceeded from the early 1930's onwards in a number of different laboratories. As a consequence of this, each

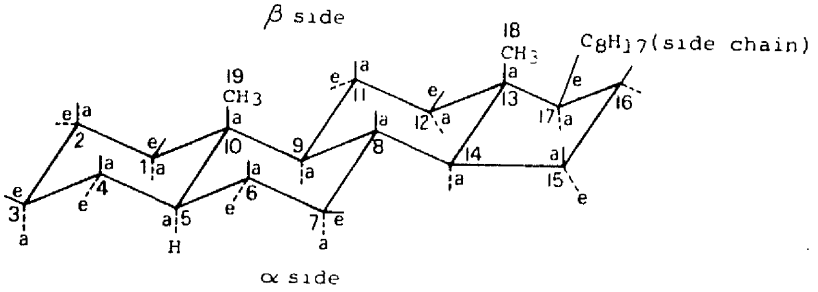
Figure 1 - Formation of Steroid Skeletons from Cholesterol


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

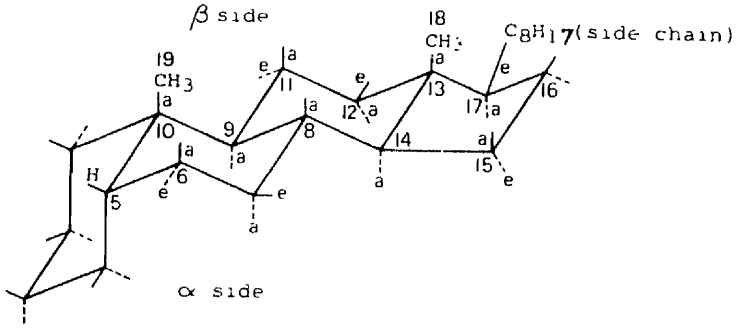
compound isolated became known by merely a trivial name. This proved quite acceptable as long as the number of steroids identified was limited. However, it became clear that not only was there a large number of steroid hormones, but also that they each had a number of metabolic products which must be named. A system for nomenclature of steroids was required, therefore, to avoid confusion. The rules for such a system were first discussed and published in 1950 and 1951. Since then, these rules have been revised, and nomenclature is now governed by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) in their "Revised Tentative Rules for Steroid Nomenclature" (IUPAC-IUB, 1969), and amendments (IUPAC-IUB, 1971).

A summary of the system for numbering carbon atoms and lettering rings is shown in Figure 2. The basic skeleton, with 17 carbon atoms and no substitutions, is known as gonane. When the gonane molecule has a methyl group substituted at carbon 13, it is then known as oestrane. For all substitutions, groups which lie below the plane of the paper are designated α, while groups lying above the plane of the paper are known as β. When there are substitutions at C20, groups lying to the left of the carbon atom are α, and groups to the right β. The nature of the substituent on the molecule is denoted by either a prefix or a suffix, which is particular for that specific type of substitution. For example, alcohols are denoted by the prefix "hydroxy-" or the suffix "-ol". When the prefix "nor-" is used, followed by a number, this means that a methyl group has been eliminated from the usual formula of that compound, the number indicating which carbon atom has been lost.





5α- cholestane (trans A:B ring junction)



5β- cholestane (cis A:B ring junction)

Some steroids are still commonly known by the trivial names they were given when they were first isolated. A list of the trivial names which are referred to in this thesis along with their correct systematic names can be found on page iv.

1.2.2 MECHANISM OF ACTION

Steroid hormones are synthesised by various different endocrine glands throughout the body and excreted into the bloodstream in which they are transported by protein molecules, such as sex steroid binding globulin and albumin, which bind the steroids with varying degrees of affinity (Daughaday & Mariz,1960, Westphal, 1970). Uptake of hormone into cells, however, is dependent on the concentration of free hormone. The equilibrium between free and carrier-bound hormone is always strongly in favour of the bound form.

Entry into cells most likely occurs by passive diffusion, although a facilitated entry process has been postulated (Milgrom et al.,1973, Gorski & Gannon, 1976). If the cell into which the steroid passes is not part of a target tissue for that particular hormone, it will quickly pass out again (Jensen & Jacobson, 1960). If, on the other hand, the cell penetrated by the hormone is in a target tissue, its exit is delayed by the presence of receptor molecules to which it is tightly and specifically bound. The possession of these receptors is one of the definitive properties of a target tissue (Folca et al., 1961, Clark & Gorski 1969, Jensen & De Sombre, 1972, Higgins et al., 1973). Receptors are found experimentally in the cell cytoplasm, where they may occur

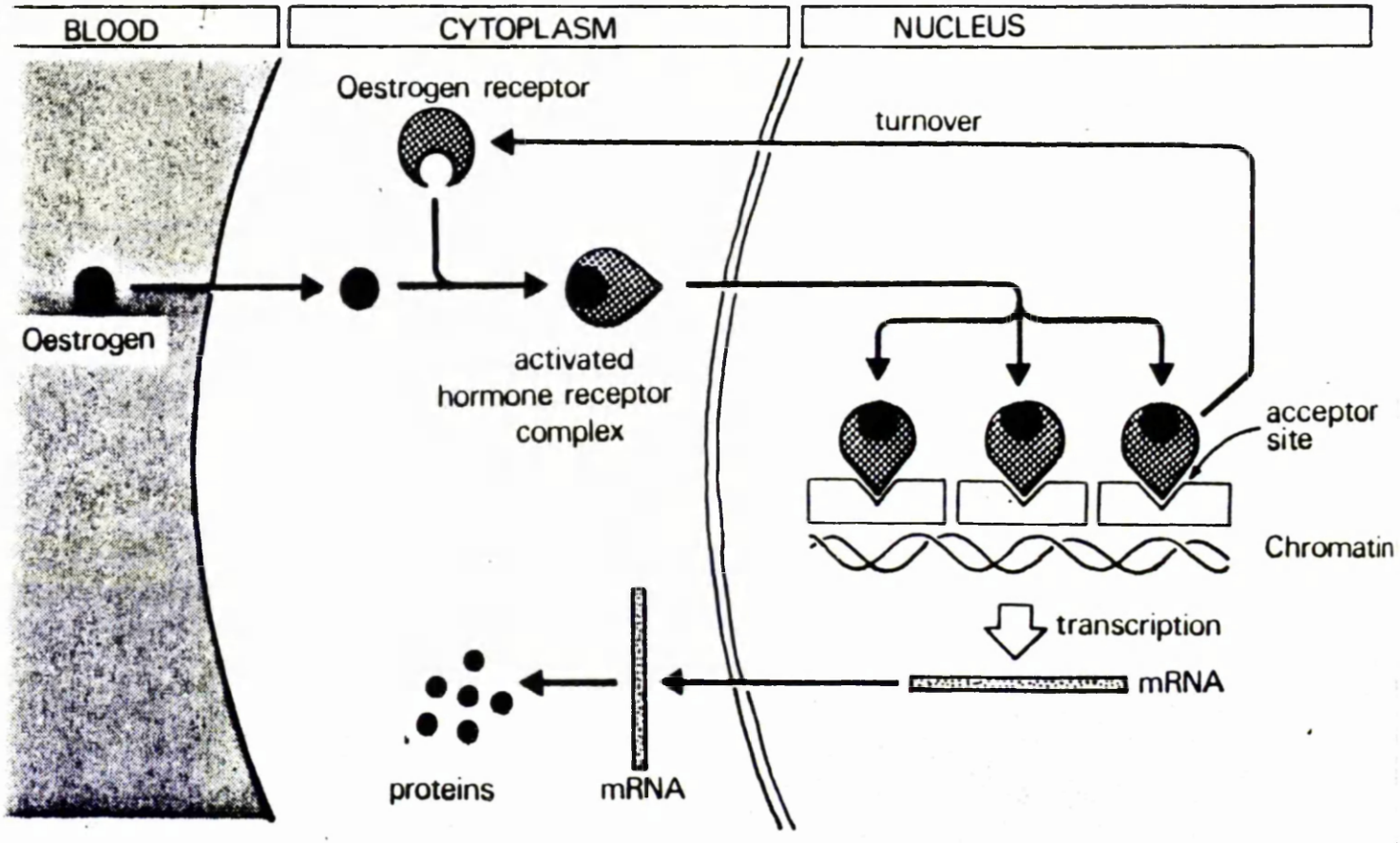
free or loosely bound to the cytoplasmic membrane (Wittliff, 1975). Their synthesis has been shown to be promoted by the presence of high levels of circulating hormone (Sarff & Gorski, 1971, Clark et al., 1977).

Most work on the action of steroid hormones has been carried out using, as a model, the interaction of oestradiol-17β with the immature rat uterus (Jensen et al., 1974). A scheme for the mechanism of action of steroid hormones in general with target tissue cells was worked out using this system and was published independently in 1968 by Jensen et al. and Gorski et al., a preliminary report having been made by Shyamala & Gorski the previous year. It is a two step mechanism, as summarised in Figure 3.

The hormone enters the cell and is bound to a receptor molecule. The receptor has been shown to be proteinaceous in nature by its sensitivity to proteolytic enzymes, but not nucleases (Toft et al., 1967). After binding the hormone, the receptor is modified in some fashion during a step referred to as "activation". This is thought to involve the addition of a further polypeptide subunit of molecular weight of the order of 50,000 (Notides & Nielsen, 1974, Leake, 1976). The heat-dependent nature of the "activation" step has been demonstrated by the fact that warming the system causes the conversion even in the absence of nuclei (Jensen et al., 1974). The actual site of this transformation is unknown, but a matter of current debate (Linkie & Siiteri, 1978, Sheridan et al., 1979).

Once activation has occurred the receptor/hormone complex is free to bind the specific "acceptor" sites on the chromatin. The specificity

Figure 3 - Mechanism of Action of Steroid Hormones


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

of these sites is thought to be due to their 3D conformation as, if chromatin is removed from its natural environment, the destruction of configuration causes loss of this property.

After the receptor/hormone complex has become bound to the acceptor sites, the various effects of the hormone's presence are seen. These effects vary according to the particular hormone, but generally involve first of all transcriptional activation, leading to an increased synthesis of RNA (Spelsberg, 1976), specifically mRNA, which is then transported into the cytoplasm, where protein synthesis occurs on cytoplasmic ribosomes (O'Malley & Means, 1974). Not all mRNA species may be synthesised at the same time, however, as was demonstrated by Palmiter et al., (1976) in chick oviduct, suggesting some intermediate step between binding to chromatin and transcriptional activity. The type of protein synthesised varies from cell to cell depending on the kind of response elicited. These events occur quite quickly after stimulation by the steroid. Cellular growth and division is seen much later. The specific actions of oestrogens on target tissues will be discussed in detail in a later section.

Once the hormone/receptor complex has been bound to the acceptor sites for a specific length of time, it is then released. The hormone is released in the same form as it had when it entered the cell, not being metabolised in any way during this process (Puca & Bresciani, 1968). The receptor appears to be recycled in some fashion, and total receptor content of the cytoplasm after oestrogen stimulation is due to a mixture of recycling and de novo synthesis (Neithercut, 1977).

Although this mode of action was elucidated specifically for the action of oestradiol-17β, it has been shown to hold true for many other steroid hormones and also for 1,25-dihydroxy vitamin D_3 (Gorski & Gannon, 1976). In the case of androgens, testosterone is metabolised within the target cell before binding to the receptor.

1.2.3 OESTROGENS

1.2.3.1 Synthesis

In Man, oestrogens may arise from a number of different sources. In the case of the premenopausal female, the main source is the ovaries, with the ovulating one contributing more than the inactive one during each cycle. The adrenal cortex also produces a small amount of oestrone, but this is not really a significant percentage of the circulating level. Another major source, predominantly for postmenopausal females is the conversion of androgens to oestrogens, (England et al., 1974) the main pathway being the transformation of androstenedione to oestrone (Gower & Fotherby, 1975). There is also a pathway for the conversion of testosterone to oestradiol, which leads to approximately 50% of the male oestrogen production. Another contribution in the male comes from the testes where a direct secretion of oestrogens has been demonstrated (Longcope et al., 1972). In the pregnant female, an additional source is the foetoplacental unit, which produces large amounts of oestrogens, predominantly oestriol (De Hertogh & Thomas, 1975).

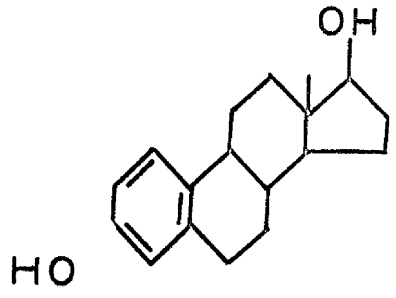
The pathway for synthesis of oestrogens, where no conversion of androgens is involved, is that shown in Figure 1. Oestrogens are all 18-carbon atom

molecules with substituents at various positions in the ring structures. The three main natural oestrogens are shown in Figure 4, along with the very potent synthetic oestrogen diethylstilboestrol,which has wide medical applications. All the oestrogens have a conjugated A ring, and the overall structure is very important for binding to receptor molecules within the cell,as will be described later.

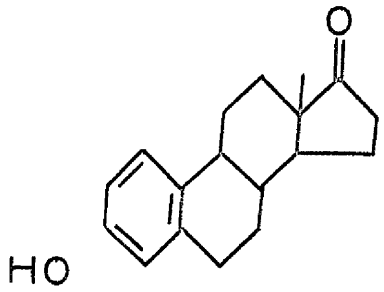
1.2.3.2 Action of Oestrogens

All studies on the action of oestrogens and the discovery of the identities of their target tissues have been greatly facilitated by the synthesis of a tritium-labelled form. Prior to this, carbon-14-labelled oestrogens were the only available tools and, to give a measureable activity at the end of the experiment, it was necessary to administer doses of hormone well in excess of the physiological level. Tritiated-hexoestrol was the first such oestrogen to be developed, and this was used in sheep, goats and humans to trace the relevant target organs (Glascock & Hoekstra, 1959, Folca et al., 1961). Jensen & Jacobson (1960) then synthesised tritiated-oestradiol, using either carrier-free tritium gas or 10% tritium in hydrogen. They used this form in studies on the fate of oestrogens in various different tissues of the body, in this way identifying the uterus, vagina and anterior pituitary as target tissues. Other target organs later identified using tritium-labelled-oestradiol, were the hypothalamus (Eisenfeld & Axelrod, 1966) and the mammary gland (Sander, 1968).

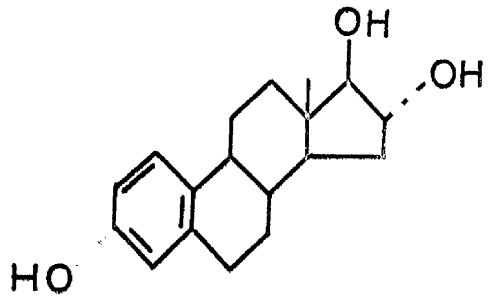
The mechanism of action of oestrogens has been accepted generally as that detailed earlier and summarised in Figure 3, although doubts are now being



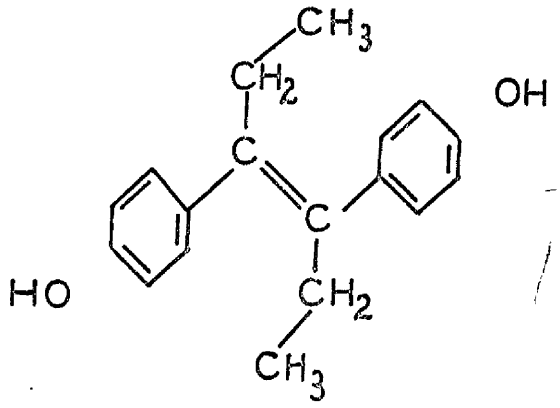
17-β oestradiol



oestrone



oestriol



diethylstilboestrol

cast upon its accuracy. The sequence of events following the binding of the hormone/receptor complex to the acceptor sites on the chromatin has been investigated extensively, and a clear timetable of steroidal stimulation has emerged similar to that summarised by Spelsberg (1974) for progesterone action on the chick oviduct.

The first indication of oestrogenic stimulation in the rat uterus is a rise in RNA polymerase B (or II) activity 15 minutes after hormone administration, followed 15-45 minutes later by a similar rise in RNA polymerase A (or I) activity Glasser et al., 1972). The rise in RNA polymerase A activity has been demonstrated to be dependent on the presence of RNA polymerase B by Borthwick & Smellie (1975) in studies using α-amanitin, a specific inhibitor of the latter enzyme. Cycloheximide was effective in the inhibition of increased RNA polymerase A activity also, indicating a requirement of some protein synthesis, presumably as a consequence of the RNA polymerase B activity.

Other "early" events following oestrogen administration are a rise in the levels of specific mRNAs, phospholipid and glycogen after 1-2 hours and then later in total RNA, DNA and protein. The effect of the hormone has been reported to be an increase in the number of initiation sites available for transcription on the chromatin (Tsai et al., 1975). The RNA synthesis is not a general effect, with mRNA and tRNA being synthesised earlier than rRNA (Billing et al., 1969a, 1969b, Jensen & De Sombre, 1972). Noteboom & Gorski (1963) and Gorski (1964) found that puromycin inhibited the rise in total RNA levels and concluded that protein synthesis must be a prerequisite for this effect. This was suggested also by Ui & Mueller (1963).

However, these findings should be treated with caution, as side effects of inhibitors could be misleading in these circumstances. Conclusions reached by Hamilton (1968) about the specificity of RNA produced in the cell should also be approached cautiously. He extracted RNA from an oestradiol-stimulated organ and found that it caused growth in a non-stimulated uterus, but the RNA extracted may have been contaminated with oestradiol. The variation in the length of time required for the stimulation of RNA synthesis is reflected in the timetable of the appearance of different proteins (Palmiter et al., 1976). Some mRNA is stimulated as early as 15-30 minutes after steroid administration and protein synthesis is detected at times from 30 minutes up to 48 hours.

In the rat uterus, oestradiol was found to give another "early" effect. This was the synthesis of a protein known as oestrogen specific uterine protein or induced protein (I.P.), first noted by Notides & Gorski (1966). It was found to be produced 30-40 minutes after oestradiol stimulation (Jensen & De Sombre, 1972), having been totally undetectable before steroid administration (Barnea & Gorski, 1970) within the sensitivity of the assay conditions described. I.P. synthesis is blocked by actinomycin D, but not by protein synthesis inhibitors (De Angelo & Gorski, 1970), showing that its synthesis must be one of the earliest events triggered off by oestrogen stimulation. Characterization has shown it to be an acidic polypeptide (Mayol & Thayer, 1970), with a molecular weight of approximately 45,000 (Iacobelli,et al.,1978).I.P. has also been discovered in vitro (Katzenellenbogen & Gorski, 1972), showing that an intact vascular or nervous system is not necessary for oestrogenic stimulation of uterine cells. Recent evidence proves that I.P. has enolase activity (Kaye et al., 1979, Kaye, 1980).

"Late" responses to oestrogen administration include a sustained high activity of RNA polymerase A, and a second rise in RNA polymerase B activity about four hours after the first response. This new peak is then sustained. Other effects are a general growth and division of cells. Clark & Peck (1976) reported that the hormone/receptor complex must remain in the nucleus for at least 4-6 hours in order to elicit these "late" responses and hence true uterine growth, as a shorter period than this represents failure to fully activate the specific acceptor sites on chromatin.

Oestradiol-17β is the active oestrogen in the human female, and can fulfil the requirement to retain the presence of the receptor in the nucleus. The other natural oestrogens in the body do not do this in the normal course of events. Oestrone was reported as being retained in neither the uterus nor vagina, and any activity it had was attributed to a conversion to oestradiol-17β (Williams-Ashman & Reddi, 1971). However, Ruh et al., (1973) reported that oestrone itself can cause I.P. synthesis, but much higher levels are required than for oestradiol, and some conversion to oestradiol does occur (about 20%). This is probably due to the lower affinity of the oestrogen receptor for oestrone, as determined by Lövgren et al., (1978), which may be responsible also for the actions of oestriol. Oestriol was, at one time, thought to be an inhibitor of oestradiol-17β action in the rat uterus (Brecher & Wotiz, 1967). The fact that it is a pregnancy hormone was thought to account for its different actions (Hisaw, 1959). It was found that it did not act like oestradiol in stimulating later events such as the second rise in RNA polymerase B activity after 4 hours (Hardin et al., 1976). However, Clark et al., (1977)

demonstrated by the use of implanted oestriol pellets, which would keep plasma levels high (as found during pregnancy), that oestriol is a potent oestrogen under these conditions, though not if injected as a single dose. These studies, along with some carried out using derivatives of oestriol (Lan & Katzenellenbogen, 1976), confirmed the idea that retention of the hormone/receptor complex in the nucleus is important for long term responses to the hormone.

Before the discovery that oestriol was not an oestrogen antagonist in high levels, it was thought that it could counteract oestradiol, thus preventing the growth of some breast tumours. This view was supported by the fact that women of oriental origin, who have a low incidence of breast cancer, had a high ratio of oestriol to oestradiol plus oestrone (the oestriol ratio). This ratio was low in Caucasian women, who exhibit a high incidence of the disease (Dickinson et al., 1974). It was then realised that this ratio may change throughout life, and, thus, would have to be measured early in life to predict the likelihood of developing breast cancer (Henderson et al., 1975). Pike et al., (1977) found daughters of breast cancer patients had high levels of oestriol, although they were at high risk of developing the condition. In addition, the overall correlation between oestriol ratio values and the incidence of breast cancer was found to be very poor (Cole et al., 1978). Combined with the discovery of the true nature of oestriol, this led to the abolition of the use of this discriminant in determining the likelihood of developing breast cancer.

1.2.4 OESTROGEN RECEPTORS

1.2.4.1 Properties

Oestrogen receptors are proteins of molecular weight around 80,000 found in oestrogen target tissues. Their protein nature has been demonstrated using purification by chromatography (King, 1968) and by their sensitivity to pronase but not nuclease (Toft et al., 1967, Korenman, 1968). They have a pH optimum of 7, and binding is destroyed by a pH of 6 and below or 9 and above (Mester et al., 1970). They are also heat-labile (McGuire & De La Garza, 1973b), prolonged exposure to temperatures above 30°C causing reduction in, and eventually abolishment of binding. It has also been shown that sulphydryl groups play an important part in the interaction between the receptor and the oestrogen (Jensen et al., 1967, Muldoon, 1971), even if only indirectly by ensuring the maintenance of the correct conformation of the receptor. Since the receptors are proteinaceous, the usual precautions must be taken in handling. Storage, especially, must be carefully controlled, as freezing and thawing destroys the receptor conformation (King et al., 1978). However, lyophilization has been shown to be a successful form of storage for receptors from many different types of tissue (Koenders et al.,1978). The amount of oestrogen receptor present in the cell has been found to depend on the level of oestrogens in the circulation (Sarff & Gorski, 1971) and also on prolactin concentration (Asselin & Labrie, 1978). The role of oestrogens in controlling their own receptors is demonstrated by the variation in uterine receptor levels during the rat oestrous cycle, although reports on the time of peak cytoplasmic receptor content vary. Feherty et al., (1970) found that levels were highest at pro-oestrous,

which was contradicted by the finding of Lee & Jacobson (1971) that the level was lowest on this day. Kielhorn & Hughes (1977) reported that the peak was found at di-oestrous, and levels were declining by pro-oestrous. White et al., (1978), in contrast to all of these reports could find no variation at all throughout the cycle. The latest report, by Fishman & Fishman (1979), places the peak at pro-oestrous and oestrous. It is clear that there is a variation, but the exact peak of receptor levels has not yet been agreed. A variation in receptor levels found in human endometrium during the menstrual cycle has been detected (Soutter et al., 1979), with a peak at day 9.

1.2.4.2 Cytoplasmic Binding

Two distinct types of binding entity have been reported in the cytoplasmic fraction of the cell (McGuire & Julian, 1971). These display high and low affinity binding. Low affinity sites have a dissociation constant of the order of 10^{-5}M, while high affinity sites display a value of 10^{-10}M - the usual circulating level of oestradiol in the bloodstream. It is the high affinity receptor which mediates the cellular activity of oestradiol (King, 1975, Feherty et al., 1971). Low affinity sites may represent contamination of the cytoplasmic preparation with plasma binding proteins. More recently, there have been reports of two different binding sites in the cytosol which have much closer affinities (Eriksson, 1978, Smith et al., 1979), the dissociation constants differing by only one power of ten. Another source of heterogeneity of the cytosol receptor has been reported by Fishman & Fishman (1979), who find that only some of these receptors will bind the antioestrogen tamoxifen, the remainder being insensitive to the drug. This again suggests two separate populations of

receptor within the cytoplasm.

The size of the cytoplasmic receptor has been investigated in sucrose density centrifugation studies, using bovine serum albumin as a marker. First reports stated that the receptor had a sedimentation coefficient of 9.5S (Toft & Gorski, 1966, Jensen et al., 1967, Jensen et al., 1968). Later reports indicated a value of 9S (Clark & Gorski, 1969, Shyamala & Gorski, 1969, Steggles & King, 1969), but the finally agreed value was 8-9S (McGuire & Julian, 1971, Shyamala & Nandi, 1972, Jensen et al., 1974). There were also reports of a 4S receptor (Steggles & King, 1969, McGuire & De La Garza, 1973a). This was found when using a high salt environment (0.4M KCl), but Stancel et al., (1973) recognised that this was not the true cellular environment and so the real form of the receptor in vivo could not be determined using this system. There are also suggestions that the 4S form is, in fact, alpha-fetoprotein - a major high affinity oestrogen binding protein in rat. The levels of this protein decline as the rat matures, but it is still evident at 20 days (Labarbera & Linkie, 1978). It is thought to combine with albumin to give the 4S binding protein and thus the 4S species measured at low ionic strength may be due completely to serum contamination of alpha-fetoprotein (Uriel et al.,1976). Muldoon (1978) has proposed that there are, in fact, two forms of the cytoplasmic receptor - 4S and 8S, the 8S form being important to conserve oestrogenic action when oestrogen levels fall, and so being necessary to ensure tissue responsiveness. A 5S form of receptor was also isolated by Muldoon (1971), and this was presumed to represent the 4S receptor in its activated form, with the additional polypeptide added at the time of transformation (Notides & Nielsen, 1974, Spelsberg, 1974) prior to or

concurrent with translocation. Linkie & Siiteri (1978) propose that the conversion of 4S to 5S receptor occurs within the nucleus. Translocation from cytoplasmic to nuclear compartments may not, in fact, be a real event, and pretransformed receptor is probably evenly distributed throughout the soluble fraction of the cell.

The oestrogen receptor displays great specificity for oestrogenic compounds. It will not interact with non-oestrogenic compounds such as oestradiol-17α, but will bind some non-steroidal compounds, such as diethyl stilboestrol or hexoestrol, if they have oestrogenic or anti-oestrogenic properties (Puca & Bresciani, 1968). This has been demonstrated by the fact that only oestrogenic and anti-oestrogenic compounds will interfere with the binding to receptor of oestradiol-17β (Puca & Bresciani, 1969). Of the natural oestrogens, oestriol binds to approximately one-fifth of the level of oestradiol-17β, while oestrone binds to around one quarter. The synthetic oestrogen diethylstilboestrol, however, binds with double the affinity of oestradiol-17β (Korenman, 1968).

Investigations into the specificity of the receptor have yielded some information on the type of configuration necessary for binding to occur. This, in turn, has led to the discovery of how the receptor binds the hormone. Hähnel and Twaddle (1974) examined the binding of a number of different steroids to the receptor. They concluded that, for binding to take place, the steroid must have an aromatic A ring, a phenolic hydroxyl group at carbon atom 3 and an oxygen function in ring D (see Figure2for nomenclature). This was confirmed by the findings of Poortman et al., (1975). The actual process by which binding of oestradiol-17β to the receptor takes place was then elucidated by

Powell-Jones et al., (1975) as an initial attachment of the phenolic hydroxyl group at carbon atom 3 to a highly specific site which facilitates attraction of a β-hydroxyl group at carbon atom 17 to a less specific site.

1.2.4.3 Nuclear Binding

The accepted mechanism of action of steroid hormones of Jensen et al.,(1968) and Gorski et al., (1968) states that once the oestrogen has become bound to the receptor, the complex as a whole travels into the nucleus in a "translocation" step. It is not yet clear how the complex gains entry to the nucleus, or indeed why (or whether) the receptor should stay in the cytoplasm until bound to a steroid. Gorski and Gannon (1976) suggested that this may indicate that the receptor is bound to something in the cytoplasm from which it is released upon binding a hormone. Another explanation is that it may be too big to enter the nucleus on its own and the hormone somehow changes its shape to allow it entry. King et al., (1976) reported that receptors may be active on their own, but require the binding of steroid to increase their efficiency. This could be by providing a key to the nucleus. More recently, there have been several reports of unoccupied receptors occurring in the nuclei of human breast tumour cells, where they are thought to be inactive, although this cannot be stated with certainty (Garola & McGuire, 1977a, Panko & MacLeod, 1978, Kato et al., 1978, Thorsen, 1979). Unoccupied nuclear oestrogen receptors have also been identified in a cell line (MCF 7) derived from a pleural effusion of a breast cancer patient (Zava et al., 1977, Horwitz & McGuire, 1978). These receptors, even though uncharged, were thought to be stimulating cellular growth in some fashion (Zava et al., 1977). Clearly, this is an area of great significance, which is not yet fully understood, and is involved in

the question of whether the hormone acts to carry the receptor to the nucleus or vice versa.

Early experiments on nuclear binding of oestrogens seemed to indicate that the hormone was bound directly to a chromatin protein (Maurer & Chalkley, 1967). However, a separate receptor was then recognised and identified as the cytoplasmic receptor (Gorski et al., 1968). More recently, Greene et al., (1977) have purified receptor and prepared antibodies to it. Using these, they have been able to confirm the similarity between the cytoplasmic and nuclear forms of the receptor in the same cells as well as compare receptors from different species.

The idea of the cytoplasmic receptor being modified to allow entry into the nucleus evolved with the models of steroid hormone action of 1968. When the disappearance of cytoplasmic receptor was found to correlate with the appearance of oestradiol in the nucleus (Shyamala & Gorski, 1969), this destroyed any ideas of the hormone binding directly to nuclear DNA or protein. Translocation of the hormone/receptor complex from the cytoplasm to the nucleus was found to occur rapidly after oestradiol administration (Williams & Gorski, 1971, Anderson et al., 1974). The level of nuclear receptors can be maintained at a maximum for 24 hours by a second administration of hormone, if given within 3 hours after the first dose, but otherwise the level will decline (Anderson et al., 1974). Temperature has been reported to be important, both in causing translocation to occur (Williams & Gorski, 1971) and in promoting binding of the hormone/receptor complex to the acceptor sites on chromatin (McGuire et al.,1972). Linkie & Siiteri (1978) reported that the type of receptor present in the

nucleus depended on temperature. If translocation occurred at 4°C, a 4S form was obtained, but at 37°C, the nuclear receptor was in the 5S form. Heterogeneity of nuclear sites was also proposed by Tseng & Gurpide (1978) while studying the interaction of the oestrogen oestetrol with human endometrium. They found that some sites bound this oestrogen more easily than others, and, further, suggested that the sites which bound it more slowly are the biologically active ones. Eriksson (1978) also found two types of binding within the nucleus, one which had translocated from the cytoplasm and another one, always present in the nucleus, which served to deal with the processing of the hormone/receptor complex upon translocation. Based on their own findings, Linkie & Siiteri proposed a revised mechanism of action of oestradiol in which the steroid enters the cell, binds to a 4S receptor, which is converted to 5S once inside the nucleus, and it is this complex which binds to acceptor sites within the nucleus.

The mechanism by which the hormone/receptor complex binds to the acceptor sites on the chromatin is not yet fully understood. It was suggested that the receptor may have a site for binding hormone and another which interacts with chromatin (Milgrom et al., 1973). The same group also proposed an enzymatic conversion of the receptor within the nucleus, which allowed it to bind to acceptor sites, but it is still not clear how binding is achieved, or the nature of that binding. The actual site of binding on the chromatin is not yet certain either, but it has been shown to involve the non-histone proteins, specifically fraction AP_3 (Spelsberg et al., 1972) in chick oviduct (O'Malley et al., 1972) and human mammary tissue (Charreau & Baldi, 1977). In fact, it probably involves both the DNA

and the non-histone proteins in active roles (King & Gordon, 1972). Spelsberg (1974) suggests two sites on the receptor, one to recognise DNA and one to bind acceptor. Yamamoto & Alberts (1975) found that, although the hormone/receptor complex does bind to DNA, the binding is non-specific. They also suggested that many specific sites could exist at different loci on the chromatin. The exact nature and specificity of these sites still remains a mystery (Yamamoto & Alberts, 1976). The study of acceptor sites *in vitro* is probably hindered by the inability to isolate chromatin while retaining its 3D structure (Higgins *et al.*, 1973). It may be that it is this conformation which holds the solution to the question of how the specificity of acceptor sites arises (Leake, 1976).

1.3 THE SIGNIFICANCE OF OESTROGEN RECEPTOR LEVELS IN BREAST CANCER

1.3.1 THE MAMMARY GLAND

The growth and differentiation of the normal mammary gland are controlled by a number of different hormones, both polypeptide and steroid in nature. The plasma level of each of these hormones is determined by the functioning of the hypothalamic-pituitary axis. The regulation of growth within the breast, then, depends not only on circulating levels of hormone, but also on the integrity of this system. In addition to this, the mammary gland has been shown to have the capacity to synthesise its own oestradiol (Adams & Li, 1975). The differentiation of the mammary gland is controlled by this hormonal milieu, the function of some of the individual hormones being unclear as yet (Wittliff *et al.*, 1978), whilst the roles of others have been elucidated (Topper, 1970). Insulin controls the formation of secretory cells, hydrocortisone the formation of secretory proteins and

prolactin causes RNA synthesis in epithelial cells. There is a synergistic effect of hormones, in that one hormone can be active in the synthesis or activity of another. Examples of such interplay are the stimulation of progesterone receptor levels (Horwitz et al.,1975a) and prolactin release (McGuire et al., 1976a) both by oestrogens. The overall functioning of the gland, for example lactogenesis (Denamur, 1971), is controlled by the combined effects of a number of different hormones. The role of sex steroids is clearly seen by the changes in morphology which are evident at puberty, during pregnancy and at the time of the menopause.

Despite this potential for sensitivity to oestrogens, the normal, non-lactating mammary gland contains very little measurable oestrogen receptor as the proportion of epithelial tissue is very small. It does have a very low level of receptors, since it has been reported to take up and retain ^{3}H-oestradiol, making it an oestrogen target organ (Puca & Bresciani, 1969, Block et al., 1975, Sander, 1968). The gland must, therefore, contain machinery to switch on the synthesis of oestrogen receptors when they are required. When a breast cell undergoes malignant transformation, two things can happen. Firstly, there may be a loss of the ability to form oestrogen receptor molecules, in which case the tissue is no longer recognised as target tissue for oestrogens, and endocrine control is no longer present (McGuire et al., 1977a). Secondly, the machinery for receptor synthesis may be "switched on" and amplified by the transformation, in which case the tissue will become very sensitive to endocrine control (Deshpande et al., 1967, Folca et al., 1961, Wittliff et al., 1972, Jensen, 1975). Oestrogen receptors have been found in some cases of benign breast lesions (Feherty et al., 1971), but

the levels are not as high as those found in malignant tissue, where excessive levels of synthesis have been activated (Sakai & Saez, 1976). The activation of receptor synthesis is not confined to female breast cancer, as binding has been detected also in male cases (Leclerq et al., 1975). It has been pointed out that one-third of male tumours are hormone-associated (mainly cancer of the prostate), while the figure for females is one-half (Miller, 1978).

The possible stimulation of oestrogen receptor synthesis at the time of transformation is not the only biochemical alteration which occurs in the breast. Enzymes responsible for hormone metabolism also appear to be effected. Pollow et al., (1977) observed that 17β-hydroxy-steroid dehydrogenase levels fell dramatically in human malignant mammary tissue, thus diminishing the amount of oestradiol converted to oestrone. This increases the exposure of the tissue to oestradiol. Brooks et al., (1978) using MCF7 cells, found that conversion of oestradiol to oestrone was increased rather than decreased. However, King et al., (1965) had found, like Pollow, that oestrone was converted to oestradiol at a much greater rate in rat mammary tumour tissue than in normal tissue from the same source and so the former tissue was exposed to greater amounts of oestradiol. King also found the tumour tissue more active in metabolising testosterone, in keeping with the more recent discovery by Deshpande et al., (1977) that tumour tissue has a specific receptor for 5α-dihydrotesteosterone. Yet another change in enzymic activity was observed with Δ^4-5β-reductase (Abul-Hajj, 1979). This form of the enzyme was found in tumours which had little or no oestrogen receptor content. The Δ^4-5α-reductase, however, was found associated with tumours containing a significant level of oestrogen

receptor. As the 5α-reductase produces metabolites which interact with oestrogen receptors, they may act to stimulate growth of the tumour. Another example of this kind of self-preservation mechanism is the ability of tumour cells to cause angiogenesis - a function unknown in normal cells (Maiorana & Gullino, 1978). The binding of glucocorticoids, which play an active role in lactation (Shyamala, 1973) does not appear, however, to be effected by the change to malignancy, although the binding protein is very similar to the oestrogen receptor (Gardner & Wittliff, 1973). Binding properties were found to be the same in both malignant and normal breast tissue, although the quantitative result might prove to be different (Goral & Wittliff, 1975).

1.3.2 HORMONAL INVOLVEMENT IN BREAST CANCER

The study of hormonal mechanisms in breast cancer has been greatly assisted by two model systems. One is the hormone-dependent rat mammary tumour induced by 7,12-dimethylbenz-a-anthracene (DMBA), an aromatic hydrocarbon administered via the gastro-intestinal tract. This tumour was first developed by Geyer et al., (1953), and later found to be capable of exhibiting hormone-dependence (Huggins et al., 1959, 1961, Young et al., 1963). This is not found in every case, however, with about 30% being autonomous. The other system is the MCF7 cell line, derived from a pleural effusion of a patient with hormone-dependent breast cancer (Soule et al., 1973). This line has been shown to contain oestrogen, progesterone, androgen and glucocorticoid receptors (Horwitz et al., 1975b), and cellular growth is sensitive to hormonal control.

1.3.2.1 Treatment in Cases of Breast Cancer

Treatment for breast cancer falls into three areas - radiotherapy, chemotherapy and hormone therapy. Each of these has associated with it considerable side effects, the worst being experienced with chemotherapy. This involves the prescription of cytotoxic drugs, which act to kill dividing cells. The cells in a mammary tumour do not divide very rapidly, and so exposure to these drugs must be maintained over an extended period of time. Intestinal cells divide rapidly and so are vulnerable to these drugs, which is why one of the worst side effects is considerable nausea, caused by the extended periods of treatment.

Hormonal therapy can take one of two general forms - either ablative or additive. Ablative therapy means removal of an endocrine gland, thus causing hormone deprival. Additive therapy involves either the administration of pharmacological doses of hormones, or the use of antihormones, both of which interfere in some way with the normal functioning of oestrogen actions within the breast. Usually, a tumour will respond much more successfully to one form than another (Huggins, 1965) and even within one general type, there may be variation in response to different treatments. Within ablative procedures, the success of a particular operation appears to depend on the hormonal status of the patient. Oophorectomy is most successful for premenopausal or menopausal women, while hypophysectomy and adrenalectomy are more suited to postmenopausal patients,with adrenalectomy the more traumatic operation (Binder et al., 1977). In addition to this, "chemical adrenalectomy" can now be achieved by the use of aminoglutethimide, which is thought to act by blocking the synthesis

of steroids at the conversion of cholesterol to pregnenolone, with an effect on aromatisation also. This can be used in cases suitable for adrenalectomy, the chemical method having much less trauma associated with it.

The mechanism by which additive therapy operates is not absolutely clear, but it is presumed to act by a feedback mechanism (down regulation), which lowers levels of oestrogen receptors. Large doses of oestrogens, progestins, androgens or glucocorticoids are used to good effect (McGuire et al., 1976b). The action of glucocorticoids in causing regression of breast tumours has been postulated to be by a dual effect (Osborne et al., 1979). One proposed aspect of action is a direct inhibition of breast cancer cell proliferation, the other being an opposition of the stimulatory effect of insulin. The efficacy of progesterone in suppressing oestrogen action was noted over thirty years ago in guinea pig (Lipschütz et al., 1939, Lipschütz & Maass, 1944). Investigations on DMBA-induced mammary tumours in the rat revealed that progesterone, when given in incremental doses along with increasing doses of oestradiol-17β gave increased tumour regression over the oestrogen alone (McCormick & Moon, 1973). Progesterone alone, however, hastened tumour appearance in this system. Progesterone can influence other hormones of the mammary gland, such as prolactin (Djicane & Durand, 1977), so the effect of progesterone on human breast tumour growth could occur by an indirect pathway. High doses of androgens, also, are successful in causing regression of breast tumours. It was not known why this was so, but there have recently been reports of androgens binding to oestrogen receptors, a step which might be involved in their action (Garcia & Rochefort, 1977, Zava & McGuire, 1978, Garcia & Rochefort, 1978, 1979). In addition, androgen receptors have

been isolated from breast tumour cells (Lippman et al., 1975, King et al., 1976). It is not clear which receptor, if either, is involved in the tumour regression caused by androgen administration.

The other form of additive therapy is the use of antihormones such as the antioestrogens nafoxidine, clomiphene and tamoxifen. Antioestrogens act to inhibit the ability of oestrogens to produce their full effect. The way in which the effect is brought about has been keenly studied, not only because antioestrogens have been found to be most effective in causing regression of breast tumours, but also because this might lead to further elucidation of the mechanism involved in the target cell response to oestrogens. Clark et al.,(1973) demonstrated two potential useful facts in studies on the interaction of nafoxidine hydrochloride (1-{2-(P-{3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl}phenoxy)ethyl}-pyrrolidine, hydrochloride, also known as Upjohn 11,100A) with rat uterus. The first observation was that the antioestrogen bound to the oestrogen receptor and caused translocation into the nucleus, but there was no replenishment of cytoplasmic receptor. The other interesting finding of their studies was that the receptor complex, once translocated, resided in the nucleus for at least 67 hours, and possibly as long as 19 days. These two factors, in combination with competition for oestrogen receptor sites (Clark et al., 1974) were thought to constitute the reason for the anti-oestrogenic action of nafoxidine. The lack of cytoplasmic receptor replenishment was noted also by Katzenellenbogen & Ferguson (1975), who suggested also that there was uptake of oestradiol into the nucleus during the time that nuclear retention of receptor was sustained, but that this was not due to translocation of cytoplasmic receptor. However, Nicholson et al., (1976) and Koseki et al., (1977) both noted while

studying the action of tamoxifen (1-{4-β-dimethyl amino ethoxyphenyl} 1,2-diphenylbut-1-ene) on mammary tumours, that replenishment of cytoplasmic receptors did in fact occur using doses of tamoxifen equivalent to those known to cause regression of tumours in humans. Nicholson (1979) suggested that the lack of cytoplasmic receptor replenishment, noted when using higher doses of the drug, could be due to a blocking of some specific genetic response. He also postulated that receptor replenishment might not be non-existent, but merely repressed to a very low level.

Horwitz et al., (1978 showed that high doses of tamoxifen, but not nafoxidine, could be oestrogenic rather than antioestrogenic, and so it is important that the dose used is regulated with care. Tamoxifen has been shown to have different effects in various tissues, and, in fact, is oestrogenic rather than anti-oestrogenic in the mouse (Terenius, 1971). Bichon & Bayard (1979) have demonstrated oestrogenic actions of the drug in the rat liver also, but the high doses administered may have led to this finding.

Koseki et al., (1977) postulated that it is the disappearance of the oestrogen receptor from the nucleus rather than its reappearance in the cytosol which is the important factor in oestrogenic response, and that this "processing" of the nuclear receptor may be important in antioestrogenic actions. Horwitz & McGuire (1978) used MCF7 cells to demonstrate the same fact. Progesterone receptor stimulation by oestrogens was found to require "processing" of the hormone/receptor complex within the nucleus rather than merely translocation. Progesterone receptor synthesis is not stimulated by antioestrogens, demonstrating a repression of late oestrogenic

responses. A rapid increase in RNA polymerase B activity does occur with tamoxifen treatment (Nicholson et al., 1977), so early responses are not effected. This has been confirmed in studies on the effects of tamoxifen on DNA synthesis in the immature rat uterus (Cowan & Leake, 1979). Mester et al., (1977) hypothesised that the reason for this might be an alteration of the oestrogen receptor by tamoxifen, causing inactivation.

A completely different mode of action for tumour regression caused by antioestrogens was proposed by Jordan & Dowse (1976). They suggested that tamoxifen inhibited the oestrogen-stimulated synthesis of prolactin, and caused tumour growth to cease by this indirect pathway. This seems unlikely, however, as even some patients who had previously undergone hypophysectomy have been found to respond to tamoxifen treatment (Moseson et al., 1978). The anti-tumour effect of tamoxifen has been demonstrated to occur through binding to oestrogen receptor rather than by altering the secretion of pituitary hormones (Tanaka et al., 1978). It has also been suggested that antioestrogens dissociate more quickly than oestrogens from the receptor, and thus do not elicit a full oestrogenic effect (Bouton & Raynaud, 1978). This theory, that rapidly-dissociating compounds are necessarily antioestrogenic, has been disproved by Nicholson et al., (1979) and Rochefort et al., (1979), who compared the actions of oestradiol, tamoxifen and its more active metabolite with their binding properties. They conclude that binding characteristics cannot be used to predict agonistic or antagonistic properties.

Although the way in which antioestrogens act is not yet fully understood, it appears that they do interact with the usual machinery for steroid

hormone action, but that the blockage of oestradiol action lies beyond the translocation step. They have proved to be an innovation in hormonal therapy of breast cancer, as they represent an additive form which does not have the usual extreme side effects or possible upsurge of tumour growth associated with other additive regimes (Moseson et al., 1978). However, they can, themselves, have some side effects (Kiang & Kennedy, 1977a).

More advances in the treatment of breast cancer are necessary as it is surprising how little the mean survival rate has changed since the early breakthrough by Beatson in 1896 with hormonal treatments (Baum, 1976, Bulbrook, 1977a). Some tumours do not appear to respond particularly well to any one form of therapy, and it has been suggested that, since this may be due to cellular heterogeneity, combined cytotoxic and endocrine therapy should be considered (Nenci, 1978).

1.3.2.2 Rationale Behind Hormonal Therapy in Breast Cancer

The first demonstration that breast tumours might be sensitive to endocrine manipulation came in 1896, when George Beatson induced regression of inoperable and advanced lesions in two young women by oophorectomy. Following this, hormonal therapies of various types were often used in cases of breast cancer, but not all patients benefitted from the treatment. Huggins & Bergenstal (1952) reported that adrenalectomy caused regression of far advanced cases of mammary tumours, but not in every patient. Luft et al., (1958) favoured the use of hypophysectomy, as this eliminated pituitary hormone involvement as well, but again there was no method of predicting which patients would benefit from the operation. As

Pearson & Ray (1960) reported, the only guide to whether a response would be found with hormonal therapy was a previous response to another form of endocrine manipulation, and even this did not give complete certainty. All the forms of hormone therapy used involved ablative procedures, which could prove highly traumatic for patients already weakened by illness, and were not without side effects. Baker et al., (1960) called for some method by which the likelihood of response could be predicted, and, on this basis, the form of therapy selected. In addition to the possible dangers of ineffective hormonal manipulation, there is also the consideration that its administration represents a delay in instituting some other therapy such as chemotherapy or radiotherapy, either of which might be of real benefit to the patient.

The discovery that organs responsive to oestrogens had the capacity to retain ^{3}H-hexoestrol, when this was injected into rats (Glascock & Hoekstra, 1959, Jensen & Jacobson, 1960), prompted Folca et al., (1961) to administer the labelled hormone to breast cancer patients about to undergo adrenalectomy. They found that response was seen in patients whose tumours concentrated large amounts of label. This phenomenon was also noted by other workers (McGuire et al., 1976b). However, the correlation between the uptake of steroid and response to hormone therapy was not strong enough to merit the adoption of this method on a routine basis.

Bulbrook et al., (1960) suggested another approach, involving the measurement of urinary steroid metabolites in breast cancer patients. The ratio of 11-deoxy, 17-oxosteroids to 17-hydroxy corticosteroids was used in an equation to find what they termed a "discriminant" (Bulbrook et al., 1962a, 1962b, Hayward & Bulbrook, 1968). A positive discriminant was found to

give a good prediction of response to adrenalectomy or hypophysectomy (60%). Ahlquist et al., (1968), however, found that no such correlation existed when these measurements were carried out, and so doubt fell on the method (Zumoff et al., 1975). Another line of approach was to measure serum levels of hormone. Oestradiol was measured, but found to be similar in all cases, whether the serum came from a healthy control or a breast cancer patient (Skinner, 1974, Thijssen et al., 1975). Progesterone levels, on the other hand, were found to be elevated in women with breast disease (Smethurst et al., 1976, England et al., 1975).

1.3.2.3 The Oestrogen Receptor Hypothesis

From the studies on the uptake of labelled oestrogen into tumours sensitive to hormonal manipulation, and the discovery of the role of oestrogen receptors in introducing the hormone into the target cell, came the concept that the presence of oestrogen receptor in a breast tumour could implicate hormonal involvement in the growth and development of that tumour. Jensen et al., (1971) suggested that hormone-dependent tumours could be identified by their content of oestrogen receptors. Feherty et al., (1971) measured receptors by labelling them with ^{3}H-oestradiol in a cytoplasmic preparation of a breast tumour. They found that almost 60% of tumours contained cytoplasmic receptors. Maass et al., (1972) used the method of incubating tumour slices with ^{3}H-oestradiol, and correlated results with subsequent response to hormone therapy. Engelsman et al., (1973) studied the response of patients to hormone therapy in relation to receptor levels. 80% of receptor-containing tumours regressed on hormone therapy, although this percentage may be artificially high due to the small number of patients involved. Only 10% of receptor-deficient tumours responded. Similar

studies carried out using various methods to measure cytoplasmic levels of oestrogen receptors revealed a somewhat lower success rate for receptor-positive biopsies. This was the subject of a workshop held by the Breast Cancer Task Force (McGuire et al., 1975a). Updated results such as those of De Sombre et al., (1978) and Byar et al., (1979) confirm success rates found in this workshop, 40-60% of receptor-positive tumours regressing on hormonal treatment.

1.3.2.4 "False-Negatives" and "False-Positives"

Although the measurement of cytoplasmic receptors had cut down the number of patients receiving unnecessary hormone therapy, a significant number of receptor-containing tumours still did not respond to this type of treatment. Patients whose tumours lack receptors, however, have very little chance of response to hormone therapy (Jensen, 1975). Due to these findings, it was decided that it was more reliable to state that no receptor signified no chance of response, as the presence of receptor could in no way guarantee a favourable response (Maass et al., 1975, King, 1975, McGuire et al., 1975b, Lippman, 1976). More recently, Roberts et al., (1978), having considered overall response rates to hormone therapy, in relation to oestrogen receptor levels, reported that, although oestrogen receptor-containing tumours are 4 to 5 times more likely to respond to hormonal manipulation than tumours containing no receptor, it is not worth carrying out surgery for the sole purpose of receptor determination. Routine biopsy of breast tumours for oestrogen receptor assay had been suggested by Jensen (1975) on the basis that oestrogen receptor level is thought to be maintained in the transition from primary to secondary disease (King, 1975, Jensen et al., 1977, Block et al., 1978). A measurement of receptor content in primary tumour tissue would thus enable

immediate treatment of secondary disease, whether a local metastasis or one of the common, and surgically inaccessible, distant metastases of bone or lung (Haagensen, 1974). The fact that receptor content is maintained during the change from primary to secondary disease might also be useful in cases of a second tumour arising, the source of which is uncertain. Kiang & Kennedy (1977b) suggest that, if the lesion were a secondary of a receptor-containing primary tumour, this could be identified. This method is not very accurate, as another primary might have the same hormonal status.

The fact that very few receptor-deficient tumours respond to hormone therapy, while a much larger percentage of receptor-containing tumours fail on hormone therapy is somewhat strange, since there are as many reasons for "false-negative" results as for "false-positives". "False-negative" results could stem from a truly receptor-deficient tumour responding to hormonal therapy by some indirect pathway, such as inhibition by tamoxifen of prostaglandin synthetase (Tisdale, 1977). There also exist many steps in the assay of receptors where they could be destroyed by incautious handling, as they are extremely thermolabile and unstable. The source of the tumour sample might also lead to an invalid result if it originated from a non-malignant piece of tissue, or a piece of tumour containing no receptor, while the majority of the rest of the tumour did contain receptors. The effect of endogenous oestrogens has also been proposed to have a masking effect on receptors in premenopausal patients. The percentage of premenopausal patients whose tumours contain cytoplasmic oestrogen receptor is significantly lower than that of post-menopausal patients (Wittliff et al., 1972). This has been attributed to

endogenous oestrogens filling the receptor sites and making them unavailable for measurement (Braunsberg et al., 1974, Sakai & Saez, 1976). Mobbs & Johnson (1976) suggested that the stage of the menstrual cycle should be taken into account when measuring receptors in order to counteract this effect.

Since the incidence of "false-positive" results is so high, it is important to realise all the possible causes of these, and try to find methods of circumventing them. Their occurrence makes the whole concept of the assay of oestrogen receptors somewhat less than perfect. All the reasons for false-positive results are not yet understood, but several possibilities exist. The first one is that only one part of the mechanism of action of oestradiol is being taken into consideration when cytoplasmic oestrogen receptor levels are measured. If there is a defect in the mechanism for transferring the hormone/receptor complex into the nucleus, then, no matter how many receptors are found in the cytoplasm, oestradiol will not be influencing the growth of the cell (Maass et al., 1975). The obvious solution here is to rule out this possibility by the assay of nuclear, as well as cytoplasmic, receptor levels (Thorsen & Stoa, 1979). This has been demonstrated to decrease the number of false-positive results (Laing et al., 1977), if only those patients whose tumours contain receptors both in the cytoplasmic and nuclear fractions are treated with hormone therapy. There are, however, still a small proportion of patients who should respond, but fail. These tumours may have a defect in the acceptor site binding within the nucleus, so that even proving the existence of nuclear receptors would not alleviate the problem.

As reported by Jensen & De Sombre (1977), malignant transformation may cause tumour nuclei to become independent of hormonal control, without shutting down receptor synthesis. In this case, several authors have suggested that what should be measured are not oestrogen receptors themselves, but rather the products of oestrogen action. The presence of these would prove, beyond any doubt, that oestrogens are functioning fully within the cell. Such products include caesin messenger RNA (Rosen & Socher, 1977), but this was found in only 70% of the hormone-dependent rat mammary tumours examined - no improvement on the kind of response rate obtained using measurement of cytoplasmic and nuclear receptors. Another marker of hormonal stimulation in the breast is the synthesis of α-lactalbumin, one of the proteins responsible for the lactose synthesis. This was found in some, but not all hormone-dependent rat mammary tumours, its absence possibly reflecting some damage to the gene responsible for its synthesis during malignant transformation (Ip & Dao, 1978, Woods et al., 1979, Hall et al., 1979). The assay for this has proved unreliable (see Results, section 3.4.5.3). Peroxidase is another protein which has been used as an indication of oestrogenic stimulation (De Sombre et al., 1975, Anderson et al., 1975, Lyttle & De Sombre, 1977a). This has been shown to be produced by oestrogen action on the rat uterus (Lyttle & De Sombre, 1977b), where it may act to cross-link the uterine proteins (Keeping & Jellinck, 1978). It has been isolated and purified successfully from hormone-dependent rat mammary tumours (De Sombre & Lyttle, 1978). However, the success of this method in predicting response to hormone therapy is in doubt, as Duffy & Duffy (1977) found peroxidase in both benign and oestrogen receptor-deficient mammary tumours. Follow up from patients treated on the basis of peroxidase levels will be necessary

to evaluate the reliability of the basic concept. The other marker of oestrogen action, which has received a great deal of attention, is the progesterone receptor. Oestradiol stimulates the synthesis of this protein within the cell (Asselin et al., 1977), and, thus, its presence was deemed a good indication of oestrogenic stimulation of growth.

Hsueh et al., (1974) attempted to measure progesterone receptor using an exchange technique for added endogenous steroid. Miller et al.,(1975) then used an electrophoretic technique. The problem encountered in the measurement of progesterone receptors was that adding ^{3}H-progesterone to a cytoplasmic or nuclear preparation of a tumour cell filled, not only progesterone receptors, but also corticosteroid binding globulin (CBG). It was thus impossible to determine how much radioactivity was due to binding to just the progesterone receptors. This was solved by the introduction, in 1974, of a synthetic progestin: 17,21-dimethyl-19-norpregna-4, 9-diene-3,20-dione (R5020). This has a high affinity for the progesterone receptor, and a very low affinity for CBG (McGuire et al., 1977b). One problem found in breast tissue is that R5020 will also bind to the cellular glucocorticoid receptor, and it was suggested that natural progesterone plus unlabelled cortisol might be a better way of eliminating the binding of progesterone to other proteins (Pichon & Milgrom, 1977). Keightley (1979), on the contrary, found that R5020 gave much more stability in binding, and less likelihood of interference from other steroids, and Duffy & Duffy (1979) report that both the natural and synthetic progestins bind to the same site and that ^{3}H-progesterone plus cortisol offers no advantages over R5020.

After the discovery of R5020,Horwitz et al., (1975a) proposed that progesterone receptor should now be assayed, in addition to oestrogen

receptors, in the cytoplasm of mammary tumours. They hypothesised that tumours containing oestrogen receptor but no progesterone receptor should not be treated with hormone therapy, as the oestrogen system within the tumour would not be fully functional. They found only 56% of tumours containing cytoplasmic oestrogen receptor also contained progesterone receptor. McGuire et al., (1976b) further reported an improved response rate to hormone therapy if both oestrogen and progesterone receptors were present. The same role of the progesterone receptor as index of oestrogen-responsiveness was noted in endometrial tissue (Martin et al., 1979). A further report from McGuire (1978) indicated that the assay of progesterone receptors might not be as successful as expected, as some tumours lacking this receptor, but containing oestrogen receptor had responded to hormonal therapy. Allegra et al., (1979b) came to the same conclusion. A new method of measuring the progesterone receptor which pays special attention to the stability of the complex formed between the receptor and R5020 has now been developed (Powell et al., 1979), in the hope that this will improve the success rate. However, a further problem in this area is the discovery by Ip et al., (1979) that the rat mammary tumour system MTW9B contains both oestrogen receptor and progesterone receptor, but is not hormone-dependent. This suggests that there may be an important step in the mechanism of oestrogen action even later than the induction of progesterone receptor synthesis, and a marker of this must be sought as a means of determining hormonal stimulation. The same conclusion can be reached from a report by Watson et al., (1979) of another mammary tumour cell line (MXT 3590) in which oestradiol directs progesterone receptor synthesis, but not tissue growth. Westley & Rochefort (1979) report the discovery of markers for this later step - three intracellular proteins produced as a result of oestrogenic stimulation, and inhibited by tamoxifen.

If the defect making the tumour a "false-positive" one does not lie in the cellular mechanism, it may be in the make up of the tumour itself. Some tumours are made up of a mixed population of cells, some of which are hormone-dependent and some independent, or autonomous (Sluyser & Evers, 1975). If the sample in which receptor content is measured is hormone-dependent, but unrepresentative of the tumour as a whole, it will contain receptors, but the tumour will only partially respond to hormone therapy. There may be a short-lived response, due to the death of the dependent cells, but this will be reversed as the autonomous cells take over. Mixed populations may represent tumours which are changing over from being hormone-dependent to autonomous, as observed by Kim & Depowski (1975) and Bulbrook (1977b).

One other possible cause of no response, where one would be expected, is that the type of hormone therapy may fail to be the best one for the particular tumour in question. Although each form of hormonal therapy may have the same overall success rate, there is evidence (McGuire et al., 1976b) that one particular form will be more successful than another in any one tumour.

The approach which has the potential to overcome all these defects, whether they make the tumour a "false-positive" or a "false-negative", would be the institution of an in vitro system to study the effects of different types of treatment on the epithelial cells of the tumour. Although this is probably the ideal method, it is not without difficulties. This area will be covered fully in Part II of this thesis.

1.3.2.5 Other Clinical Aspects of Oestrogen Receptors

Although the measurement of oestrogen receptors was intended to be used as an index by which response to hormonal therapy could be predicted, it has been suggested that it may also be of use in determining the likelihood of a tumour's response to chemotherapy and also the patient's prognosis. From data on the relationship between disease free interval and oestrogen receptor content (Block et al., 1978, Hähnel et al., 1979), it appears that receptor-containing tumours grow less aggressively than tumours lacking receptor (Meyer et al., 1977). On the basis that chemotherapy acts by attacking rapidly-growing cells, the relatively faster growing receptor-deficient cells should respond more successfully to this form of treatment (McGuire, 1978). Two reports have been published recently dealing with this topic. One, from Kiang et al., (1978), states that, in fact, more receptor-containing tumours respond to chemotherapy (80%) than do those tumours lacking receptor (37%). Unfortunately, the other report (Lippman et al., 1978) states the exact opposite, with the receptor-deficient tumours more likely to respond favourably. Kiang et al. also suggest that hormone therapy may act against subsequent attempts to apply chemotherapy by destroying chemosensitive factors. Both of these papers have defects in the way tumours were selected for the study or how they were evaluated for response, making each subject to suspicion. The problem of which one of these reports, if either, is right will be solved when more groups evaluate their results in terms of the relationship between chemotherapy response and oestrogen receptor content. The answer to the problem will prove very important to all those involved in the treatment for breast cancer.

1.4 OESTROGEN RECEPTORS IN NON-TARGET TISSUES

1.4.1 OCCURRENCE

Although most of the preliminary work on the nature and action of oestrogen receptors was carried out using the immature rat uterus as a model, oestrogen receptors have been located in many different tissues. These tissues vary widely from more to less expected sources. More expected locations are human endometrium (Tseng & Gurpide, 1978, Soutter et al., 1979), cervix (Sanborn et al., 1978) and placenta (McCormack & Glasser, 1978) and rat ovary (Saiduddin & Zassenhaus, 1977). Less expected receptor-containing locations have been the kidney (Bojar et al., 1975), pancreas (Rosenthal & Sandberg, 1978), liver (Duffy & Duffy, 1978) and human and canine prostate (Ekman et al., 1979, Dubé et al., 1979). Oestrogen receptors have also been detected in the rat adrenal gland, showing that oestradiol does act directly on this organ, as was hypothesised (Calandra et al., 1978). The important questions which arise from these findings are, whether the receptors are functional in these various tissues, and would their presence in carcinoma tissue of the same organs justify the use of hormonal therapy.

1.4.2 PREDICTIVE VALUE OF OESTROGEN RECEPTORS IN NON-TARGET TISSUE TUMOURS

In addition to the normal tissues detailed above, oestrogen binding proteins have also been demonstrated in kidney carcinoma tissue (Bojar et al., 1975), human prostatic carcinoma (Ekman et al., 1979) and human colonic tumours (McLendon et al., 1977, Alford et al., 1979). Other sites where oestrogen binding proteins have been identified are malignant melanoma (Fisher et al., 1976), leukaemia (Lippman et al., 1973) and endometrial carcinoma (Pollow et al., 1975). Whether these proteins represent true receptors

and whether their presence indicates that the tumour will respond to a form of hormonal therapy, is not yet clear. Some follow up of patients with these lesions treated with hormone therapy is necessary.

The idea that tumours of the intestinal tract may be hormone-dependent is particularly appealing. Berg (1975) suggested that hormone-dependent tumours may be linked to diet. The same is thought to be true of colorectal cancer, the prevalence of which in particular areas is thought to reflect the kind of food eaten. Another line of reasoning is that cancer of the gastro-intestinal tract is the most common form of cancer linked to breast carcinoma, which is itself known to be hormone-dependent in a number of cases (Hermann 1972). The presence of oestrogen receptors in colonic tumour tissue should thus give a good indication that the tumour might respond to endocrine manipulation.

Oestrogen receptors are not the only steroid receptors to be found in these solid tumours of non-endocrine tissues. Androgen and progesterone receptors are also in evidence, either of which could act in some form of hormonal sensitivity. Since these receptors can be mimicked by plasma proteins, care must be taken to avoid contamination with plasma. If, however, measurement of cellular receptor can be clearly identified, the relevance of its presence must be elucidated.

As Friedman et al, (1978) state, the presence of receptors may merely be an indication of longer potential survival, but only further investigation in this field will elucidate the true relevance of their synthesis.

1.5 OBJECTIVES

The aim of the investigations involved in this part of the project was to study the actions of steroid hormones, and oestradiol in particular, in normal and neoplastic tissues. The approach adopted was investigation of the nature and distribution of oestrogen receptors in the various fractions of these tissues, and a reliable, routine method of doing this was sought. It was hoped that, once this method was successfully established, it would prove useful not only in the better understanding of steroid mechanisms, but also aid in the treatment of cancer patients by more accurately predicting their likelihood of responding to a hormonally-based therapeutic regime.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 FINE CHEMICALS were obtained as follows:-

DTT (dithiothreitol)	The Boehringer Corporation (London) Ltd
Norit A activated charcoal (untreated powder)	Sigma, London
Bovine serum albumin (fraction V)	Sigma, London
Phenyl methyl sulphonyl fluoride (PMSF)	Sigma, London
Dimethyl sulfoxide (DMSO)	Sigma, London
Deoxyribonucleic acid (DNA) (calf thymus type V sodium salt, highly polymerized)	Sigma, London
Dextran blue (MW 2,000,000)	Sigma, London
Monothioglycerol	Sigma, London
Dextran T70	Pharmacia, Sweden
Sephadex G25	Pharmacia, Sweden
Triton X-100	Koch-Light Laboratories, Colnbrook, England
Trasylol (Aprotinin in isotonic solution containing 0.9% benzyl alcohol)	Bayer, Germany

All other chemicals used were, wherever possible, AnalaR reagents, supplied by BDH Chemicals Ltd., Poole, Dorset.

2.1.2 BUFFERS

N-2-hydroxypiperazine-N'-2-ethane sulphonic acid (HEPES) was obtained from The Boehringer Corporation (London) Ltd.

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

2.1.3 STEROIDS

(6,7-^{3}H) oestradiol-17β (40-80 mCi/ml) was obtained from The Radiochemical Centre, Amersham.

Oestradiol-17β and diethylstilboestrol (DES) were routinely obtained from Sigma, London, although initial trials were carried out using materials supplied by NIH Steroid Reference Collection.

2.1.4 SCINTILLATION MATERIALS

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

Toluene (AR grade)

2,5-diphenyloxazole (PPO)

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

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

2.1.5 LIVESTOCK

Rat uterine tissue was obtained from female Albino Wistar rats (Glasgow University colony).

2.1.6 HUMAN TISSUE

Tumour tissue of various kinds was kindly supplied by the following Health Board hospitals:-

Victoria Infirmary, Glasgow

Western Infirmary, Glasgow

Gartnaval General Hospital, Glasgow

Royal Infirmary, Glasgow

Stobhill General Hospital, Glasgow

Royal Beatson Memorial Hospital, Glasgow

Southern General Hospital, Glasgow

Hairmyres Hospital, East Kilbride

Monklands General Hospital, Airdrie

Belvidere Hospital, Glasgow

Ballochmyle Hospital, Mauchline, Ayrshire

Normal breast tissue was kindly supplied by Canniesburn Hospital, Bearsden.

Breast tumour tissue for quality control comparison was sent from the Imperial Cancer Research Fund, London.

Lyophilized breast tumour and calf uterine material was supplied by Christie Hospital, Manchester.

2.1.7 MISCELLANEOUS

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

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

Glass/glass tissue grinders were obtained from either Kontes, USA or Cowie Scientific, Middlesbrough, England.

Glassware was washed and rinsed in glass distilled water as the assay of receptors was found to be adversely affected by the presence of divalent metal ions.

All solutions were made up in glass distilled water.

2.2. METHODS

2.2.1 TISSUE HANDLING

2.2.1.1 Collection

Tissue was collected fresh from the operating theatre, where a piece was selected from the area adjacent to the section removed for pathological examination. Collection was into an empty sterile container which was then transported on ice to the laboratory for analysis.

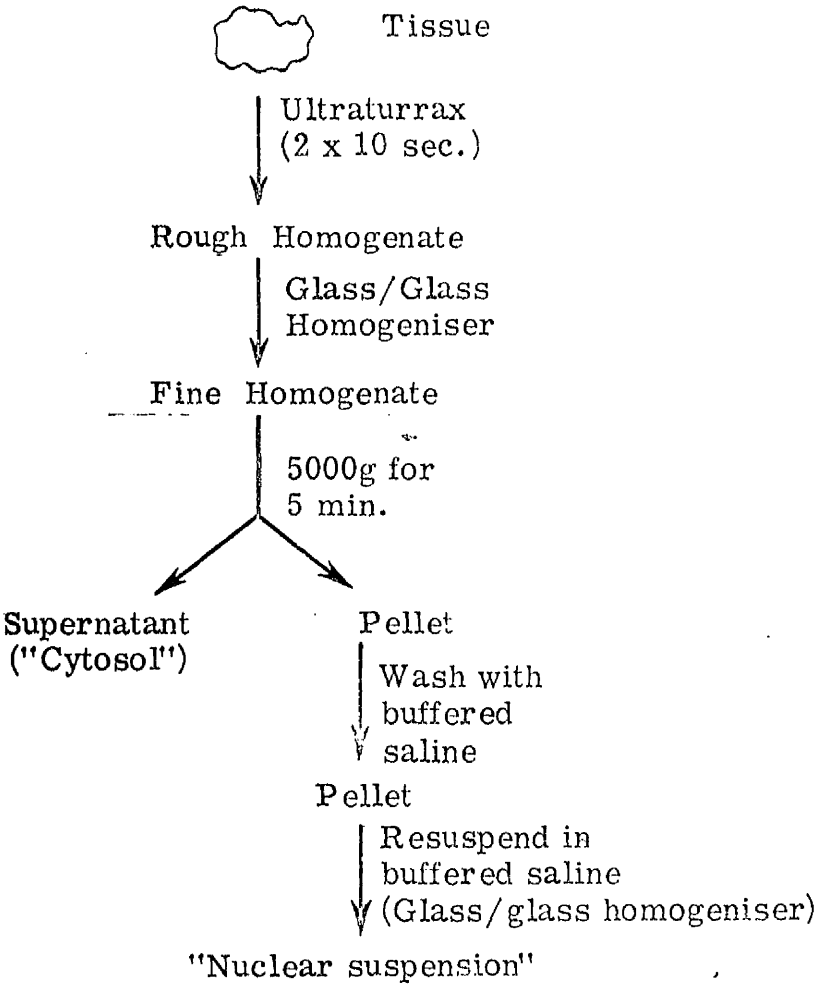
2.2.1.2 Storage

Wherever possible, analysis of oestrogen receptor content was carried out on the same day as collection. However, when it was not possible to assay the tissue on the same day, storage of the sample was at -20°C in a medium of 50% (v:v) glycerol:0.25M sucrose, 1.5 mM $MgCl_2$, 10 mM HEPES (N-2-hydroxy-piperazine-N'-2-ethane sulphonic acid) (pH 7.4) (see Results, section 3.1.1). Before assay, the tissue was rehydrated in 0.25M sucrose, 1.5 mM $MgCl_2$, 10 mM HEPES (pH 7.4) at 0°C for 15-30 minutes.

2.2.1.3 Preparation of Fractions for Receptor Assay (See Figure 5)

Tissue was dissected free of all possible adhering fat as this was found to interfere with receptor measurement. A sample of at least 150 mg

Figure 5 - Preparation of Tissue Fractions for Receptor Assay


Tissue
Ultraturrax
(2 x 10 sec.)
Rough Homogenate
Glass/Glass
Homogeniser
Fine Homogenate
5000g for
5 min.
Supernatant
("Cytosol")
Pellet
Wash with
buffered
saline
Pellet
Resuspend in
buffered saline
(Glass/glass homogeniser)
"Nuclear suspension"

(see Results, section 3.3) was then selected from as near as possible to the cut made for the pathological specimen (see "Collection" above). This sample was homogenised in 1.5 mM EDTA (ethylene diaminetetra acetic acid), 0.25 mM DTT (dithiothreitol), 10 mM HEPES (pH 7.4) (HED buffer) at a concentration of 50 mg/ml. Homogenisation was carried out using 2 x 10 second bursts at a setting of 150 on an Ultra-Turrax homogeniser (Model TP 18/2), followed by the use of a glass/glass tissue grinder (see Results, section 3.1.2). The homogenate was kept cool throughout this process as any heating which occurred would be detrimental to the receptors.

The homogenate was then centrifuged at 5000 g for 5 minutes in a Sorvall RC5 Superspeed Refrigerated Centrifuge at 4°C. The resultant supernatant ("cytosol") was retained in this crude form for receptor assay, as results obtained using a higher speed supernatant (100,000 g) were not found to be significantly different in relation to DNA content. The pellet was washed once with 0.15 M NaCl, 10 mM HEPES (pH 7.4), and finally resuspended in the original homogenisation volume, using the saline solution. A glass/glass tissue grinder was used again at this stage, to ensure an even suspension of the nuclear material ("nuclear suspension"). As in the case of the cytosol, the purification of the nuclear suspension by centrifugation through 2.4 M sucrose, in no way enhanced the results of the receptor assay, and so these forms, although crude, were adopted for routine receptor measurement.

2.2.2 PREPARATION OF ^{3}H-OESTRADIOL SOLUTIONS

Stock ^{3}H-labelled oestradiol-17β, the purity of which had been checked by LH-20 chromatography, was prepared at a concentration of 10^{-7}M in absolute

alcohol, and this was stored at -20°C. Appropriate amounts of this were aliquotted out to give a series of seven concentrations of ^{3}H-oestradiol-17β ranging from 4 x 10^{-10}M - 4 x 10^{-9}M. Another two aliquots to give 4 x 10^{-9}M were measured out, and to these were added either unlabelled oestradiol-17β or diethylstilboestrol (DES) both in absolute alcohol, in 100-fold excess. The ethanol was evaporated down in all cases in a stream of compressed air, and 1 ml of HED buffer added to give oestradiol concentrations in the range quoted. The ^{3}H-oestradiol-17β solutions were then stored at 4°C for no longer than one week.

2.2.3 ASSAY OF RECEPTORS

2.2.3.1 Incubation of tissue fractions with steroid.

50 µl aliquots of each ^{3}H-oestradiol-17β solution prepared as detailed above were mixed with 150 µl aliquots of cytosol or nuclear suspension in polystyrene test tubes. This gave a final range of concentrations of steroid in the tubes of 1x, 1.5x, 2x, 4x, 6x, 8x 10^{-10}M, ^{3}H-oestradiol-17β and 10^{-9}M ^{3}H-oestradiol-17β±10^{-7}M unlabelled oestradiol or DES. The tubes were then incubated at 4°C for 18 hours or 20°C for 2 hours (see Results, section, 3.1.3.1). A set of control tubes was also set up for the cytoplasmic assay which were identical to those above except that HED buffer was substituted for cytosol. The incubation conditions were as for the test sets.

2.2.3.2 Separation of unbound from bound steroid

This was achieved by different methods for cytosol and nuclear assays.

(a) Cytosol

(i) Sephadex column chromatography

After incubation, 100 µl of the cytosol/steroid mixture was removed from the tube and mixed with 25 µl of dextran blue, which served as a marker dye. This was applied to the top of a Sephadex G25 column made in a pasteur pipette using a glass wool plug as support, and kept at 0°C throughout. The sample was washed through the column using 10 mM HEPES, 1.5 mM EDTA (pH 7.4) (HE buffer). Four-drop fractions were collected directly into plastic scintillation vials, measuring both background and radioactivity representing bound ^{3}H-oestradiol-17β. The column was then washed with HE buffer ready for re-use. 10 ml Triton-toluene scintillant (1400 ml toluene/PPO (2,5-diphenyloxazole) (5g/l)/POPOP (1,4-di-{2-(5-phenyloxazole)}-benzene) (0.24 g/l): 600 ml Triton-X100:200 ml absolute alcohol) was added to each vial, and the vials counted.

(ii) Dextran-coated charcoal adsorption

After incubation, 900 µl HE buffer was added to each tube, and the tubes mixed. 500 µl of dextran-coated charcoal solution (DCC), (0.15% w/v Norit A charcoal, 0.0015% w/v dextran T70 in 0.25M sucrose, 1.5 mM EDTA, 10 mM HEPES (pH 7.4)) was then added, and the tubes mixed again. The charcoal "stripping" was continued for 15 minutes at 0°C, with periodic mixing of the tubes. At the end of this time, the charcoal was pelleted by centrifugation at 1000 g for 5 minutes. 1 ml aliquots of each supernatant were transferred to plastic scintillation vials, 10 ml Triton-toluene scintillant added to each vial, and the vials counted.

Background and total values were obtained from the control set of tubes.

(b) Nuclear

After incubation, 100 µl aliquots from each tube were added to 5 ml aliquots of 0.15 M NaCl, 10 mM HEPES (pH 7.4). Each one was then poured down the

chimney of a millipore filter apparatus on to a prewetted glass fibre filter. The tube was then washed out with another 5 ml of saline, and the washing poured on to the filter as before. The chimney was washed down with 3 x 4 ml aliquots of saline, which was sufficient to wash any unbound material from the filter. The chimney was then removed from the filter and the edges of the filter carefully washed with saline. The filter was removed from the Millipore apparatus and placed in a plastic scintillation vial. 50 µl aliquots of the original incubation mixtures were added to dry filters in scintillation vials, to give a measure of the total counts present. All vials were then placed at 60°C overnight to allow the filters to dry. After cooling, 10 ml toluene/PPO (5g/l) were added to each vial, and the vials counted.

2.2.3.3 Counting of radioactive samples

All vials from cytosol and nuclear assays were counted on a Philips or a Searle Mk 11 Liquid Scintillation Analyser. The efficiency of counting was approximately 25% for cytosol samples and 35% for nuclear samples.

2.2.4 CALCULATION OF RESULTS

Results from the above assays were analysed by the method of Scatchard (1949). The Scatchard equation can be derived from the Langmuir adsorption isotherm by making various assumptions. The Langmuir adsorption isotherm may be expressed:

$$\frac{P}{x/m} = \frac{1}{k_1 k_2} + \frac{P}{k_2} \qquad (1)$$

(p = pressure, x = amount adsorbed, m = mass of adsorbent, and k_1 and k_2 are constants, k_2 being an index of the total capacity of the system).

Where p = total available ligand; in conditions of a large excess of ligand, p $\doteq$ free ligand (F).

In a closed system, the amount of adsorbent is constant, so m is constant and equation (1) can be rewritten:

$$\frac{P}{x} = \frac{1}{k_1 k_2} + \frac{P}{k_2}$$

where x = amount bound (B)

The equation can now be rewritten:

$$F/_B = \frac{1}{k_1 k_2} + \frac{F}{k_2}$$

$$= \quad F/_B = \frac{1 + k_1 F}{k_1 k_2}$$

$$= \quad B/_F = \frac{k_1 k_2}{1 + k_1 F}$$

$$= \quad B = \frac{k_1 k_2 F}{1 + k_1 F}$$

$$= \quad B + Bk_1 F = k_1 k_2 F$$

$$= \quad B = k_1 k_2 F - Bk_1 F$$

$$= \quad B/_F = k_1 k_2 - k_1 B$$

If k_1 is a binding constant, and k_2 the total number of binding sites, the above equation can be rewritten:-

$$B/F = nk - kB$$

This is the Scatchard equation.

If B/F is now plotted against the concentration of oestradiol-17β bound, a straight line is obtained, with gradient equal to -Ka or -1/KD, and an X-axis intercept from which may be derived the total number of receptors.

A more simple derivation is achieved by examining:-

$$\underset{(F)}{H} + \underset{(RT-B)}{R} \rightleftharpoons \underset{(B)}{HR}$$

$$Kd = \frac{F(RT-B)}{B}$$

$$= \quad B/F = \frac{RT}{Kd} - \frac{B}{Kd}$$

Thus, by this method, a single plot gives a measure of both the number of receptors and the dissociation constant of the binding, which is a measure of the affinity with which the receptor binds the ligand. The specificity of the receptor for the binding of oestradiol-17β was measured by comparing the binding of ^{3}H-oestradiol-17β alone and in the presence of a 100-fold excess of unlabelled oestradiol or DES (see Results, section 3.3.1.3). DES is preferred in this role, as it will not bind to proteins of a non-cellular nature, which would bind oestradiol with high affinity. The presence of DES as a competitor eliminates incorrect measurement which would result from contamination by such proteins, such as sex steroid binding globulin

(King & Mainwaring, 1974). Since non-specific binding is principally non-saturable at the concentrations being considered in this assay system, the amount of radioactivity bound with or without competitor would be identical. Specific binding is saturable, however, and very few molecules of labelled steroid would be bound by a specific receptor in the presence of a large excess of competitor.

2.2.5 EXPRESSION OF RESULTS

2.2.5.1 Protein Determination

The protein content of each cytosol preparation was determined by the method of Lowry et al.,(1951).

2.2.5.2 DNA Determination

The DNA of each nuclear suspension was determined by the modification by Katzenellenbogen & Leake (1974) of the method of Burton (1956).

2.2.5.3 Final form of results

Scatchard analysis yielded levels of receptor for both cytosol and nuclear preparations expressed as fmol/ml. When protein and DNA analysis had been completed, cytosol levels were expressed in units of fmol/mg cytosol protein and nuclear levels in fmol/mg DNA.

3. RESULTS

3.1 OESTROGEN RECEPTOR ASSAY CONDITIONS

To design a reliable assay for oestrogen receptors in both cytoplasmic and nuclear fractions of the cell, with possible applications to routine measurement, assisting in the choice of therapy for breast cancer patients, several parameters had to be determined. Experiments to establish the optimal conditions for such an assay system were carried out using principally rat uterine or human breast tissue, but the source of tissue will be defined in each case. The uterus of the immature rat has been used widely in the elucidation of oestrogenic actions, but variation between this model and the human breast does exist. This fact will be considered when interpreting the significance of results.

3.1.1 TISSUE STORAGE

Since it is not always practicable to assay the oestrogen receptor content when fresh tissue is made available, satisfactory storage conditions which would preserve the functional integrity of the receptors were sought. Different forms of storage were thus tested by examining the effects of such storage on the receptor content of tissue, relative to that of the same tissue when fresh. The results of these studies showed that, although storage of tissue at -20°C did not appear to be harmful to the cytosol receptors after one short period of storage (48 hours), repeated freezing and thawing or lengthy periods at this temperature, might prove so. This was not, however, the experience of Keightley *et al.*, (1978), who found myometrial cytosol receptor was stable when stored at -20°C or -70°C for

up to one week. The nuclear receptors in breast tumour tissue appeared much more sensitive to the freezing and thawing process, being totally abolished.

When tissue was stored at -20°C in a solution composed of sucrose buffer (0.25M sucrose, 1.5 mM Mg Cl_2, 10 mM HEPES, pH 7.4)/50% glycerol (v:v), which did not permit freezing of the material, no apparent adverse effect on the receptors was noted. This latter method was adopted for storage of all types of receptor-containing tissue where necessary, as it was judged less harmful than any other storage regime.

To assess the time period over which receptor viability could be maintained under these conditions, a large piece of breast tumour (approximately 750 mg) was divided into several portions of approximately 150 mg each. The oestrogen receptor content of these was assayed after various lengths of storage. Table 1 demonstrates that the receptors, when tissue was retained in this storage solution, were stable for up to three weeks. However, the results in Table 1 may be fortuitously good, as variation in receptor content across tissue can occur (see Section 3.2.1). Rat uteri stored in this way have been found to contain functional receptor after up to six months. Breast tissue might also be stored for this length of time if necessary, although full investigation of the effects of long-term storage on this tissue has not been carried out (preliminary results from our laboratory show that receptor was stable in stored breast tumour tissue for up to three months).

Storage in solid CO_2 and lyophilization of material have been found to maintain oestrogen receptor presence (see section 3.3). These processes

Table 1 Oestrogen Receptor Content of Breast Tumour Tissue Stored Under Sucrose Buffer (0.25 M Sucrose, 1.5 mM $MgCl_2$, 10 mM HEPES, pH 7.4)/ 50% Glycerol (v/v) for Varying Lengths of Time

A large piece of breast tumour tissue was divided into portions of approximately 150 mg. The oestrogen receptor content of one portion was determined immediately as described in the methods section. The other slices were stored in sucrose buffer/50% glycerol (v/v) at -20°C for varying lengths of time before rehydration at 0°C in sucrose buffer, followed by oestrogen receptor determination as for the fresh portion.

Time of storage (Days)	Oestrogen receptor level (fmol /mg cytosol protein)
0	396
9	316
14	421
18	400

do not interfere with receptor binding to oestradiol, thus permitting measurement of the receptor level. However, whether the receptor remains functional after such treatment has still to be determined. The effects of storage of tissue in liquid nitrogen on oestrogen receptor levels have not been investigated fully. This form, followed by pulverization, is used by many workers for the preparation of tissue fractions. However, samples of breast tumour tissue stored in this way have often yielded a high level of cytosol receptors, while no nuclear binding could be detected. This method of storage, like freezing in buffer at low temperatures, thus has a selective deleterious effect on the nuclear receptors, emphasising that they may be less stable than the cytoplasmic form (see section 3.1.3.1).

3.1.2 TISSUE FRACTIONATION

The method used to prepare cytosol and nuclear fractions from all types of tissue handled was as detailed in section 2.2.1.3. As stated therein, the fractions prepared are crude in nature, but, as noted also by McGuire et al., (1977c) when preparing breast tumour cytosol for oestrogen receptor analysis, no purification process significantly enhanced the yield of receptors. In this project, this was found to be the case for both cytosol and nuclear fractions. In addition to purification attempts, studies were also carried out to ensure that oestradiol binding observed in the nuclear fraction represents a separate nuclear receptor and does not merely reflect an insufficient degree of washing the nuclear pellet, leading to contamination of a cytoplasmic origin. To establish this, human breast tumour tissue was homogenised and centrifuged as described in section 2.2.1.3 to produce a cytoplasmic fraction and nuclear pellet. The pellet was split into two equal portions at this point and washed with either saline or Triton X-100

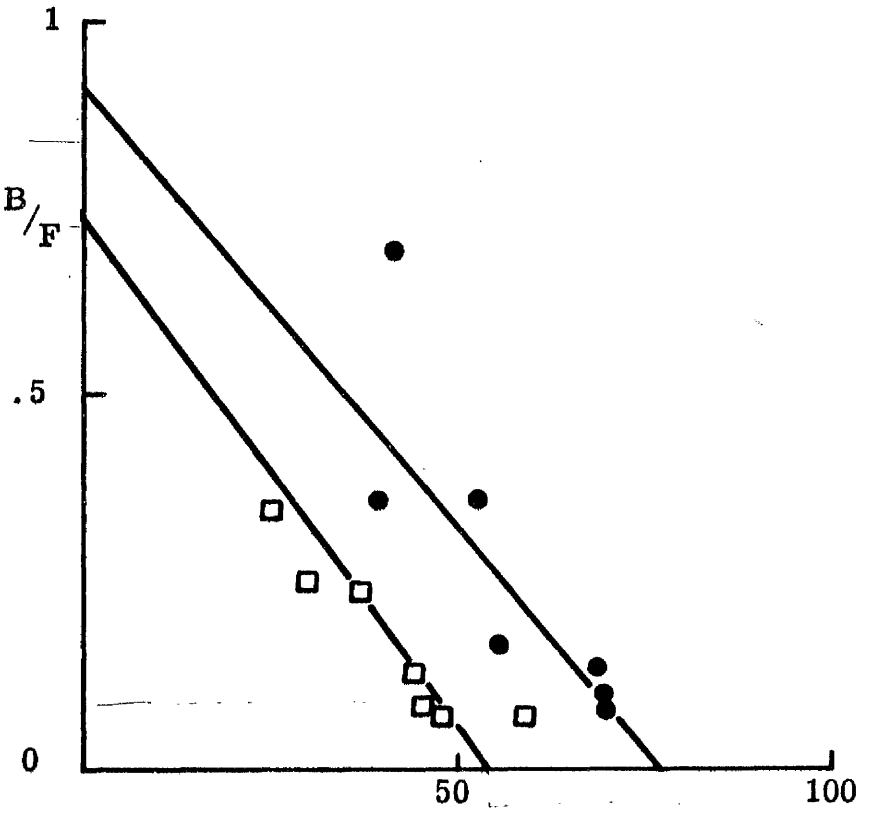
(0.1% in homogenisation buffer). The Triton X-100 should remove any cytoplasmic material from the nuclear pellet.

The results in Figure 6 show that the use of this detergent has slightly decreased the level of receptors in the nuclear fraction, as determined by Scatchard plot analysis. It is also clear, however, that the dissociation constants of binding, which are derived from the slopes of the lines (see Section 2.2.4), are virtually identical. From the very small difference in receptor level made by the inclusion of a detergent wash, and from the finding that, in some tissue, receptor is detected exclusively in the nuclear fraction (see section 3.4.1), it is clear that there is in fact a separate nuclear receptor for oestradiol. The satisfactory abolishment of cytoplasmic material from the nuclear fraction is also demonstrated, emphasising the acceptability of the method of preparation. Thus, a step incorporating a Triton X-100 wash of the nuclear pellet to remove any residual material of non-nuclear origin could be added to the standard procedure for the fractionation of tissue, although little effect on the level of nuclear receptors would result.

The presence of a thiol reagent during the preparation of tissue fractions is required to protect the sulphydryl groups reported to participate in the interaction between receptor and oestradiol (Jensen et al., 1967, Muldoon, 1971). McGuire & De La Garza (1973b) found that the thiol reagent dithiothreitol (DTT) was satisfactory for this purpose, 8S receptor being completely lost in its absence at all temperatures and still very labile at high temperatures even in its presence. In my experience, DTT successfully maintained the integrity of receptors in rat uterine and human breast and endometrial tumour tissue at 0°, 4°, 15° and $20^{\circ}C$ for up to 24 hours,

Figure 6 - Effect of Washing with Triton X-100 on Nuclear Receptor Level

The nuclear pellet of breast tumour tissue was prepared as in Section 2.2.1.3. Before resuspension, it was washed with either 0.1% Triton X-100 (□-□) or saline(●-●). Nuclear suspensions were formed from each pellet, and the receptor concentration in them determined by Scatchard analysis.


1
B/F
.5
0
50
100
conc. bound (p molar)

and for a short time at 37°C. Keightley et al., (1978), however, have subsequently found no difference in binding characteristics with or without DTT in studies on myometrial receptor.

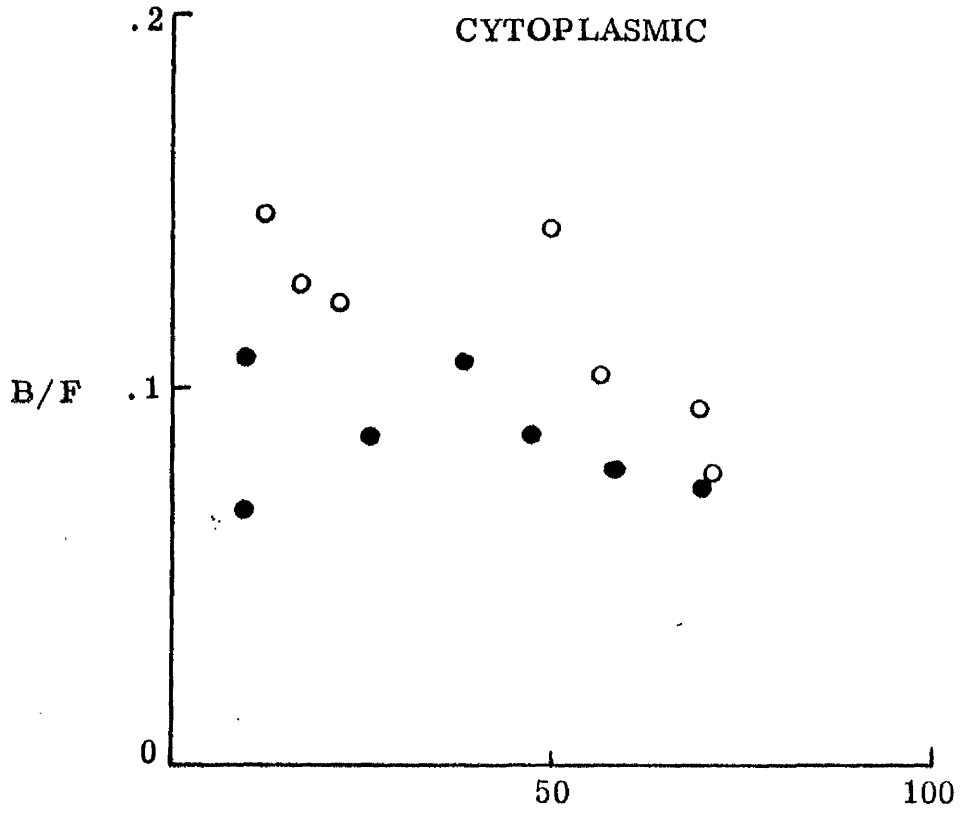
Failure to detect oestrogen receptors in human colonic carcinoma tissue (see Section 3.4.7) was thought possibly to reflect some inadequacy of DTT not previously encountered. A comparison was drawn, therefore, between the efficacy in colonic tissue of 0.25 MM DTT and 10 mM monothioglycerol, another thiol agent. Figure 7 shows a representative result of such a comparison. Neither agent is successful in producing a Scatchard plot, which would suggest the fault lies not in the thiol reagent employed, but in the receptor deficiency of the tissue. 0.25 mM DTT and 10 mM monothioglycerol were equally effective in protection of the receptor in breast tissue. DTT was preferred due to ease of handling. This concentration of DTT (0.25 mM) is high enough to protect the receptor without interfering in the determination of the cytoplasmic protein content.

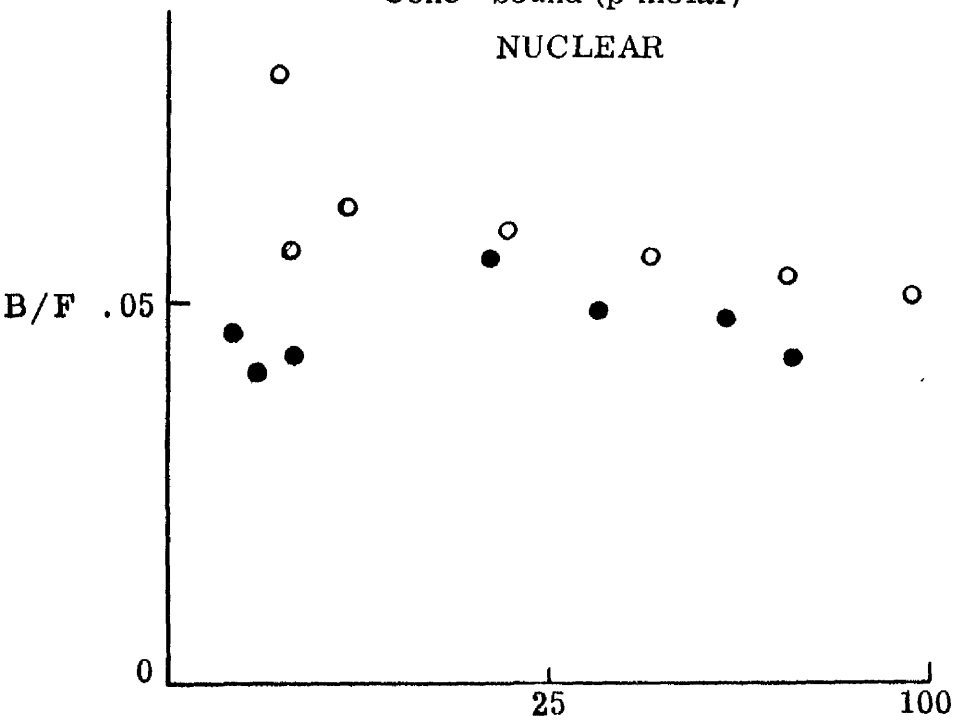
Another necessary constituent of the homogenisation buffer was ethylenediaminetetracetic acid (EDTA). This was found to be essential, especially where nuclear binding was being measured, as the nuclear receptor appeared to be inhibited by the presence of magnesium and calcium ions. EDTA has been reported to be harmful to cellular organelles (Wittliff, 1975), but this does not appear to effect the system under study here.

A further consideration in the preparation of tissue fractions was the selection of the type of homogeniser for the fractionation process. Initial, rough chopping of the tissue was carried out using an Ultra-turrax homogeniser,

Figure 7 - Comparison of DTT (0.25 mM) and Monothioglycerol (10 mM) Effectiveness in Detection of Oestrogen Binding in Biopsies from Human Colonic Carcinoma Tissue

Colonic carcinoma tissue was homogenised in HEPES-EDTA buffer, pH 7.4, containing either 0.25 mM DTT (O) or 10 mM monothioglycerol (●). Cytoplasmic and nuclear fractions were prepared as described in Section 2.2.1.3, and their receptor content determined by Scatchard analysis.


CYTOPLASMIC
.2
B/F
.1
0
50
100
Conc bound (p molar)


NUCLEAR
B/F
.05
0
25
100
Conc. bound (p molar)

but further, finer homogenisation was then required to produce an even homogenate. The two types of homogeniser available comprise a glass tube with either a ground glass or teflon-coated pestle. Experiments carried out on different tissue types comparing the two forms of homogeniser showed little difference in human colonic or endometrial tissue or rat uterine tissue, but with human breast tumour tissue, the yield of receptors was greatly depleted after teflon/glass homogenisation, in agreement with a finding reported by Wittliff (1975). Consequently, glass/glass homogenisation was used with all types of tissue prior to receptor measurement, although teflon/glass homogenisation yielded a better endometrial nuclear preparation, as determined by histological examination.

3.1.3 BINDING OF STEROID TO RECEPTORS

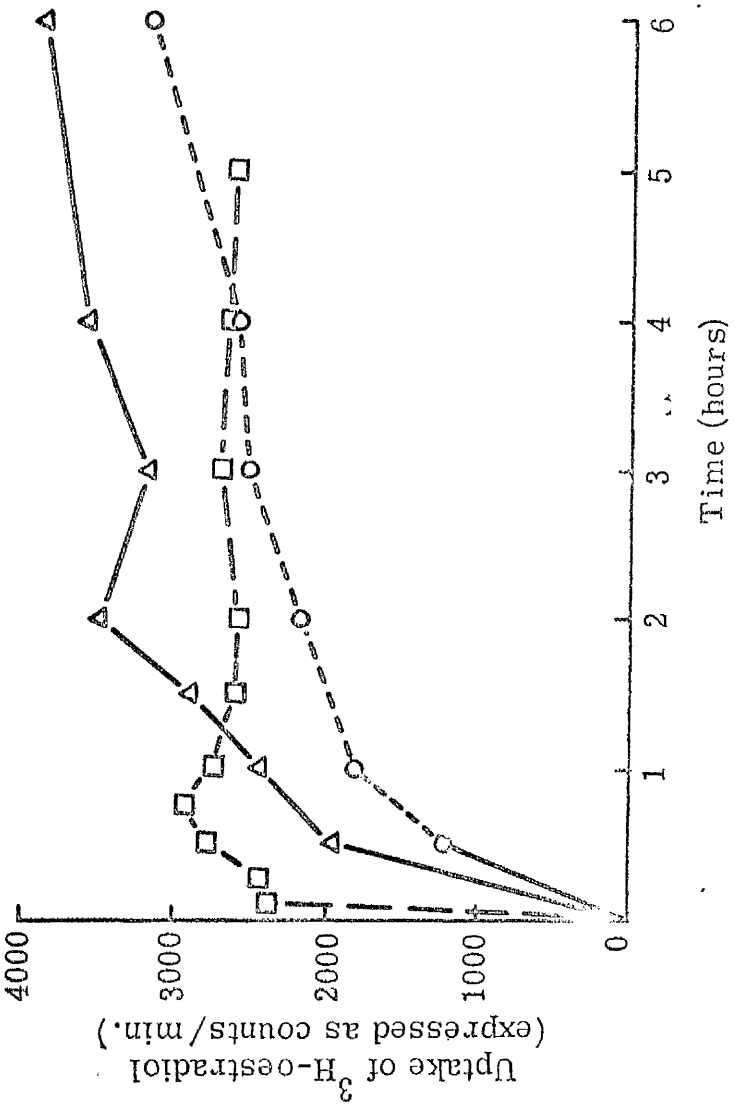
In order to construct a system where the maximum binding of oestradiol would occur, and thus the most accurate measure of receptor content result, the optimal values for the time, temperature and range of oestradiol concentrations were required.

3.1.3.1 Time and Temperature

The thermolabile nature of the receptors and the stability of the hormone/receptor complex were investigated in a series of experiments in which either the uptake or displacement of labelled oestradiol was measured at different temperatures, over various lengths of time. Figure 8 shows the uptake of labelled steroid into the uterine nuclear fraction of a mature rat sacrificed on the day of pro-oestrous, when receptor content is thought to be at its highest although some controversy surrounds this timing, as discussed in the Introduction. A clear contrast can be seen between the

Figure 8 - Uptake of ^{3}H-Oestradiol-17β into the Nuclear Fraction of Mature Rat Uterus

Nuclear suspension from a mature rat uterus was prepared as in Section 2.2.1.3. Aliquots were incubated with ^{3}H-oestradiol-17β (10^{-9} M) at 0°C (O--O), 20°C (Δ - Δ) and 37°C (□ --□). Samples were removed at various time points, applied to GFC filter discs, and unbound steroid removed by washing, as described in Section 2.2.3.2.


Uptake of ^{3}H-oestradiol
(expressed as counts/min.)
4000
3000
2000
1000
0
1
2
3
4
5
6
Time (hours)

situation at 37°C compared to 20°C and 0°C. At 37°C there is a rapid initial uptake of oestradiol into the nuclear fraction, which then appears to decline gradually. At the lower temperatures, there is a much more gradual uptake of label to a level which is maintained for up to 6 hours.

The same experiment was carried out on the nuclear fraction of human breast tumour tissue (see Figure 9). In this case,the instability of the receptor at 37°C is even more pronounced, with the same rapid uptake observed in the rat tissue being followed by a dramatic fall, presumably due to receptor degradation. At the lower temperatures of 4°C and 15°C, the receptor was stable for as long as 24 hours.

Figure 9 illustrates the situation in the nuclear fraction of tissue from a postmenopausal patient. When the same experiment was carried out on tissue from a premenopausal patient, a clearly different result was obtained, as shown in Figure 10. The receptor in the nuclear fraction of the premenopausal patient's tissue appears to be much more stable at 37°C than was that from the postmenopausal example. The rapid uptake of ^{3}H-oestradiol into the nuclear fraction is similar in both cases, but the premenopausal level rises to a maximum higher than that obtained at 4°C or 15°C and is maintained at this value, even up to 24 hours. This suggests that the receptor is much more stable in the highly active situation found in a premenopausal woman, where circulating levels of oestrogen are high. This could also account for the intermediate level of resistance to heat found in the receptors of the uterine nuclear fraction from a cycling rat. The study was carried out on only one premenopausal patient, however, due to the scarcity of large biopsies of premenopausal tissue containing receptors. It is realised that variation could occur between individuals,

Figure 9 - Uptake of ^{3}H-Oestradiol-17β into the Nuclear Fraction of Postmenopausal Breast Tumour Tissue

Nuclear suspension was prepared from breast tumour tissue of a postmenopausal patient as described in Section 2.2.1.3. Aliquots of the suspension were incubated with ^{3}H-oestradiol-17β (10^{-9} M) at 4°C (O - O), 15°C (▲-▲) and 37°C (△-△). Samples were removed at various time points, applied to GFC filter discs, and unbound steroid removed by washing as described in Section 2.2.3.2. The insert shows the Scatchard plot of nuclear binding, which yielded a receptor concentration of 2560 fmol/mg DNA and a Kd of 1.1×10^{-10}M.

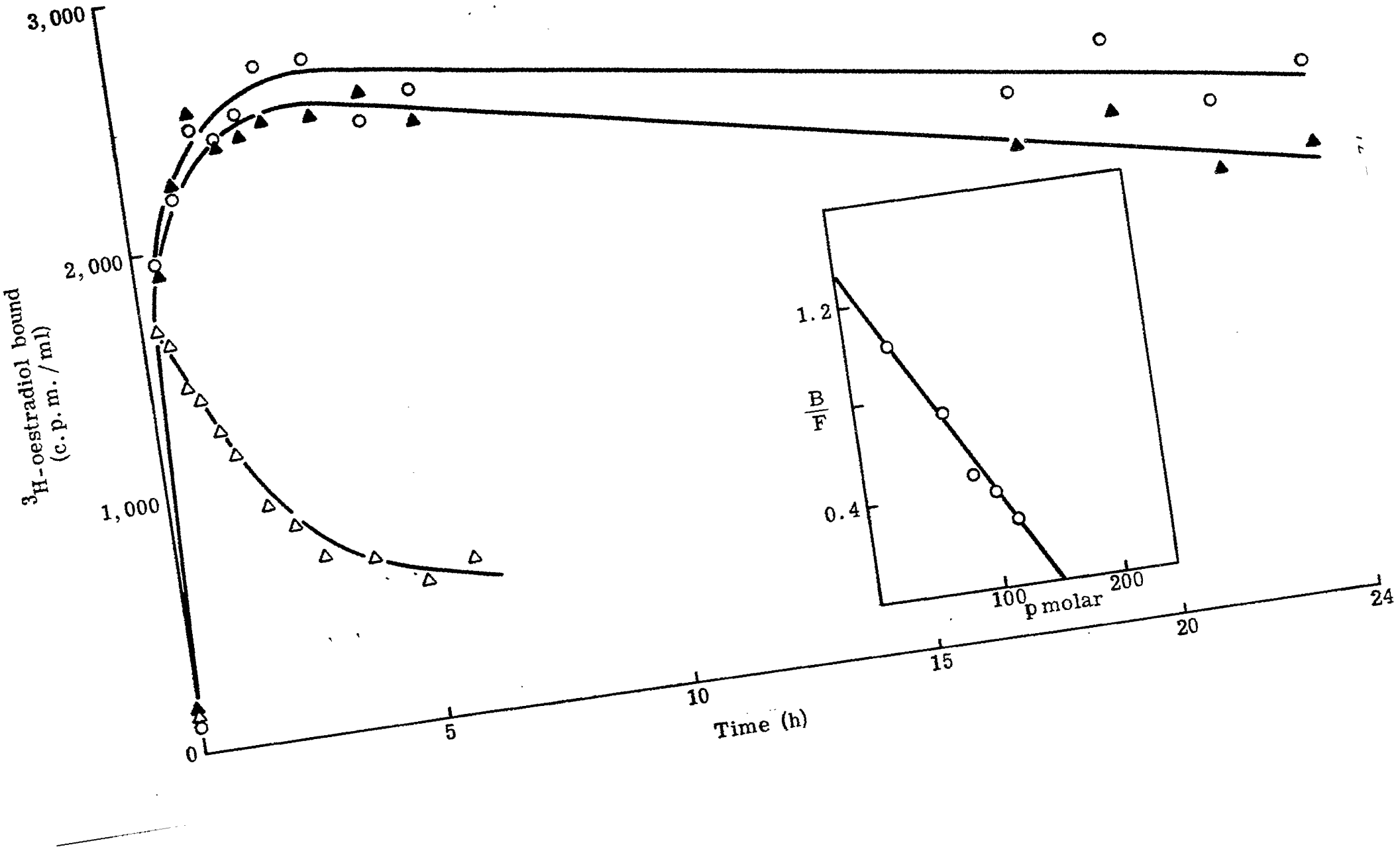
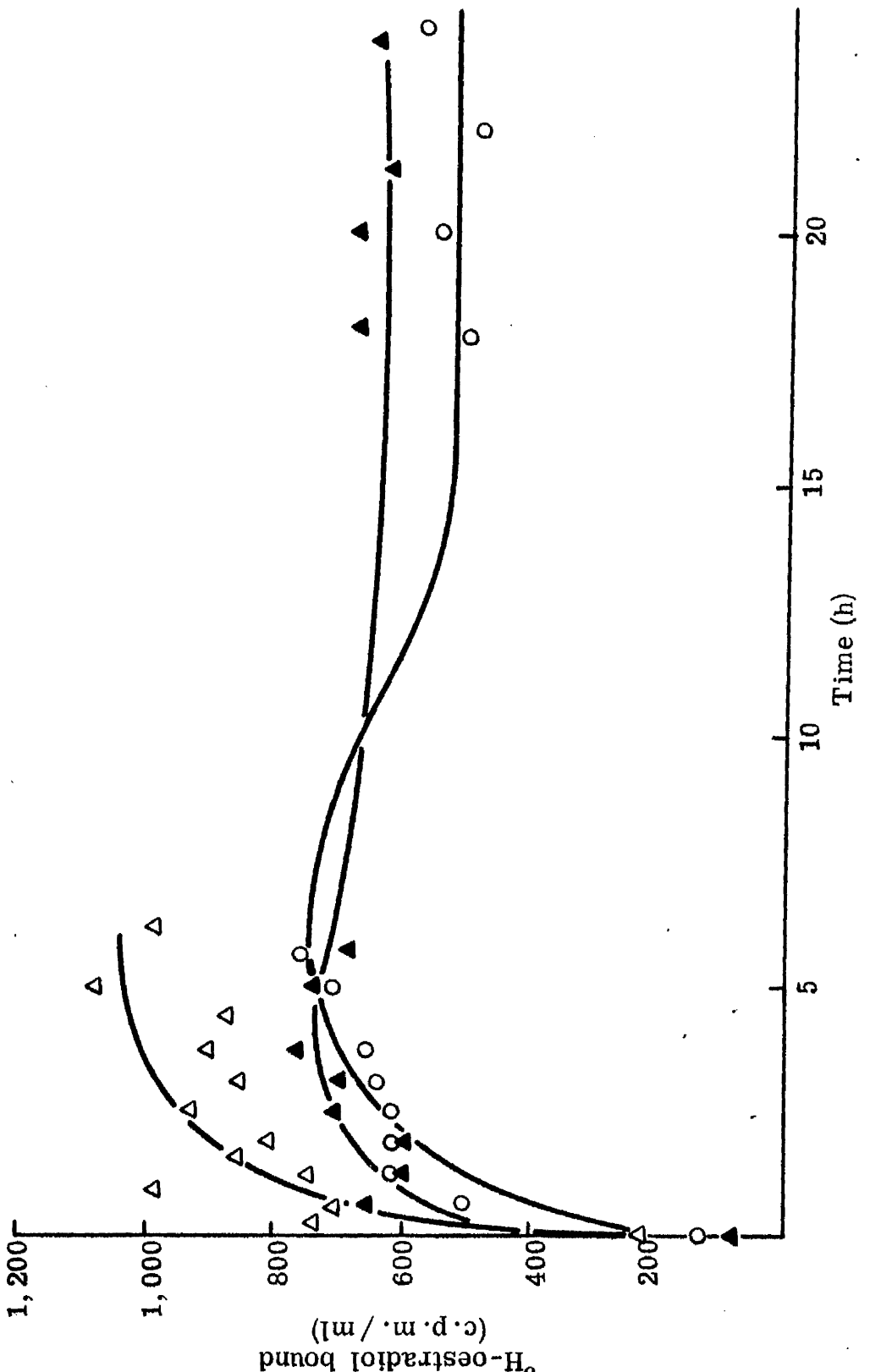

3,000
2,000
1,000
0
^{3}H-oestradiol bound (c.p.m./ml)
5
10
15
20
24
Time (h)
1.2
0.4
B/F
100
200
p molar

Figure 10 - Uptake of ^{3}H-Oestradiol-17β into the Nuclear Fraction of Premenopausal Breast Tumour Tissue

Nuclear suspension was prepared from breast tumour tissue of a premenopausal patient as described in Section 2.2.1.3. Aliquots of the suspension were incubated with ^{3}H-oestradiol-17β (10^{-9} M) at 4°C (O - O), 15°C (▲ - ▲) and 37°C (Δ - Δ). Samples were removed at various time points, applied to GFC filter discs, and unbound steroid removed by washing as described in Section 2.2.3.2


^{3}H-oestradiol bound
(c.p.m./ml)
1,200
1,000
800
600
400
200
5
10
15
20
Time (h)

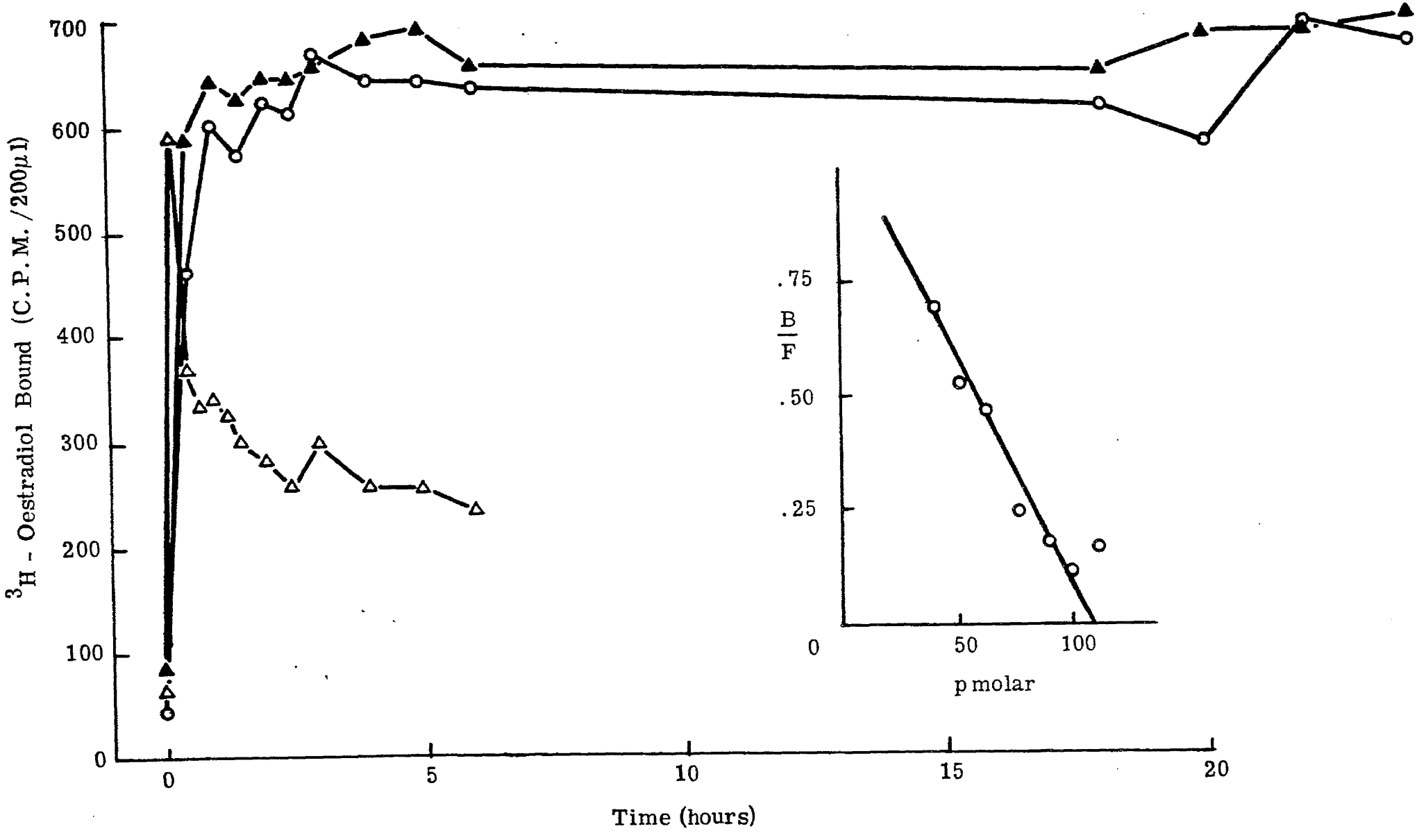
and the results shown in Figure 10 may reflect an atypical situation. Further investigation may serve to confirm the increased stability of the premenopausal receptor, or may reveal the existence of variability from one patient to another.

The situation in the cytoplasmic fraction of human breast tumour tissue was also investigated. Cytosol was prepared from a receptor-positive breast tumour from a postmenopausal patient. The uptake of ^{3}H-oestradiol into the cytosol was measured at 4°C, 15°C and 37°C as shown in Figure 11. As with the postmenopausal nuclear fraction, the uptake declines rapidly at 37°C, but is maintained at the lower temperatures for up to 24 hours, again reflecting the instability of the binding at the elevated temperature.

It has been suggested that any assay conditions which have been described for the detection of cytosol oestrogen receptors could measure only "unfilled" sites, and not those bound to ligand. It is considered that these unfilled receptors would be less common in the cycling female, and this has been used as an explanation for the lower levels of receptor found in the cytoplasm of premenopausal, as opposed to postmenopausal, breast tumour tissue (see Section 3.4.6) (Sakai & Saez, 1976). It has been proposed that circulating levels of oestradiol should be taken into account when measuring cytosol receptor because of this (Mobbs & Johnson, 1976, Nagai et al., 1979), although results obtained here suggest that this would be of no great value (see Section 3.4.5.5.). However, Korenman & Dukes (1970), Feherty et al., (1971) and Liskowski & Rose (1976) all argue that, using a dextran-coated charcoal method similar to that used in this project, all cytoplasmic sites are measured, and that this proposal is invalid. There is also opposition

Figure 11 - Uptake of ^{3}H-Oestradiol-17β into the Cytosol Fraction of Postmenopausal Breast Tumour Tissue

A cytoplasmic preparation was made from breast tumour tissue of a postmenopausal patient as described in Section 2.2.1.3. Aliquots were incubated with ^{3}H-oestradiol-17β (10^{-9} M) at 4°C (O - O), 15°C (▲- ▲) and 37°C (Δ- Δ). Samples were taken at various time points, and unbound steroid removed by adsorption on to dextran-coated charcoal, as described in Section 2.2.3.2. The insert shows the Scatchard plot of cytoplasmic binding, which yielded a receptor concentration of 63 fmol/mg cytosol protein and a Kd of 0.99×10^{-10} M.


700
600
500
400
300
200
100
0
^{3}H - Oestradiol Bound (C.P.M./200µl)
0
5
10
15
20
Time (hours)
.75
B/F
.50
.25
0
50
100
p molar

to the idea that "filled" nuclear receptors are being measured by an exchange technique similar to that of Anderson et al., (1972) at low temperatures of $0^{o}C$ or $4^{o}C$. Katzenellenbogen et al., (1973) stated that the exchange rate at these low temperatures is very little in uteri of immature rats. Following this, unfilled nuclear receptors were discovered firstly in MCF7 cells (Garola & McGuire, 1977a) and then in breast tumour tissue (Panko & MacLeod, 1978, Kato et al., 1978, Thorsen, 1979). On the basis of this, the concept suggested for cytosol binding by Chamness et al., (1975) that incubation at $0^{o}C$ or $4^{o}C$ caused filling of empty receptors, while exchange occurred only at $30^{o}C$ is now proposed to hold true for nuclear binding also (Garola & McGuire, 1977a, Panko & MacLeod, 1978, Thorsen, 1979). However, the salt extraction of the receptors employed in these studies may alter their properties relative to those displayed when the receptor is bound to chromatin.

In order to clarify the situation as to whether exchange is possible during incubation at different temperatures, a further group of experiments was designed which measured the dissociation of ^{3}H-oestradiol from both cytosol and nuclear fractions of breast tumour tissue in the presence of excess unlabelled steroid. Cytosol and nuclear fractions were prepared as described in section 2.2.1.3 and incubated with $2 \times 10^{-9}M$ ^{3}H-oestradiol, the cytosol at $0^{o}C$ and the nuclear at $37^{o}C$ both for 30 minutes in order to fill all the receptor sites. Unbound steroid was then removed, either by the action of dextran-coated charcoal stripping or by washing; for the cytosol and nuclear fraction respectively. Incubation of the fractions was then carried out in the presence of $10^{-7}M$ unlabelled oestradiol at $4^{o}C$ and $20^{o}C$, and the dissociation of labelled steroid measured by the amount of activity remaining in aliquots sampled at various times.

The result of such an experiment performed on the nuclear fraction of post-menopausal breast tumour tissue is shown in Figure 12. Both at $4^{o}C$ and $20^{o}C$, considerable exchange of the unlabelled for labelled steroid has occurred. The same situation is observed when the experiment is performed on the cytoplasmic fraction of the same tissue (Figure 13). Again, much exchange of the unlabelled for labelled oestradiol occurs at both temperatures. That this is exchange rather than heat or protease digestion of the receptor can be demonstrated by the fact that the same concentration of receptors is recoverable in each fraction by a second exchange at the end of 24 hours. Figure 14 shows a comparison of the displacement of ^{3}H-oestradiol by unlabelled oestradiol in the two tissue fractions at $4^{o}C$ or $20^{o}C$. At each temperature, approximately the same amount of interaction occurs in both fractions. However, slightly more displacement occurs at $20^{o}C$ than at $4^{o}C$ and, by 2 hours the level of exchange at $20^{o}C$ is as high as it reaches within 24 hours at $4^{o}C$, although this is only 80%. This observation could be of value in designing a more rapid method of assaying oestrogen receptor content. It has been confirmed by comparing the Scatchard plots obtained after incubation of cytosol and nuclear fractions from breast tumour tissue with oestradiol at $20^{o}C$ for 2 hours and at $4^{o}C$ for 18 hours. These findings are also consistent with those obtained from immature rat uterine cytosol which was preincubated with $10^{-9}M$ unlabelled oestradiol and then incubated with ^{3}H-steroid as above. The resultant Scatchard plots are shown in Figure 15. Virtually no difference in receptor level or dissociation constant of binding can be seen between the two situations.

The results from this set of experiments confirm that exchange of added for bound steroid may occur in both the cytoplasmic and nuclear fractions of

Figure 12 - Dissociation of ^{3}H-Oestradiol-17β from the Nuclear Fraction of Breast Tumour Tissue in the Presence of Excess Unlabelled Oestradiol at 4°C and 20°C

Nuclear suspension was prepared from breast tumour tissue as described in Section 2.2.1.3. Preincubation was carried out in ^{3}H-oestradiol-17β (2×10^{-9} M) at 37°C for 30 minutes, followed by removal of unbound material by washing in HED buffer. Aliquots were then incubated with unlabelled oestradiol-17β (10^{-7} M) at 4°C (X - X) and 20°C (□-□). Samples were removed at various time intervals, applied to GFC filter discs, and unbound steroid washed away as described in Section 2.2.3.2. The activity remaining at each time point was expressed as a percentage of the initial value.

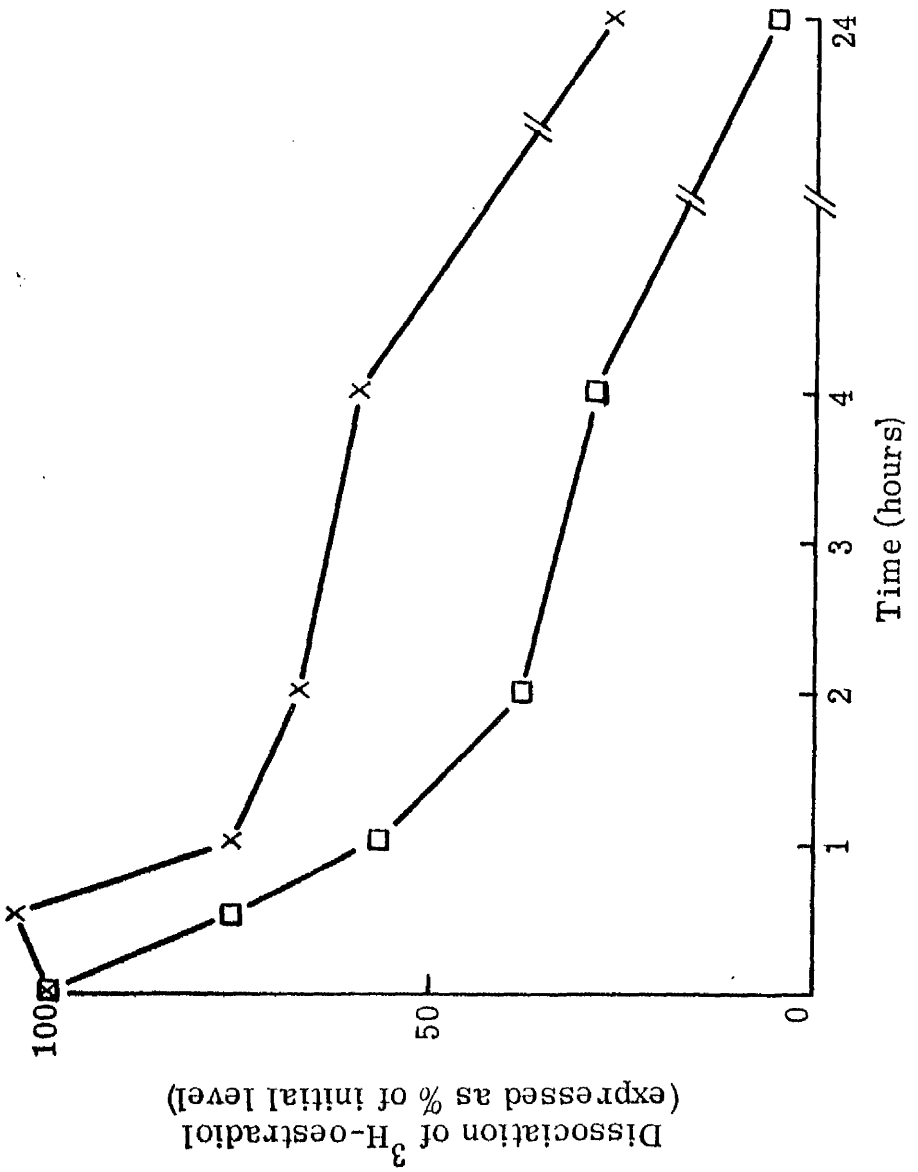

Dissociation of ^{3}H-oestradiol
(expressed as % of initial level)
100
50
0
1
2
3
4
24
Time (hours)

Figure 13 - Dissociation of ^{3}H-Oestradiol-17β from the Cytosol Fraction of Breast Tumour Tissue in the Presence of Excess Unlabelled Oestradiol at 4°C and 20°C

Cytosol was prepared from breast tumour tissue as described in Section 2.2.1.3. Preincubation was carried out in ^{3}H-oestradiol-17β (2×10^{-9} M) at 0°C for 30 minutes, followed by removal of unbound steroid by dextran-coated charcoal stripping as described in Section 2.2.3.2. Aliquots were then incubated with unlabelled oestradiol-17β (10^{-7} M) at 4°C (X - X) and 20°C (□ - □). Samples were removed at various times, and any unbound steroid adsorbed on to dextran-coated charcoal as before. The activity remaining at each time point was expressed as a percentage of the initial value.

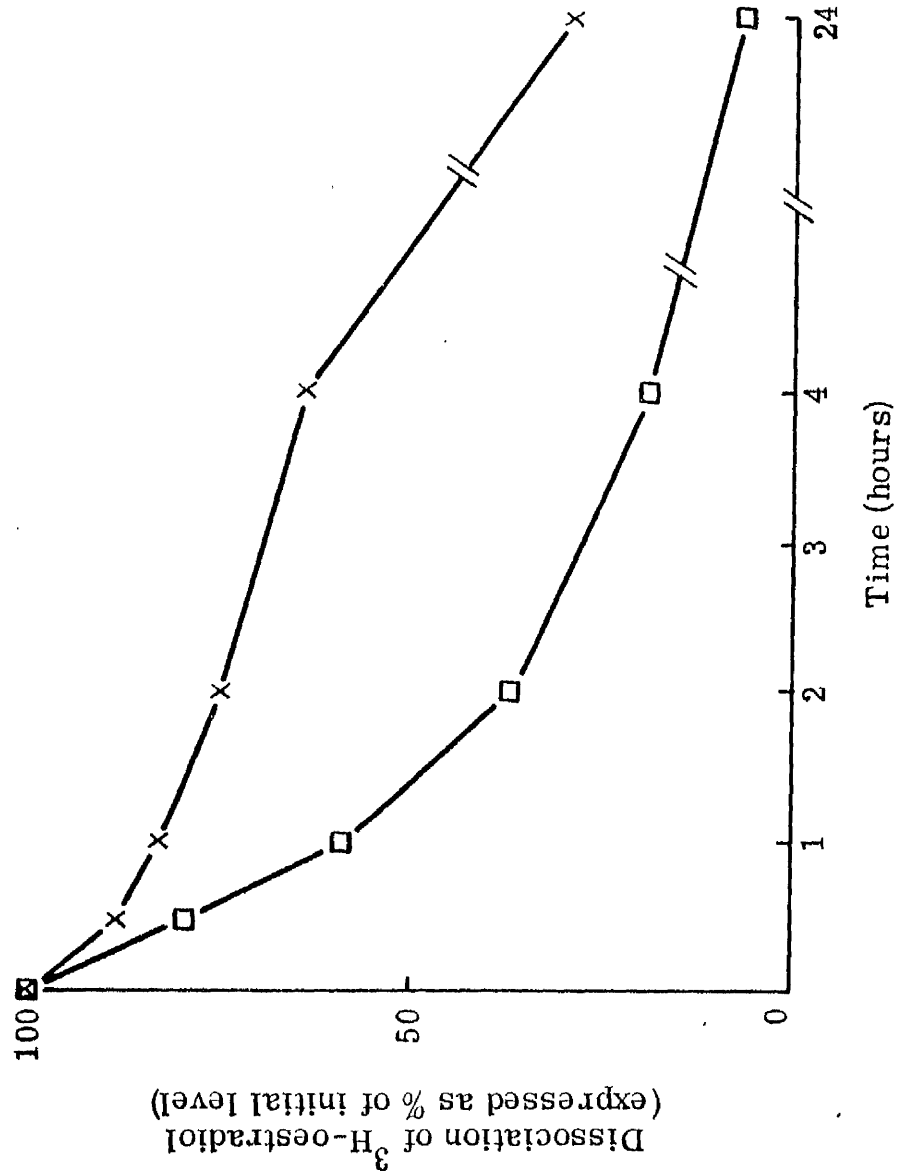

Dissociation of ^{3}H-oestradiol
(expressed as % of initial level)
100
50
0
1
2
3
4
24
Time (hours)

Figure 14 - Comparison of Dissociation of ^{3}H-Oestradiol-17β from the Cytosol and Nuclear Fractions of Breast Tumour Tissue in the Presence of Excess Unlabelled Oestradiol at 4°C and 20°C.

Breast tumour tissue was fractionated by the procedure described in Section 2.2.1.3. The cytoplasmic and nuclear fractions were then preincubated with ^{3}H-oestradiol-17β (2×10^{-9} M) for 30 minutes at 0°C and 37°C respectively, followed by removal of unbound steroid by dextran-coated charcoal stripping of the cytosol or washing of the nuclear fraction. Aliquots of each were incubated with unlabelled oestradiol (10^{-7} M) at 4°C and 20°C. Samples were removed at various time intervals and unbound steroid removed by the methods described in Section 2.2.3.2. Dissociation, in each case, was expressed as percentage of initial activity. ▲---▲ represents dissociation from the cytosol at 4°C, X - X that from the nuclear fraction at 4°C, ■---■ that from the cytosol at 20°C and □ - □ that from the nuclear fraction at 20°C.

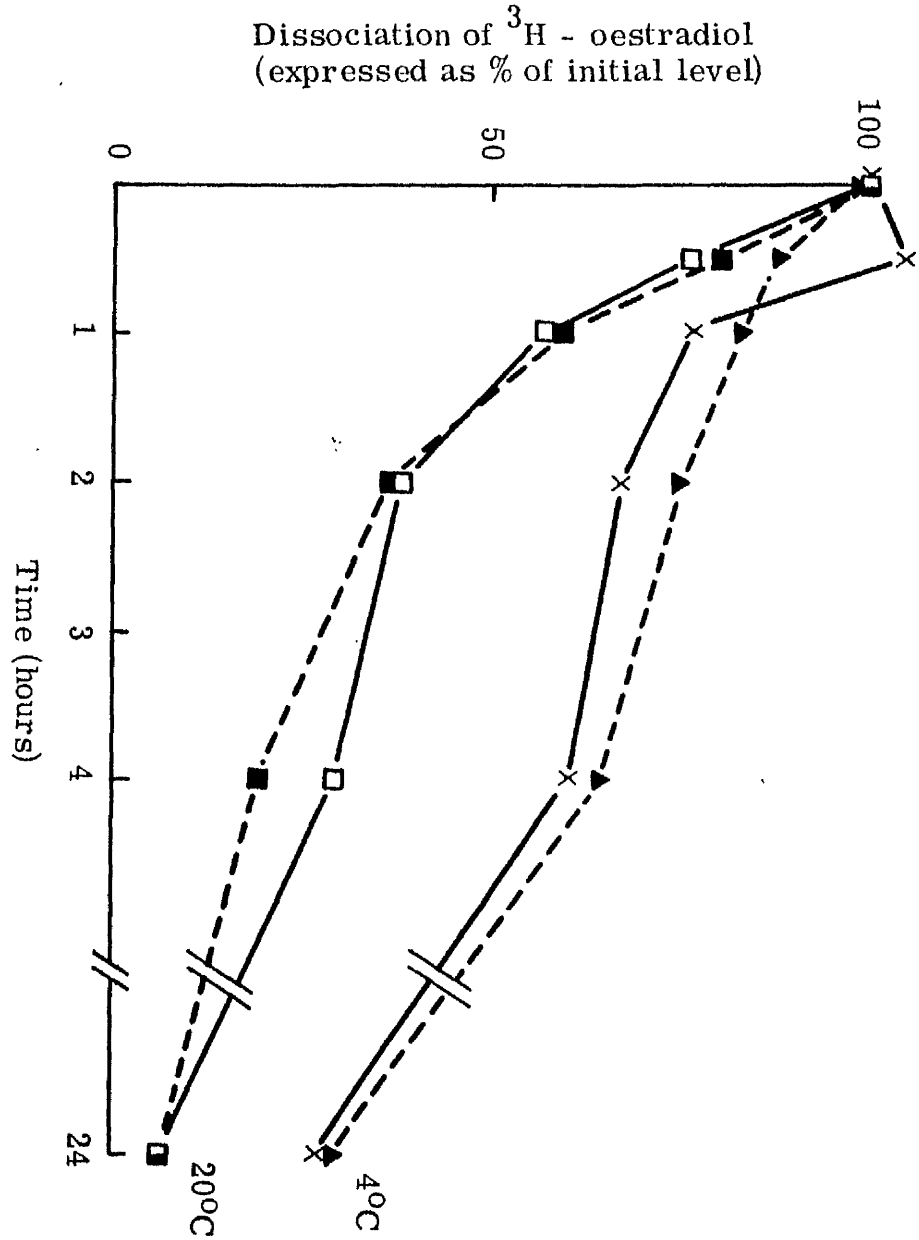
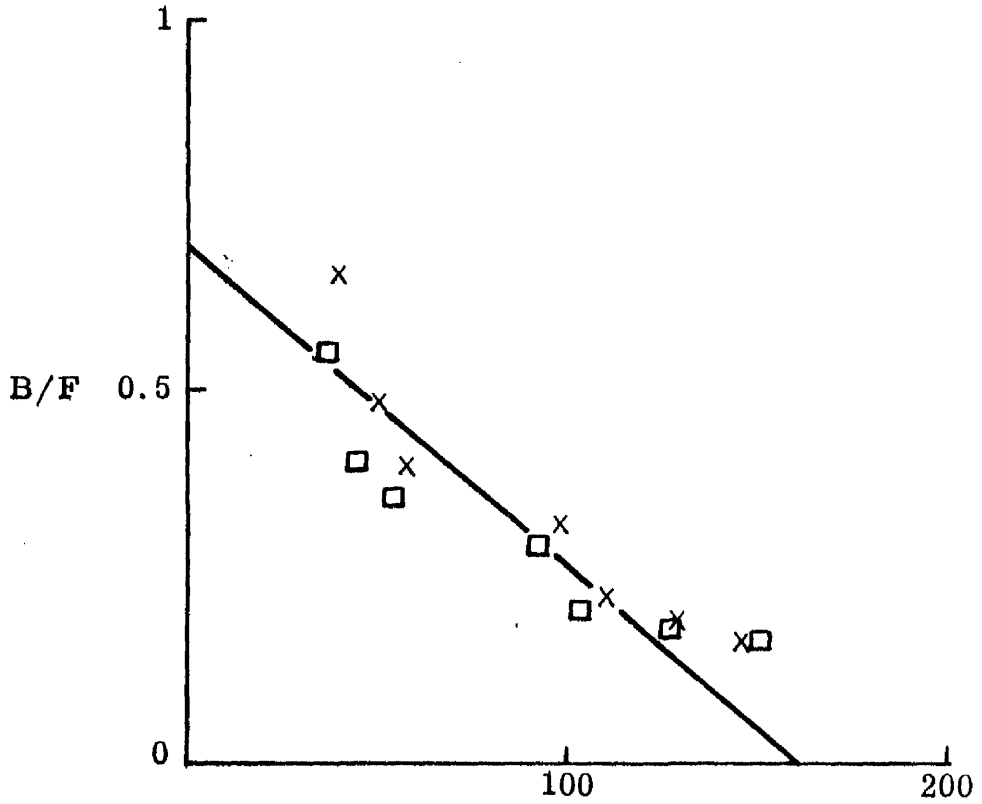

Dissociation of ^{3}H - oestradiol
(expressed as % of initial level)
0
50
100
1
2
3
4
24
Time (hours)
20°C
4°C

Figure 15 - Comparison of Scatchard Analysis of ^{3}H-Oestradiol-17β Binding at 4°C and 20°C to Rat Uterine Cytosol Pre-incubated with Unlabelled Oestradiol

Cytoplasmic fraction from immature rat uterus was prepared as described in Section 2.2.1.3. Pre-incubation was carried out with unlabelled oestradiol-17β (2×10^{-9} M) at 0°C for 30 minutes, and unbound steroid removed by dextran-coated charcoal stripping. The cytosol was then incubated with a range of ^{3}H-oestradiol-17β concentrations from 10^{-10} M - 10^{-9} M at 4°C for 18 hours (X - X) and 20°C for 2 hours (□-□). Unbound labelled steroid was removed as described in Section 2.2.3.2, and the receptor concentration determined by Scatchard analysis.


1
B/F 0.5
0
100
200
Conc bound (p molar)

breast tumour tissue at $4^{o}C$, although slightly less than at the higher temperature of $20^{o}C$. This, in turn, suggests that during the assay of cytoplasmic and nuclear receptors in breast tumour tissue, at low temperature total receptor concentration is determined, and not merely the level of "unfilled" sites. However, the possibility remains that a small proportion of total filled sites remains unexchanged after 18 hours at $4^{o}C$. A further observation which can be made is that, in the case of postmenopausal breast tissue at least, no alteration in stability of the hormone/receptor complex results from its binding to nuclear acceptor sites.

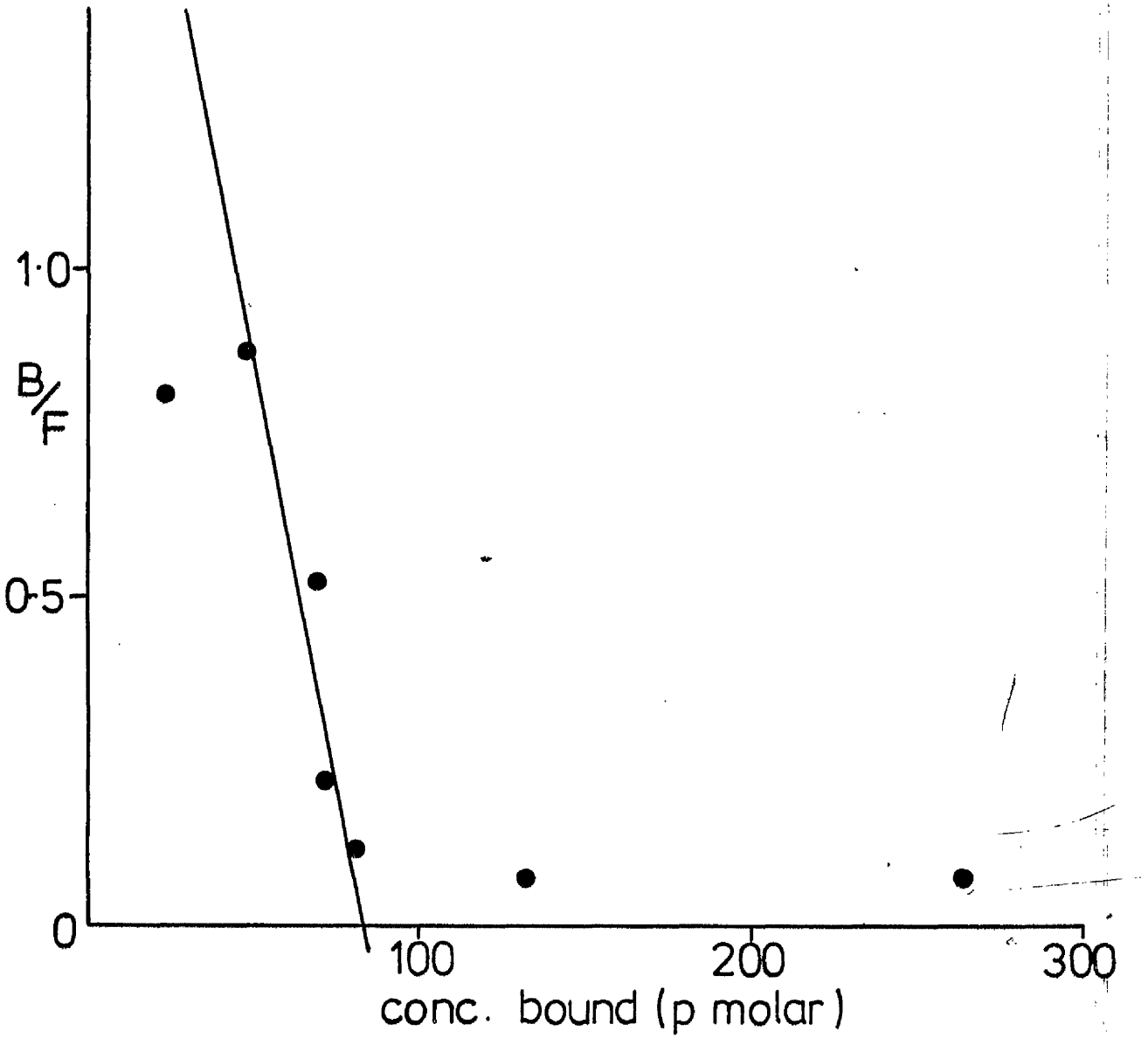
For standard measurement of oestrogen receptors in breast tumour tissue, it was concluded that incubation with ^{3}H-oestradiol should be carried out at either $4^{o}C$ for at least 18 hours or $20^{o}C$ for 2 hours. Both of these times give only 80% exchange of added oestradiol for any endogenous oestradiol, but careful observance of these conditions on a routine basis will ensure that the same amount of exchange can occur on each occasion. As observed by Hawkins et al., (1975), the use of these lower temperatures is advisable, because, as demonstrated in the experiments on the uptake of ^{3}H-oestradiol, at $37^{o}C$, equilibrium is not properly established before receptor degradation commences.

3.1.3.2 Range of Oestradiol Concentrations

To determine the most suitable range of oestradiol concentrations for the detection of oestrogen receptors, Scatchard plots were constructed from the results of binding experiments using wide ranges centred about the value of $2 \times 10^{-10}M$, which represents the circulating level of oestrogens in the human body. Figure 16 shows a representative example of such a plot. The range used here is $5 \times 10^{-11}M - 4 \times 10^{-9}M$ ^{3}H-oestradiol. The best

Figure 16 - Scatchard Plot of Results Obtained from an Oestrogen Receptor Assay Carried Out on Breast Tumour Cytosol Using a Range of ^{3}H-Oestradiol-17β Concentrations of 5×10^{-11} M - 4×10^{-9} M.

Receptor analysis was carried out on the cytosol of a breast tumour biopsy, adopting the procedure summarised in Figure 19. ^{3}H-oestradiol-17β was added in concentrations from 5×10^{-11} M to 4×10^{-9} M in order to assess the optimal range for receptor detection. Scatchard analysis was performed on the results obtained, and a plot constructed.


1·0
B/F
0·5
0
100
200
300
conc. bound (p molar)

range of concentrations can be chosen from the sloping part of the graph. At the end of the plot nearest to the origin, the concentrations are too low to detect all the receptor present. Chamness & McGuire (1975) have suggested that there is also more chance of receptor degradation when the concentration of ligand is low, accounting for the apparent decrease in binding. At the other end of the range, the concentrations used more than saturate the amount of receptor present, and the line levels off due to the introduction of non-specific, or low affinity, binding as observed also by McGuire (1975). The optimal range for the measurement of oestrogen receptors thus lies in the mid-part, and expansion of this part should give a more meaningful plot. From these studies, oestradiol in the range 10^{-10}M - 10^{-9}M was chosen as being most effective in the detection of oestrogen receptors from all tissue types examined.

3.1.3.3 Determination of Specificity

In order to ensure that the binding demonstrated in these assays is due to specific oestradiol receptor, not only should a Scatchard plot be constructed to enable determination of the dissociation constant of the interaction, but also the specificity for oestrogen binding should be checked. Specific binding is, by definition, saturable by physiological levels of steroid. Non-specific binding, however, is unsaturable at these concentrations.

To determine which kind of binding is present, or what percentage of binding is specific, the incubation of both tissue fractions with labelled oestradiol should be set up in duplicate with an excess of an unlabelled oestrogen added to one set of incubations. Since the unlabelled steroid is in excess, this should fill the majority of the saturable specific sites, and any labelled oestradiol bound would then represent non-specific binding. By comparison of the bound activity in the presence and absence

of excess unlabelled steroid, the level of specific oestrogen receptors could be measured.

This operation was carried out using as competitors both unlabelled oestradiol-17β and the synthetic oestrogen, diethylstilboestrol (DES). The advantages of using the latter oestrogen is that it will not interact with non-cellular binding proteins such as sex hormone binding globulin (SHBG) (King & Mainwaring, 1974), and so the presence of these substances can be detected. The use of unlabelled oestradiol as the competitor would not be effective in this respect, as it would bind to SHBG as readily as ^{3}H-oestradiol. The role of plasma proteins as a source of erroneous results has been widely recognised, and alternative competitors have been suggested. These include another synthetic oestrogen (R2858) (Okret et al., 1978), and 5α-dihydrotestosterone (Ratajczak & Hähnel, 1976, Hähnel & Twaddle, 1979), but DES appears to perform equally efficiently as either of these. Hähnel & Twaddle (1979) also suggested that the influence of SHBG would never be so great as to change the classification of tissue from receptor-negative to -positive, which has been partially confirmed by studies carried out here, comparing the level of receptor using either excess unlabelled oestradiol-17β or diethylstilboestrol as competitor. It was found that, in the nuclear fraction, no difference was encountered using either competitor, although a small difference was found in levels of receptor detected in the cytoplasmic fraction using these two competitors. This difference was sufficiently small to discount concern about the contamination of cytoplasmic preparations by plasma proteins. This conclusion holds for breast tissue, but may not be true for other types of tissue of a more sanguineous nature, such as colonic carcinoma. Due to this fact, it is advisable that DES, rather than oestradiol, should be used as

cold competitor with all tissue types.

In order to assess the amount of competition required, assays were carried out on breast tumour cytosol and nuclear fractions using 100-fold excess of DES at all concentrations of ^{3}H-oestradiol. It was found that the use of competitor at only the highest concentration of ^{3}H-oestradiol (10^{-9}M) was just as effective in determining the specificity of binding as adding it at every concentration, and this system was adopted routinely although for an exact, quantitative study, competition at each concentration should be used. A comparison was made also between 100- and 1000-fold excess competitor, but the higher level did not increase the efficiency of the determination.

3.1.3.4 Protease Activity

A further consideration in the maintenance of the receptor during binding studies is the possibility of attack by proteases. The presence of these enzymes would cause the receptor content of the tissue to be underestimated. Proteases were found to interfere particularly with an assay system for nuclear receptors using protamine sulphate precipitation (Zava et al., 1976). For this reason, the assay system was altered, and the protease inhibitor Trasylol included (Garola & McGuire, 1977b), although the original method may have been invalid since salt extraction of nuclear receptors was used. Clark & Peck (1976) reported that it is the nuclear receptors resistant to KCl extraction which are important in long-term responses to oestrogens. To determine the effect of proteases on the receptor level in either the cytoplasmic or nuclear fraction of breast tumour tissue in the present system, studies were carried out on the uptake of ^{3}H-oestradiol into these fractions in the presence and absence of the protease inhibitors phenylmethylsulphonyl-

fluoride (PMSF) (1mM) and Trasylol (5000 units/ml), either alone or in combination. The results of one such study are shown in Figure 17. The level of uptake of labelled steroid with or without these agents is not significantly different in either tissue fraction. Thus, no one regime used here could be said to give an increase in the amount of steroid bound by either the cytosol or nuclear fraction of breast tumour tissue. The binding properties of these fractions are not being effected adversely by proteases, and the use of protease inhibitors is probably not required in this assay system.

3.1.4 SEPARATION OF UNBOUND FROM BOUND OESTRADIOL

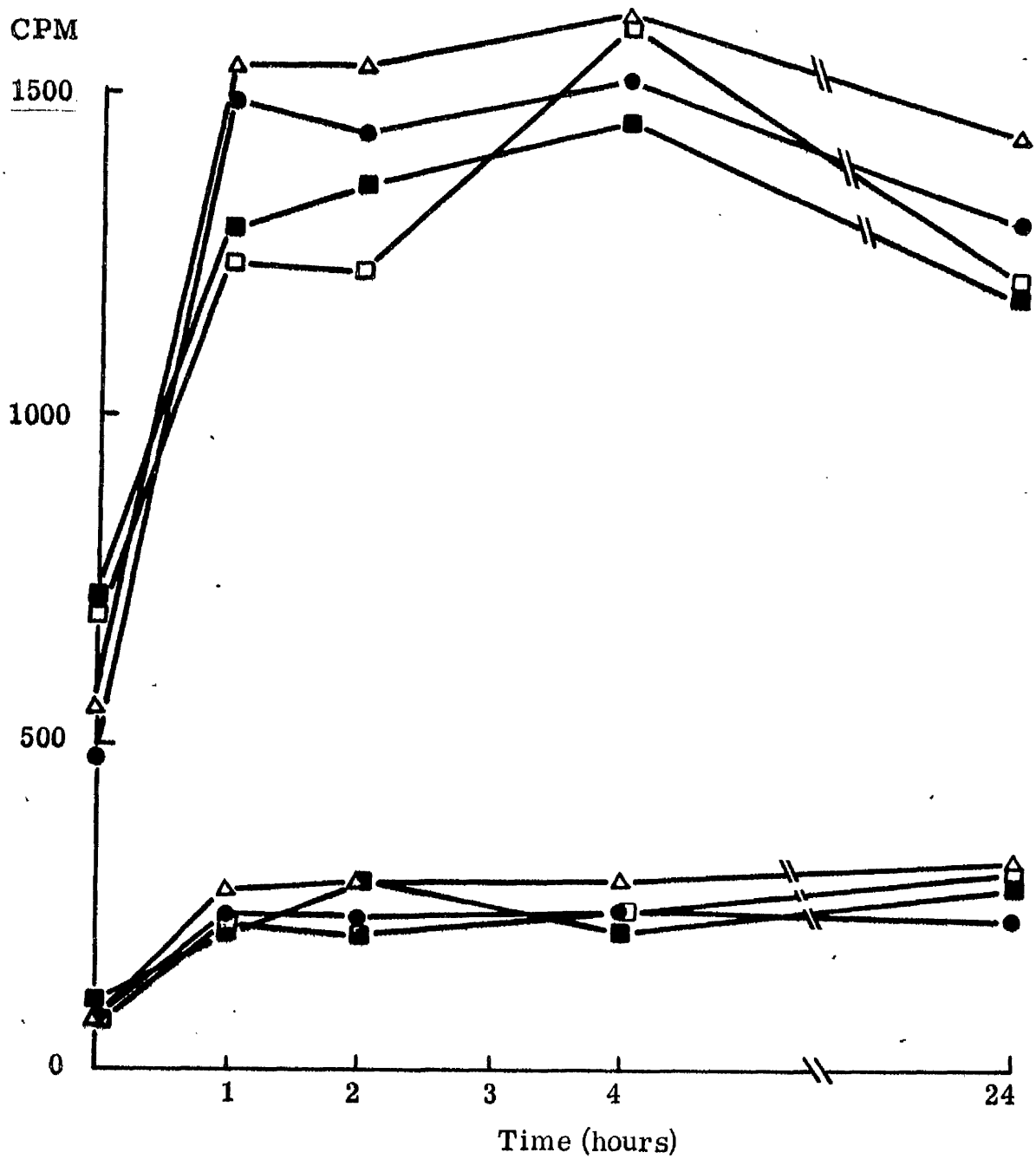
After incubation of labelled oestradiol with tissue fractions, the unbound steroid must be separated from the bound in order to determine the amount of binding which has occurred during the interaction of receptors with oestradiol. Due to the different nature of the cytoplasmic and nuclear fractions, different methods had to be employed to achieve removal of unbound material in each case.

3.1.4.1 Cytoplasmic Fraction

In initial attempts to separate unbound oestradiol, Sephadex G25 chromatography was used in the case of the cytoplasmic fraction. This method required much supervision, as four-drop fractions were counted into scintillation vials, and the columns had to be recharged by washing before another sample could be applied to them. A simpler and quicker method was found to be adsorption of the unbound steroid by dextran-coated charcoal (DCC). Steroid not involved in receptor interaction can be adsorbed by the charcoal, being able to penetrate the dextran coating (Nugent & Mayes, 1966). DCC has the

Figure 17 - Uptake of ^{3}H-Oestradiol-17β into the Cytosol and Nuclear Fractions of Breast Tumour Tissue in the Presence or Absence of PMSF (1 mM) or Trasylol (5,000 units/ml)

Cytosol and nuclear fractions of breast tumour tissue were prepared as described in Section 2.2.1.3, but at twice the normal concentration. Each was divided into aliquots which were diluted 1:1 with HEPES-EDTA buffer (● - ●), Trasylol (10,000 units/ml) (■-■), PMSF (2 mM) (Δ-Δ) or Trasylol (10,000 units/ml) + PMSF (2 mM) (□-□), and incubated with ^{3}H-oestradiol-17β (10^{-9} M) at 20°C. Samples were removed from each at various time intervals, unbound steroid removed as described in Section 2.2.3.2 and the uptake of oestradiol recorded. The upper group of lines refers to the cytosplasmic and the lower one to the nuclear fraction.


CPM
1500
1000
500
0
1
2
3
4
24
Time (hours)

additional advantage that it will strip steroid from loose binding interactions, and thus decrease the level of low affinity binding. The use of DCC has subsequently been criticized, however, as having an unacceptably high sensitivity level and requiring too much tissue (Hazato et al., 1979). This was not found to be the case here, where as little as 5 fmol/mg protein of receptor could be detected reproducibly and the aspect of the assay system limited by the supply of tissue was simply the range of oestradiol concentrations which could be used to construct the Scatchard plot (see Section 3.3).

A comparison was drawn between results obtained using Sephadex chromatography and dextran-coated charcoal to separate bound and unbound steroid. This determined that the level of binding detected by the two methods was virtually identical. Due to the ease of operation with the charcoal stripping method, this was preferred to Sephadex chromatography.

Agargel electrophoresis has been reported to be very useful in this context, as it also separates plasma proteins from receptors (Wagner, 1972, Hawkins et al., 1975), but the workers involved have returned to the DCC method. Polyacrylamide gel electrophoresis was also suggested as an alternative (Ritzen et al., 1974), but, like the agargel form, the method is much more complicated than the use of DCC.

The concentration of charcoal reported as being most efficient in removing unbound steroid from solution varies, (Korenman, 1968, Katzenellenbogen et al., 1973, Hawkins et al., 1975) and so this concentration along with the proportion (v:v) to use in order to achieve maximum stripping, were investigated. Solutions of ^{3}H-oestradiol in buffer were prepared at

concentrations of 10^{-10}M, 10^{-9}M and 10^{-8}M. Aliquots were then incubated with varying amounts of either 0.25% or 0.15% DCC (w:v) suspended in a sucrose-containing buffer. Figure 18 shows the relative efficiencies of these two DCC suspensions in removing the oestradiol. One point illustrated in Figure 18 is how easily a piece of contaminated charcoal can be disturbed and picked up during the aliquotting of the charcoal stripped supernatant. A large error can result from this, as shown in the graph demonstrating the efficiency of 0.15% DCC. Great care must be taken to ensure that the charcoal pellet is not disturbed, and that any fine pieces of charcoal floating on top of the supernatant are not transferred to the scintillation vial along with the liquid. Both concentrations of DCC in Figure 18 appear equally efficacious in the removal of free steroid, with 0.5 - 1 volumes giving maximum effect. This information was then used in preparing the most efficient assay system for oestrogen receptors (see Figure 19).

3.1.4.2 Nuclear Fraction

Once the charcoal had adsorbed the unbound steroid in the cytoplasmic incubation mix,it could be centrifuged down, and in this way removed from solution. This method could not be employed with the nuclear fraction, however, since it is in the form of a suspension of nuclear material. Instead, the method used was to trap the particles of nuclear material on a filter paper and wash away any unbound steroid under suction. Glass fibre filter discs were preferred to paper ones for this purpose, as they collected the nuclear material more efficiently, while not retaining any unbound steroid after washing with 15 ml of saline.

Once the material had been applied to the filter and washed, the filters

Figure 18 - Comparison of the Efficiencies of Two Concentrations of Dextran-Coated Charcoal (DCC) in Adsorbing Free Oestradiol-17β

^{3}H-oestradiol-17β was prepared at concentrations of 10^{-10} M (● - ●), 10^{-9} M (▲ - ▲) and 10^{-8} M (□ - □) in HED buffer. To aliquots of each of these were added various volumes of dextran-coated charcoal (0.15% or 0.25%). 0.15% DCC was prepared by equilibrating charcoal (0.15% w/v) with Dextran T-70 (0.0015% w/v) in 0.25 M sucrose, 1.5 mM MEDTA, 10 mM HEPES (pH 7.4). 0.25% DCC was prepared in the same way, using corresponding amounts of charcoal and Dextran T-70. Incubation of steroid with DCC was carried out for 30 minutes at 0°C with shaking, followed by centrifugation at 1000 g for 5 minutes. The amount of oestradiol not adsorbed was determined by the radioactivity remaining in solution.

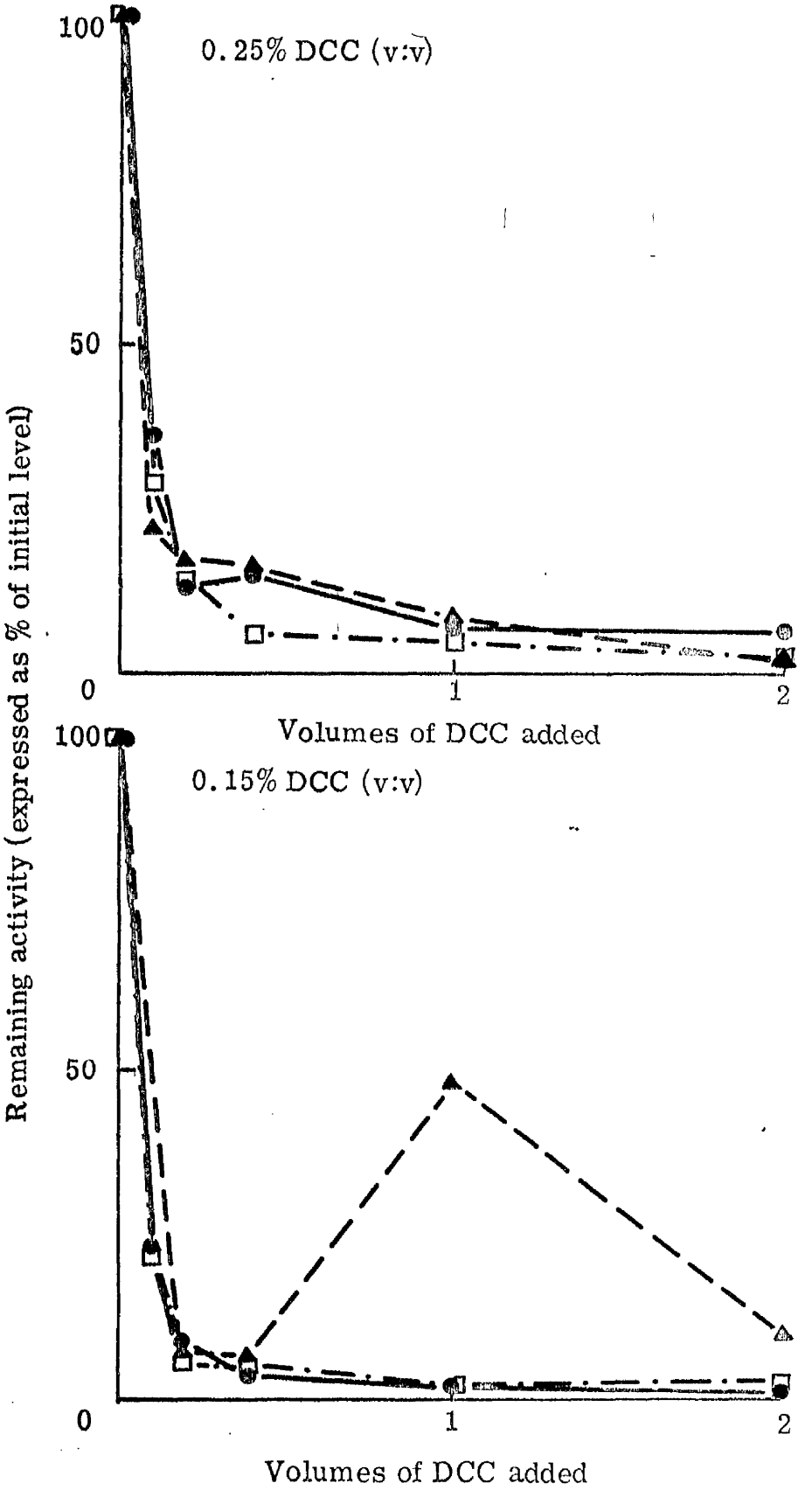
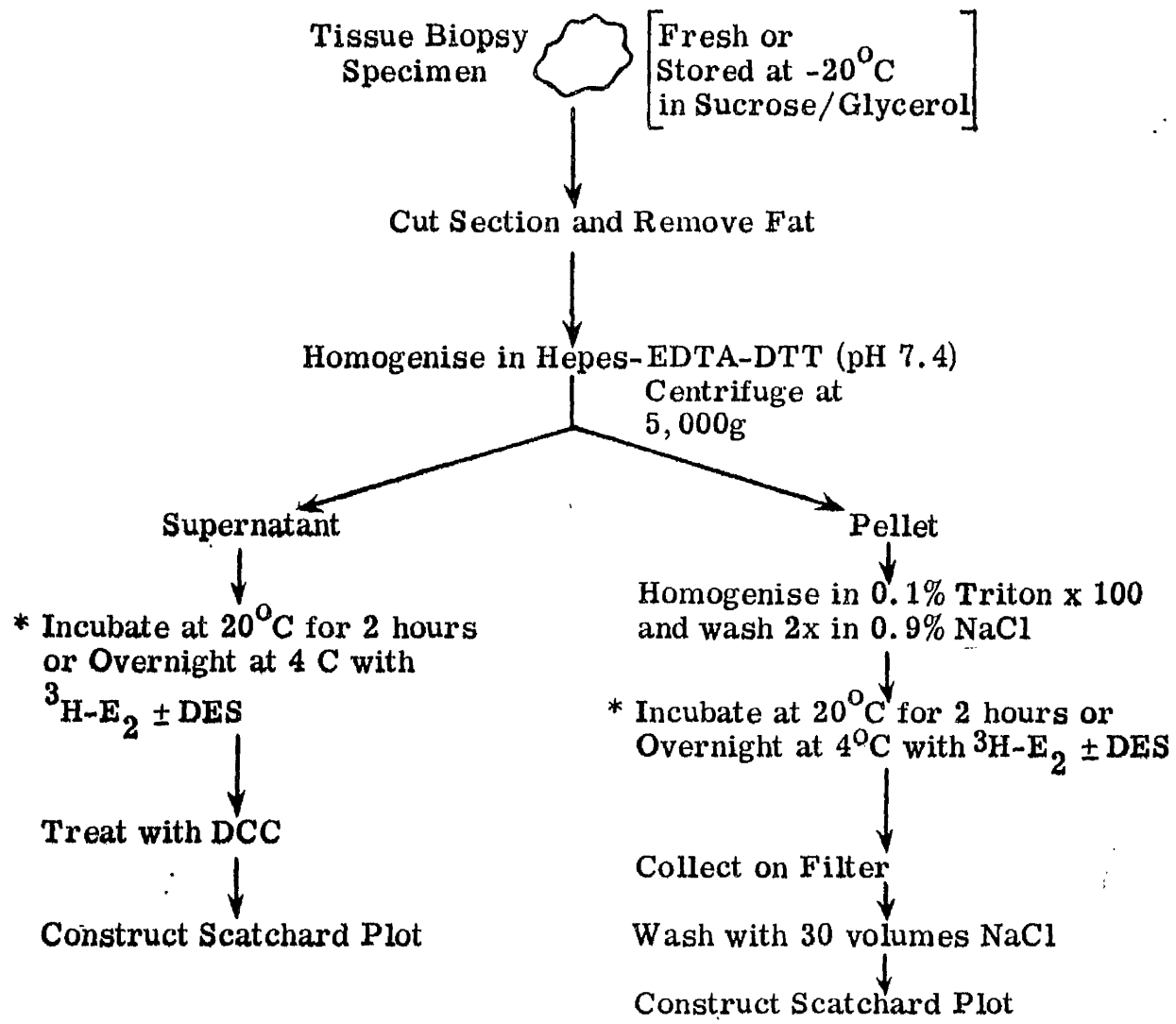

0.25% DCC (v:v)
100
50
0
1
2
Volumes of DCC added
0.15% DCC (v:v)
100
50
0
1
2
Volumes of DCC added
Remaining activity (expressed as % of initial level)

Figure 19 - Assay System for the Determination of Oestrogen Receptor Levels



* Incubation in the presence of Trasylol/PMSF (5000 units/ml and 100 mM) may give a minor improvement.

were dried before counting. This was carried out at 60°C for 18 hours, although incubation at this temperature for up to 60 hours did not diminish the activity. Solubilization of the activity by incubation with hyamine hydroxide (1M in methanol) at 60°C for 20 minutes gave no enhancement of the level of activity measured.

Having taken all the considerations described in the preceding sections into account, it was possible to construct an assay system for the measurement of oestrogen receptors, allowing optimal sensitivity whilst having potential for routine application. This is summarised in Figure 19.

3.2 REPRODUCIBILITY OF THE OESTROGEN RECEPTOR ASSAY

To examine the reproducibility of results obtained from the receptor assay summarised in Figure 19, studies were conducted on the variation of receptor content across one piece and within a bulk homogenate preparation of breast tumour tissue.

3.2.1 VARIATION ACROSS TISSUE

A piece of breast tumour tissue was sliced into six parallel portions of approximately 150 mg each. Separate cytoplasmic and nuclear fractions were prepared and oestrogen receptor levels assayed independently in each one. The results of this study are shown in Table 2. It can be seen that considerable variation in the numerical value of the receptor occurs in both the cytoplasmic and nuclear fractions of the tissue. However, very little variation in the value of the dissociation constant occurs. This means that each piece of this tissue assayed here would be pronounced

Table 2 Variation in Oestrogen Receptor Level and the Dissociation Constant of its Binding to Oestradiol-17β in the Cytosol and Nuclear Fractions of Six Parallel Sections from a Breast Tumour Biopsy

A large piece of breast tumour tissue was divided into six parallel slices of approximately 150 mg. Each was treated as described in Section 2.2.1.3 to yield a cytosol and nuclear fraction. The oestrogen receptor content and Kd of binding for each fraction was determined by Scatchard analysis, as described in the methods section. (R_c - cytoplasmic oestrogen receptor, R_n - nuclear oestrogen receptor).

Slice No.	R_c		R_n	
	fmol/mg protein	Kd ($X10^{-10}$M)	fmol/mg DNA	Kd ($X10^{-10}$M)
1	124	0.9	1933	3.8
2	171	1.0	1395	2.0
3	484	1.0	658	2.5
4	146	1.6	658	2.9
5	423	1.0	1778	2.6
6	526	1.2	753	2.7

receptor-positive, and so the assay conditions used can be said to produce reproducible results at a qualitative level. Hawkins et al., (1977) state that the receptor variability across a breast tumour is very high compared with other tumours, and suggest a requirement for replicate sampling, but this does not appear to be necessary with the assay system developed in this project, if receptor presence, rather than absolute amount, is the parameter sought.

3.2.2 VARIATION WITHIN ONE HOMOGENATE

A large piece of breast tumour tissue (approximately 1 g) was homogenised and cytosol and nuclear fractions prepared from the bulk homogenate. These fractions were then used to set up six separate oestrogen receptor assays from each. The results of this study are shown in Table 3. Here, the variation in receptor level is small ($\sim\pm$ 20%) for both cytoplasmic and nuclear fractions. In common with the results presented in Table 2, very little variation in the dissociation constant value occurs. This is further evidence, therefore, that the assay conditions produce reproducible results.

3.3. QUALITY CONTROL OF THE RECEPTOR ASSAY SYSTEM

In order to assess the assay systems being used by various workers throughout the country for the determination of oestrogen receptor levels in breast tumour tissue, portions of tissue from the same tumour were frozen in solid CO_2, and despatched to the appropriate groups for receptor measurement by their own methods. In addition to solid tumour samples, some homogenates were prepared also, and frozen portions circulated in the same way. The findings of this study have been published (King et al., 1978, 1979a). The

Table 3 Variation in Oestrogen Receptor Level and the Dissociation Constant of its Binding to Oestradiol-17β in the Cytosol and Nuclear Fractions Prepared from One Bulk Homogenate from a Breast Tumour Biopsy

Cytosol and nuclear fractions were prepared from a large piece of breast tumour tissue (approximately 650 mg) as described in Section 2.2.1.3. Each fraction was then used to set up six parallel assays for oestrogen receptor as described in the methods section. (R_c - cytoplasmic oestrogen receptor, R_n - nuclear oestrogen receptor).

Assay No.	R_c		R_n	
	fmol/mg protein	Kd($X10^{-10}$M)	fmol/mg DNA	Kd($X10^{-10}$M)
1	362	1.2	1442	1.7
2	368	1.3	1695	2.1
3	358	1.4	1305	1.3
4	345	1.2	1137	0.9
5	389	1.3	1389	1.2
6	373	1.4	1316	0.9

assay system established in the course of this project compared favourably with those of other workers.

One important observation to emerge from this trial was that one piece of tumour assayed in this laboratory using full Scatchard analysis was found to contain specific binding on the grounds of successful competition by DES, but no high affinity binding could be detected using the criterion of a linear Scatchard plot. From the evidence provided by the competition data alone, this tumour would have been pronounced receptor-positive, but, by the inclusion of incubation with a full range of oestradiol concentrations and the construction of a Scatchard plot, an abnormality in the receptor was detected, which renders the tumour unlikely to respond to hormonal therapy on the grounds of receptor content. This clearly demonstrates the advantage of constructing a Scatchard plot whenever the supply of material allows, although this is not a universally accepted conclusion. Johnson et al., (1975) and Johnson & Nakamura (1977) suggest that it is possible to obtain as accurate a result by constructing a Scatchard plot bearing only two points. An error in either of these points, no matter how small, would tend to change the receptor concentration and the Kd radically using this method. McGuire et al., (1977c) compared various methods, and determined that a single concentration of ^{3}H-oestradiol with and without unlabelled competitor was sufficient for accurate receptor measurement, which is contradicted by the finding reported above. This "single spot" assay was also favoured by Mobbs & Johnson (1976), except where a very high level of receptor is involved. Hawkins et al., (1975), however, employed a full Scatchard analysis using seven different concentrations of ^{3}H-oestradiol (very similar to the system established in this project). Slightly more tissue is required by the present system (150 mg is needed to construct a Scatchard

plot and check specificity) than for a one spot assay, but invaluable additional information is supplied which is very important in defining the exact nature of the steroid binding observed. The need for this extra data is emphasised further in studies on oestrogen receptor content of colonic carcinoma tissue (section 3.4.7), where a one-spot assay has been found to reveal a binding agent, the identity of which is not confirmed as a true oestrogen receptor by Scatchard analysis. Chamness & McGuire (1975) stressed that caution should be applied to the interpretation of Scatchard plot results, as they concluded, in a review on the subject, that many errors could, and do, arise from incorrect use of the data.

Another quality control study currently in operation has the added objective of assessing the feasibility of storing tissue homogenates in lyophilized form prior to oestrogen receptor assay. This has been successfully tried Koenders et el., (1978), using a variety of tissue sources. In the present study portions of lyophilized material from breast tumour tissue and calf uterus have been circulated to workers for oestrogen receptor measurement. Preliminary results from this study confirm the consistency of the assay system described in this thesis with those of other workers and also demonstrate that lyophilization abolishes neither cytoplasmic nor nuclear oestrogen receptor level from either of the two tissue types used.

3.4 OESTROGEN RECEPTORS IN HUMAN TISSUE

For either cytoplasmic or nuclear fraction of any tissue type to be termed "oestrogen receptor-positive", three criteria must be satisfied. The first of these is that it must be possible to construct a Scatchard plot from the results of a receptor assay, which yields an unambiguous straight line.

Secondly, the Kd value derived from that straight line must be of the order of 10^{-10}M, in human tissue at least, with values in the range 0.5 - 5 x 10^{-10}M being acceptable. The last condition is that specificity of binding must be established by competition with excess unlabelled oestradiol-17β, or, preferably, diethylstilboestrol (see Section 3.1.3.3). If these three criteria are met, this should establish beyond any doubt that the binding detected is indeed characteristic of cellular, high-affinity, specific oestrogen receptor. Receptor can be detected down to a level of 5 fmol/mg protein in the cytosol or 25 fmol/mg DNA in the nuclear fraction (equivalent to 100 receptor molecules/cell) although the vast majority of receptor-containing samples have receptor levels greatly in excess of these figures.

Where the supply of tissue is not great enough (i.e. less than 100 mg) to enable a Scatchard plot to be constructed, the first and second of these criteria cannot be met, and the third one must be satisfied alone. This is not so satisfactory, as explained in section 3.3. In cases where a sufficient supply of material is available, and Scatchard analysis thus made possible, failure of the binding to satisfy any of the criteria detailed above leads to the classification of the tissue as "receptor-negative".

3.4.1 RECEPTOR PROFILES IN BREAST TUMOUR TISSUE

Using the assay system established in sections 3.1 - 3.3, cytoplasmic and nuclear oestrogen receptor levels were measured in 1000 samples of mammary tumour tissue. This total represents primary and secondary tissue, and comes from all age groups of women, of varying menopausal status.

Table 4 shows a summary of cytoplasmic receptor content from these assays.

Table 4 Summary of Cytoplasmic Oestrogen Receptor Presence in 1000 Biopsies of Breast Tumour

For definition of receptor-"positive" and -"negative" see Section 3.4. The 1000 biopsies include samples of primary and secondary disease from both pre- and post-menopausal patients.

Receptor Content	No. of Patients	Percentage
Positive	461	46
Negative	539	54

To be classed as "receptor-positive", the tumour cytosol must satisfy the criteria detailed above. No "cut-off level" of receptors is set below which tissue would be regarded as "receptor-negative". Approximately half of the cytosols assayed were found to contain receptor displaying the required characteristics. This is approximately the same level of receptor-positive biopsies as reported by other workers (e.g. Walt et al., 1976, Allegra et al., 1979a), though lower than some (Roberts et al., 1978, Hawkins, et al., 1979, King et al., 1979b).

Table 5 shows a summary of the receptor patterns which emerged when both nuclear and cytoplasmic receptor levels were considered together. The majority of biopsies were found to contain no receptor in either fraction, while approximately one third of those samples assayed contained receptor in both fractions. Using the property of containing both types of receptor, rather than a cut-off point for cytosol receptor alone, a lower percentage (30%) of potentially responsive patients is identified than in studies using cytosol receptor measurement as the only index (e.g. McGuire et al., 1975, King et al., 1979b, Byar et al., 1979). In the present study, those tumours containing cytosol, but no nuclear receptor, were initially thought to be unlikely to respond to hormone therapy.

The interesting groups in Table 5 are those with receptor in only one fraction, no detectable level being present in the other. These represent abnormalities in the receptor, which would not have been discovered had receptor content been assessed in the cytoplasmic fraction alone. This would have led to a possible mistake in the interpretation of the best therapeutic regime for these two groups of patients. In the case of patients whose tumours contained cytoplasmic, but no nuclear oestrogen

Table 5 Analysis of Cytoplasmic and Nuclear Oestrogen Receptors in 1000 Biopsies of Breast Tumour

The presence (+) or absence (o) of both cytoplasmic (R_c) and nuclear (R_n) oestrogen receptors are summarised. For definition of "positivity" and "negativity" see Section 3.4. The 1000 biopsies include primary and secondary samples from both pre- and post-menopausal patients.

Receptor Content (R_c/R_n)	No. of Patients	Percentage
+/+	343	34
o/o	479	48
+/o	118	12
o/+	60	6

receptor, the absence of nuclear receptor suggests that either the receptor is abnormal and unable to translocate into the nucleus, or the translocation mechanism within the cell is not functioning correctly. Another explanation could be that the level of circulating oestrogen was either very low or completely non-existent, meaning that there was no stimulus for the receptor to bind to the chromatin. This is unlikely, since the presence of oestradiol is necessary to promote synthesis of its own receptor, and cytoplasmic levels would be undetectable also in the absence of circulating steroid. There is also direct evidence (see Section 3.4.5.5) that circulating oestradiol can be present in such cases. The first two defects could be caused by malignant transformation of the cells. In either event, although oestradiol can be bound within the cell, it cannot enter the nucleus, or even cause the receptor to enter the nucleus, and thus will not elicit any of the typical cellular responses to hormonal stimulation. A patient with such a tumour should, thus, receive no benefit from hormone therapy, unless by an indirect pathway. This type of situation has also been noted by Thorsen & Stoa (1979), but they found that 25% of their samples contained cytoplasmic receptor alone - a higher proportion than that reported here (12%). However, their study was carried out on only 16 patients.

To try to elucidate the type of defect present in these cells, tissue with detectable oestrogen receptors in the cytosol only was homogenised, the cytosol fraction prepared and incubated with unlabelled oestradiol. Unbound steroid was removed, and cytosol incubated with nuclear material from immature rat uterus or receptor-positive breast tumour tissue. In this way, translocation into these normal chromatins could be achieved if the

receptors were not defective. After incubation between the cytosol and nuclear fractions, they were separated again, and Scatchard plot analysis carried out on each one to determine whether any change in receptor levels had occurred.

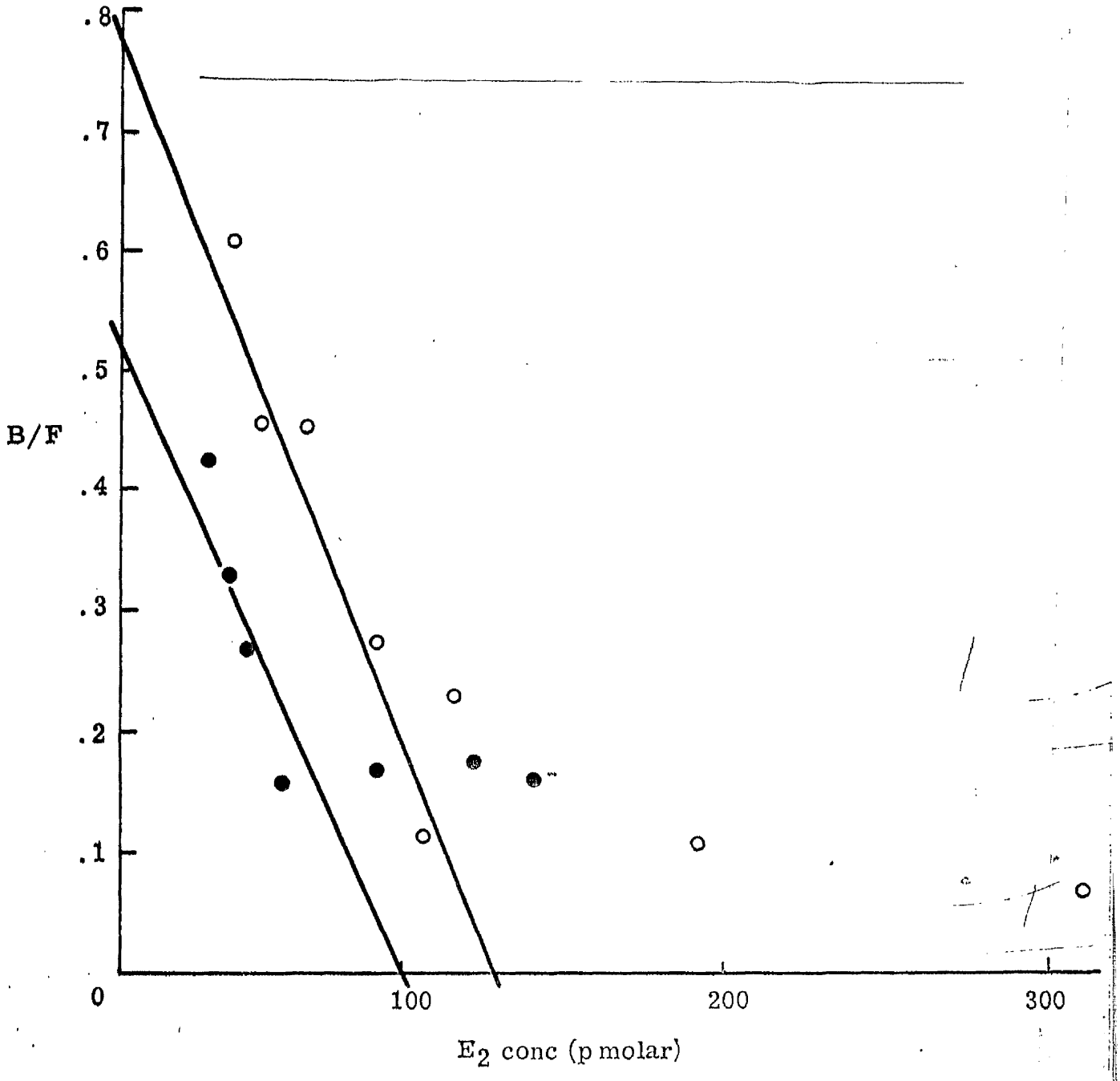
Figure 20 shows results from such an experiment. No translocation of receptor could be detected in any system, using as the criterion a rise in receptor levels. Thus, the fault in these samples containing cytosol receptor only may lie with the receptor's inability to become transformed. This experiment was conducted only once with each system, however, due to the lack of suitable material, so the conclusions are merely tentative.

The other incidence of abnormal receptor is that where it is found exclusively in the nuclear fraction of the tissue. This phenomenon has been reported by other workers also (Panko & MacLeod, 1978), but the significance of the receptor's presence in the nucleus is not understood fully as yet. It is not possible, therefore to predict how such a tumour would respond to hormone therapy. It should, however, become possible after observing the response of some such tumours which are subjected to hormonal therapy. As discussed later (see Section 3.5.1), this approach leads to the suggestion that the nuclear receptors are not functional without their cytoplasmic counterparts, and the tumours in this group are not likely to respond to hormone therapy.

Tissue containing cytoplasmic receptor only was reassessed, examining the absolute value of these receptor levels. Histogram 1 shows the comparison of cytoplasmic receptor concentrations in 118 such biopsies and 118 samples containing receptor at both levels, randomly selected. The majority of samples containing no nuclear receptor have cytoplasmic levels lower than

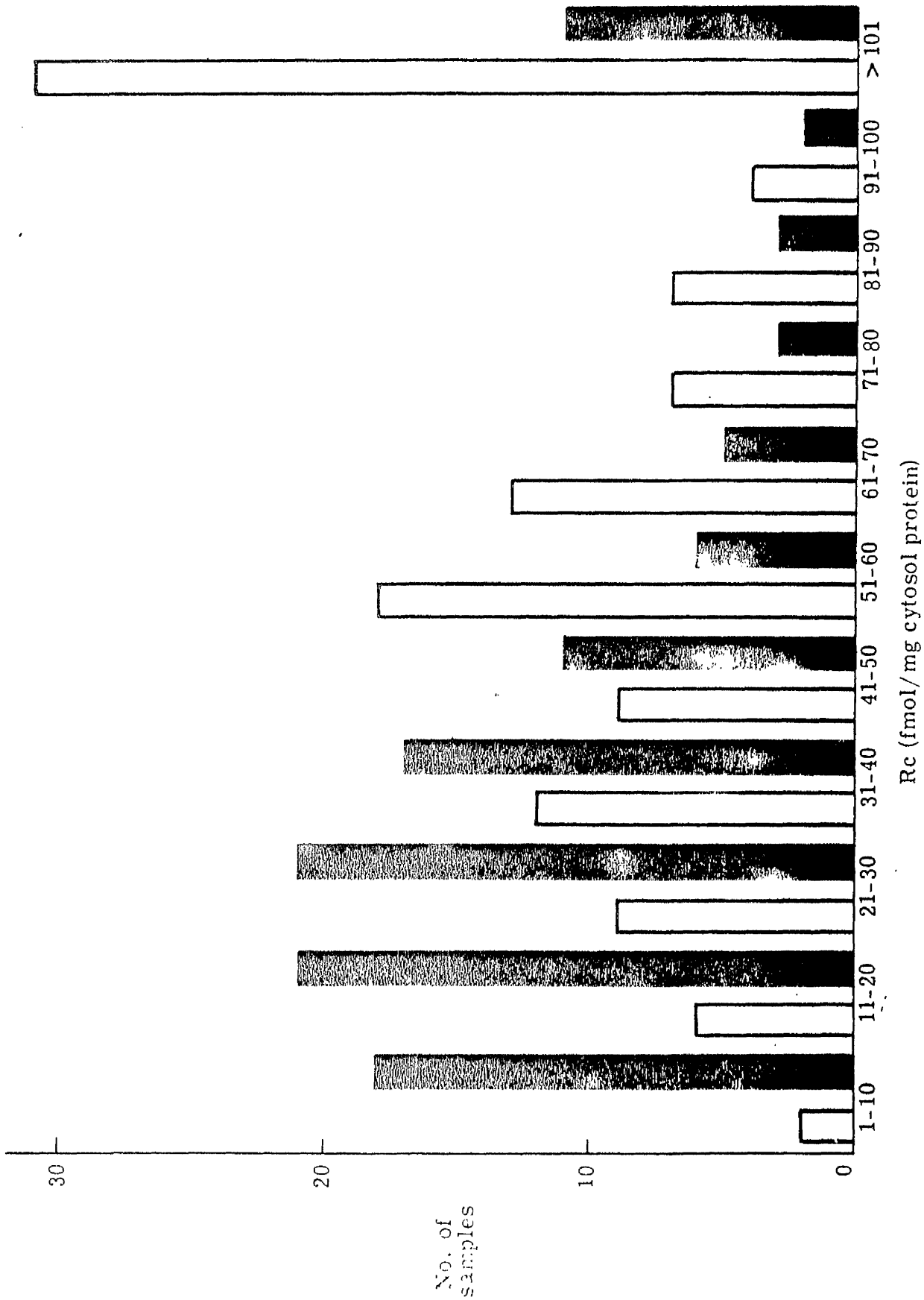
Figure 20 - Measurement of Oestrogen Receptor Levels in Rat Uterine Nuclear Fraction Before and After Incubation with Cytosol from +/o Breast Tumour Tissue

Cytosol was prepared as described in section 2.2.1.3 from breast tumour tissue (+/o) known to contain receptors in the cytoplasmic fraction alone. This was incubated with 2×10^{-9} M unlabelled oestradiol-17β at $0^{o}C$ for 30 minutes, and then unbound steroid removed by DCC stripping as described in section 2.2.3.2. The stripped cytosol was used to resuspend the nuclear pellet from immature rat uterine pellet, and this suspension incubated at $37^{o}C$ for 30 minutes. The nuclear material was pelleted again, and washed before Scatchard analysis of the receptor content was performed, using ^{3}H-oestradiol-17β. The receptor content of the nuclear material before (O - O) and after (● - ●) incubation with the tumour cytosol was compared to determine whether any translocation had occurred between the two fractions.


.8
.7
.6
.5
.4
.3
.2
.1
B/F
0
100
200
300
E_2 conc (p molar)

Histogram 1 - Comparison of the Distribution of Cytoplasmic Oestrogen Receptor Levels in +/+ and +/o Breast Tumour Biopsies

The levels of cytoplasmic oestrogen receptor were compared in the same number of cases of +/+ and +/o (shaded) breast tumour tissue.


No. of samples
0
10
20
30
1-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
>101
Rc (fmol/mg cytosol protein)

30 fmol/mg cytosol protein, the mean value in the case of +/+ biopsies being significantly higher than this. However, there are a number of cases where +/o values are in excess of 30 fmol/mg cytosol protein, and also some +/+ samples with cytoplasmic receptors of a lower level. Thus, a low titre of cytosol receptors could not be taken alone as an indication of a tumour's inability to respond to hormonal therapy. Further evidence of this is that tumours containing low levels of cytosol receptor, but also nuclear receptors have, nonetheless, responded to hormonal manipulation. On this basis, a cut-off point for cytosol receptor level, below which no tumour would be predicted to respond to hormonal therapy would not be advocated.

The inclusion of both cytoplasmic and nuclear determinations of oestrogen receptor levels thus splits breast tumour tissue into four categories. Cytoplasmic assay alone would not detect the lack of nuclear binding found in some cases, whereas measurement of only nuclear receptors would fail to recognise those biopsies exhibiting nuclear binding alone. Together, these categories constitute a high enough percentage of total biopsies to make it essential that they be identified, as both could lead to application of inapplicable therapeutic regimes if they were not fully investigated.

Receptor levels were also determined in some cases of male breast cancer. This has also been reported by other workers (Leclerq et al., 1975). It was found that, as in the female occurrences, the levels of receptor varied from tumour to tumour, some containing none at all. The number of male biopsies examined was too small to enable any statistically significant conclusions to be drawn.

3.4.2 REPRODUCIBILITY OF OESTROGEN RECEPTOR STATUS FROM ONE LESION OF A TUMOUR TO ANOTHER

A very important aspect of the measurement of oestrogen receptors in breast tumour tissue is whether the receptor status determined in the primary disease will be duplicated in subsequent lesions. This is also of importance when considering more than one metastatic site. If the receptor level is to be used in the selection of the ideal therapeutic regime for the patient, then the knowledge of whether the presence, or indeed absence, of receptors is maintained during the transition from primary to secondary disease, and, further, will remain consistent in several metastatic lesions is essential. If these facts could be assumed, then inoperable lesions could be treated with the most potentially effective therapy on the basis of the receptor status determined in the primary disease or another metastic lesion.

To determine whether this is the case, a comparison was drawn between receptor levels found in tissue from the same patient biopsied from different lesions. In some cases, this represents a transition from primary to secondary disease, while in some, the samples come from different metastatic sites. Table 6 shows the results obtained in 32 cases where a primary biopsy was followed some time later by a secondary. In 63% of cases, the receptor status is the same for both samples, no matter what length of time had elapsed between operations. However, there are a significant number of instances where the exact receptor status is not reproduced. The loss of receptors over time could be understood, since it has been reported that hormone-dependent tumours do become autonomous (Kim & Depowski, 1975), although the time taken for this process to occur appears to vary. The appearance of receptors where none could be detected before is less easily explained,

Table 6 Cytoplasmic and Nuclear Oestrogen Receptor Status in Biopsies of Primary and Secondary Breast Disease from the same Patient

The receptor content of the cytoplasmic (R_c) and nuclear (R_n) fractions of primary breast tumour was compared with that of a secondary lesion from the same patient. The time interval between first diagnosis and recurrence of disease was also noted.

Patient	Age	Time between biopsies (months)	Primary Status (R_c/R_n)	Secondary Status (R_c/R_n)
517535	59	35	o/o	o/o
529941	41	11	o/o	o/o
490197	64	19	+/+	+/+
543284	58	19	o/o	o/o
206073	40	10	o/o	o/o
335662	56	17	+/+	o/o
554902	78	8	o/+	+/o
517288	59	8	+/+	+/+
512434	44	14	o/o	o/o
338381	52	21	+/o	o/+
190658	Unknown	13	+/o	o/+
533839	38	21	o/o	o/o
550668	43	31	o/o	o/+
559665	64	7	+/o	o/o
528446	68	16	+/+	+/+
528171	68	2	o/o	o/o
297738	46	11	o/o	o/o
519488	49	12	o/o	o/o
420564	52	21	o/o	+/+
348637	57	5	o/o	o/o
525191	77	12	o/o	o/o
498101	43	6	o/o	o/o
227746	75	23	o/o	o/o
551907	64	12	+/+	+/+
263806	80	4	o/o	o/o
526290	44	19	+/+	+/o
341527	52	5	o/o	o/o
518777	49	23	+/+	o/o
297515	71	18	+/+	+/o
305543	67	15	o/+	+/o
409965	46	32	+/+	+/+
653306	Unknown	23	+/+	o/+

if the initial absence were not caused by pre-operative treatment, although this phenomenon has also been reported by Korsten et al., (1975) and Liskowski & Rose (1976). The present findings do not agree completely with the conclusions of King (1975) or Jensen et al., (1977), who found that the receptor status in the primary consistently determined the status and response of the secondary lesions but the number of patients in their studies was much less than is reported here. In the majority (75%) of instances here, where the secondary receptor status varied from that of the primary, defective receptor was involved, in that one or both biopsies contained either cytoplasmic or nuclear binding alone. Since, in these situations, the receptor is obviously abnormal in some manner, the fact that it is also apparently unstable is not entirely surprising. This would suggest that only results where receptor status is the same in both tissue fractions are of positive value in this respect. Overall, however, the results in Table 6 suggest that, in the majority of cases, receptor status and, therefore, hormone-responsiveness are the same in the primary and secondary lesions. There are exceptions, which means that the concepts of routinely assaying primary tissue to aid in therapy of a secondary (Jensen, 1975) or determining whether a second tumour is another primary or a secondary on the basis of receptor content (Kiang & Kennedy, 1977b) should be treated with caution.

Table 7 shows the comparison of receptor levels in two or more secondary biopsies from the same tumour. The receptor content of some of these tumours was also determined at the primary level, and so they are also included in Table 6. The reproducibility of receptor status between biopsies in this case is very similar to that found in the transition of primary to secondary disease (70% in Table 7). Again, the majority of variability (83%) appears to occur where abnormal receptors are involved, emphasising

Table 7 Cytoplasmic and Nuclear Oestrogen Receptor Status in Biopsies of more than one Secondary Deposit from the same Breast Cancer Patient

The receptor content of the cytoplasmic (R_c) and nuclear (R_n) fractions was compared between the first metastasis and any subsequent secondary sites. The time elapsed between biopsies was noted.

Patient	Age	Time between biopsies (months)	1st metastatic sample (R_c/R_n)	2nd metastatic sample (R_c/R_n)	3rd metastatic sample (R_c/R_n)
529941	41	2	o/o	o/o	-
503664	48	13 & 9	o/o	o/o	o/o
543284	58	4	o/o	o/o	-
612828	unknown	23	+/o	o/o	-
517288	59	14 & 10	+/+	+/+	o/o*
576240	47	18	o/o	o/o	-
190658	unknown	6	o/+	+/+	-
416889	60	14	o/o	o/o	-
297738	46	6	o/o	+/+	-
560179	64	10	o/o	o/o	-
519488	49	7	o/o	o/o	-
420564	52	1	+/+	+/o	-
482442	49	11 & 12	+/o	o/o	+/+
525191	77	7	o/o	o/o	-
249687	unknown	10	o/o	o/o	-
170263	72	25	o/o	o/o	-
AF/V	unknown	14	+/+	+/+	-
526290	44	10	+/o	o/o	-
518777	49	3	o/o	o/o	-
544403	45	30	+/+	+/+	-

*patient withdrawn from tamoxifen only 10 days previously (see text)

that care is required in interpreting the therapeutic requirements of further lesions from such tumours.

In some cases, the treatment the patient was receiving (the antioestrogen tamoxifen) was found to effect the ability to detect receptor. In two specific instances, patients receiving this drug had assays performed on their tissue, from which it was concluded that no receptors were present, but, after tamoxifen therapy was discontinued, further biopsies were performed and found to contain both cytoplasmic and nuclear receptors. One of the patients was given a further course of tamoxifen and again receptor became undetectable. The time between biopsies here was 3-4 weeks. Another patient, on whom several biopsies had been performed, all containing receptor, was administered tamoxifen. The tumour continued to grow, and another biopsy was submitted for receptor assay 10 days after the last tamoxifen treatment. This contained no receptor at all. Although the plasma half-lives of the drug have been reported by Nicholson et al., (1979) as 7-14 hours and 7 days, it appears to have a relatively prolonged effect, probably due to an action on receptor synthesis. This demonstrates that previous, as well as current, therapy should be taken into account when interpreting receptor levels.

3.4.3 RECEPTOR LEVELS IN CASES OF BILATERAL BREAST CANCER

Receptor measurements have been carried out in a few cases of bilateral breast cancer. In each case, the disease in the second breast was a new primary tumour, and did not represent metastatic spread. Although the number of instances encountered is very low, (4 cases out of 900), it is clear that, while some second tumours have the same receptor status, and

thus potential to respond to hormone therapy, as the first, others have entirely different abilities to bind oestradiol. This suggests that the stimuli responsible for the malignant transformations in the two tissues are not always consistent in their effect on the receptor-synthesising mechanism within the cells, some activating the machinery and some not. This would rule out any suggestion that breast disease in the second breast could be treated on the basis of receptor levels found in the first.

3.4.4 RECEPTOR LEVELS IN "NORMAL" BREAST TISSUE AND BIOPSIES OF BENIGN BREAST TUMOURS

Receptor measurement was performed on several samples of "normal" breast tissue, obtained from patients undergoing plastic surgery, and tissue from benign tumours of the breast. The assay system was the same as that used for receptor measurement in breast tumour tissue in all respects. In every case, binding was undetectable using this system. Since the lower limit of sensitivity is 5 fmol/mg cytosol protein, or 25 fmol/mg DNA in the nuclear tissue fraction, this finding was taken to mean that no receptors were present. This is in agreement with a report of Block et al., (1975), that normal breast tissue exhibited hardly any uptake of oestradiol, presumably due to lack of receptors. Wittliff et al., (1972) and Sakai & Saez (1976) acknowledged that binding was reduced in normal breast or benign disease relative to malignant tumours, but that some was present. Feherty et al., (1971) and Hawkins et al., (1975), however, both detected receptor activity in benign tissue, but reported widely varying occurrence figures of 7% and 21% respectively, and much lower concentrations than those encountered in tumour tissue. The failure to measure any receptors at all in this type of tissue in the present study may reflect an insufficient

number of attempts if a level as low as 7% (Feherty et al., 1971) is accurate, but this seems statistically improbable. A problem in the analysis of "normal" tissue derived from anaplastic reduction material is that it is very fatty. However, tissue used in the present study was carefully selected to represent mammary epithelium, free of fat. Failure to do so could lead to binding of oestradiol by fat being misinterpreted as an indication of receptor presence.

3.4.5 CORRELATION OF OTHER FACTORS WITH RECEPTOR STATUS IN BREAST TUMOURS

To explore the possibility of other guides to prediction of response to hormone therapy by breast tumours, the connection between receptor levels and various other parameters was examined. Although the receptor assay in its present form is both straightforward and reproducible, another system requiring less tissue, or indeed no tissue at all might be preferable in some cases. Any parameter which did fluctuate with receptor levels could act also as a confirmation of results obtained by receptor assay.

3.4.5.1 Tissue Protein and DNA Content

One area examined as a possible guide to receptor content was the protein and DNA content of the tissue fractions. The protein levels were found to vary very little from tissue to tissue - on average within only a three-fold range (approximately 2-6 mg/ml) - although levels outside this range were occasionally encountered. It is advisable to keep protein values within as small a range as possible to allow direct comparison of receptor levels from one sample to another. In contrast to the experience with protein values, the DNA content was not so consistent, and varied over a

15-fold range (9-127 µg/ml). No correlation could be drawn between receptor levels and either protein or DNA content of the relative tissue fractions. The ratio of protein to DNA content was also considered in relation to the receptor status, but, again, no pattern could be detected.

3.4.5.2 Tumour Histology and Clinical Staging

The overall histology of the breast tumour biopsies was compared with the oestrogen receptor content. No particular histological feature was found which was consistent with receptor status. This has been reported also by Johansson et al., (1970) and Rosen et al., (1975). When studies were carried out on the connection between clinical staging and receptor status, no stage was found to be associated with any one receptor classification more than another. This is demonstrated in Table 8, where 133 patients, both pre- and post-menopausal were considered. The observation that no correlation exists is in agreement with those of Maass et al., (1972, 1975). Maynard et al., (1978) and Maynard & Griffiths (1979) find a definite connection between histological grade and receptor content in post-, but not pre-menopausal women. They found that the better-differentiated tumours rarely lacked receptor, but that no correlation could be identified between any detailed histological feature and receptor status, confirming the present findings.

3.4.5.3 Lactalbumin Content

Samples of tissue to be assayed for oestrogen receptor content were split, and a portion despatched to Dr. A. Howell, Queen Elizabeth Hospital, Birmingham for detection of lactalbumin, using an immunoperoperoxidase staining technique. A comparison was drawn between receptor status and lactalbumin absence or presence. Although the level of lactalbumin, when present,

Table 8 Comparison of Receptor Status and Stage of Tumour in 186 Primary Breast Tumour Biopsies

Receptor content of the cytoplasmic (R_c) and nuclear (R_n) fractions of breast tumour biopsies was compared with the stage of tumour. Stage I disease represents a tumour of less than 2 cm, with no nodal involvement and no metastases. Stage II describes any size of tumour up to 5 cm, which may also have nodal involvement but no metastases. In Stage III disease, tumours are greater than 5 cm in size, have no metastases, but may have little or extensive nodal involvement. Stage IV tumours are of any size, may, or may not, have any degree of nodal involvement, but display distant metastases.

R_c/R_n	Number of Patients			
	Stage I	Stage II	Stage III	Stage IV
+/+	25	27	4	1
o/o	37	54	6	3
+/o	7	7	o	1
o/+	6	8	o	o

varied from sample to sample, giving rise to a grading system for positivity, this did not coincide reproducibly with the presence or absence of receptor, as shown in Table 9. Thus, this test could not be applied to the prediction of receptor status or likelihood of response to hormonal therapy.

3.4.5.4 Plasma Receptor Levels

Receptor status of plasma from breast cancer patients was compared with that of tumour tissue from the same patients, following a report by Tisman & Wu (1977) that plasma receptors could be detected in some cases of breast cancer. This would provide the basis of a much simpler system to monitor patients, if receptor could be detected reproducibly and correlated with response to hormone therapy. Accordingly, blood, with anticoagulant added, was collected immediately preoperatively from breast cancer patients. The blood was centrifuged, and the plasma collected. The plasma was assayed in an identical fashion to the tissue fractions. Figure 21 shows the Scatchard plots obtained from oestrogen receptor assays on the cytoplasmic and nuclear fractions of breast tumour tissue and plasma from the same patient. This was typical of the type of plot obtained using 15 plasma samples, regardless of the receptor content of the tumour, the menopausal status or the sex of the patient and is in accordance with the findings of Leclerg et al., (1973), who could not detect any oestradiol receptor in plasma. From the Scatchard analysis, it is clear that something is binding oestradiol, but it is not the same high-affinity, specific receptor encountered in breast tumour tissue, and its presence is not consistent with the presence of that receptor in solid tissue. The same effect on the Scatchard plot was noted by Ratajczak & Hähnel (1976) studying binding in the presence of plasma.

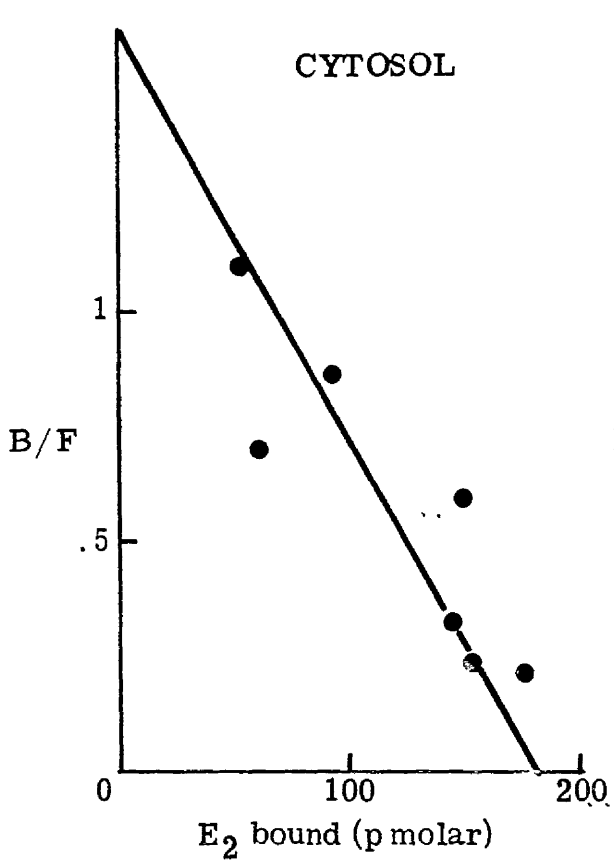
Table 9 Comparison of Receptor Status and Lactalbumin Content of 26 Breast Tumour Biopsies

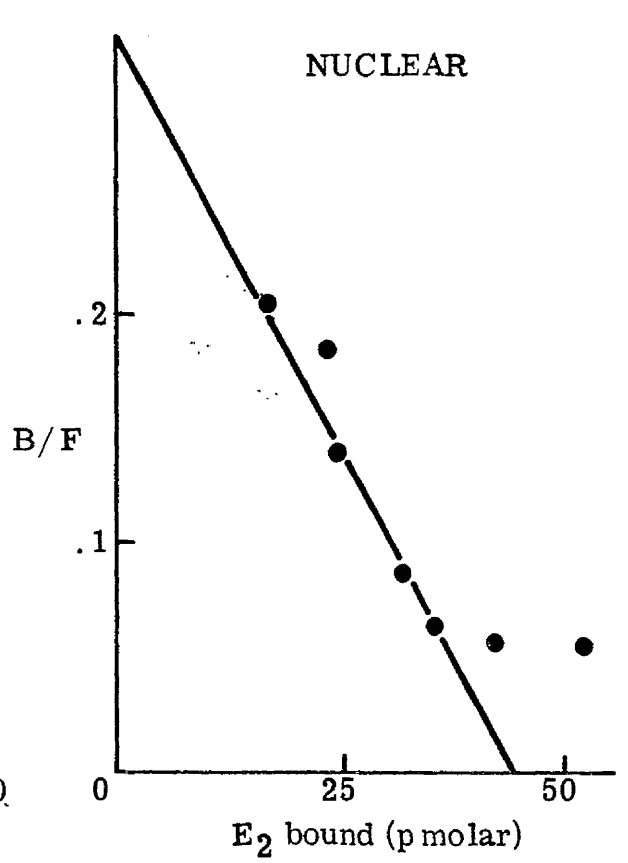
The overall receptor status of 26 breast tumour biopsies was compared with the lactalbumin content, as determined by immunoperoxidase staining. Lactalbumin grading was given as negative, grade I (small number of positive cells, small areas), grade II (moderate number of positive cells, not all of section) or grade III (many positive cells throughout the section). (R_c - cytoplasmic oestrogen receptor, R_n - nuclear oestrogen receptor).

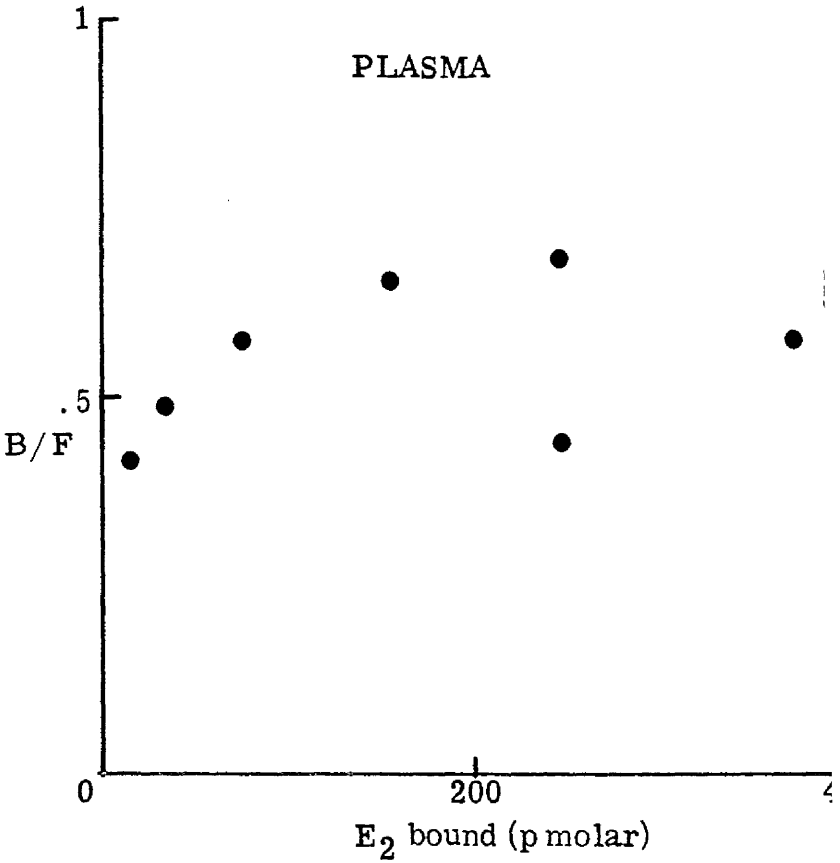
Receptor Status (R_c/R_n)	Lactalbumin Content			
	Negative	Grade I	Grade II	Grade III
+/+	3	4	2	1
o/o	3	2	1	2
+/o	2	2	1	1
o/+	-	-	2	-

Figure 21 - Scatchard Plots of ^{3}H-Oestradiol-17β Binding in the Cytosol and Nuclear Fractions of Breast Tumour Tissue and in Plasma from the Same Patient

Cytosol and nuclear fractions were prepared from breast tumour tissue, as described in Section 2.2.1.3. Blood, collected immediately preoperatively from the same patient and stored in a heparinized container, was centrifuged at 1000 g for 10 minutes at 0°C to yield plasma. The receptor content of all three fractions was determined by incubation with a range of ^{3}H-oestradiol-17β concentrations followed by removal of unbound steroid with DCC in the case of the cytosol and plasma or by washing of the nuclear material. The resultant Scatchard plots are shown of cytoplasmic, nuclear and plasma binding of steroid.


CYTOSOL
B/F
1
.5
0
100
200
E_2 bound (p molar)


NUCLEAR
B/F
.2
.1
0
25
50
E_2 bound (p molar)


PLASMA
B/F
1
.5
0
200
E_2 bound (p molar)

3.4.5.5. Plasma Steroid Levels

The correlation between tissue receptor levels and plasma steroid concentrations in breast cancer patients was studied. Blood was collected immediately preoperatively, during operation and postoperatively from these patients and radioimmunoassay performed to measure plasma levels of oestrogens, progestins and glucocorticoids by Dr. J.K. Grant, Department of Steroid Biochemistry, Glasgow Royal Infirmary. No correlation could be identified between the levels of any of these steroids and the levels of receptor present in the tumour tissue. This finding, along with the failure to detect any receptors in blood plasma (see previous section), rules out the possibility of using a simple blood test in the identification of the receptor status of tumours.

3.4.5.6 Correlation of Nodal Status with Oestrogen Receptor Status

When a mastectomy is performed in cases of breast cancer, any enlarged axillary lymph nodes are biopsied at the same time to test for tumour content. The nodes may or may not be cleared, depending on the findings of these investigations. Two recent reports are at variance over the correlation between nodal involvement in breast cancer and receptor levels in the tumour. Hähnel et al., (1979) report that absolutely no correlation exists, while Allegra et al., (1979a) find that receptor-positive tumours are likely to be associated with nodes lacking tumour content. The latter case might be expected, since both receptor positivity and absence of nodal involvement have been reported independently as giving a better prognosis for the patient.

The receptor results in the present study were examined in relation to the nodal status of the patient, where this had been determined. Table 10 shows

Table 10 Comparison of Receptor Status and Nodal Status in 110 Primary Breast Tumour Biopsies

The overall receptor status of 110 breast tumour biopsies was compared with the tumour involvement of the lymph nodes. Node-negative patients had no nodal infiltration by tumour, while those in the node-positive category had nodes with some degree of tumour content. (R_c - cytoplasmic oestrogen receptor, R_n - nuclear oestrogen receptor). The percentage occurrence in each nodal category is indicated for each receptor category.

R_c/R_n	Node -ve		Node +ve	
	No. of Patients	%	No. of Patients	%
+/+	16	47	18	53
o/o	22	42	30	58
+/o	4	36	7	64
o/+	8	62	5	38

the results of such a comparison carried out on 110 patients, both pre- and post-menopausal. No clear correlation appears to exist between receptor status and nodal status in these patients, in agreement with the report of Hähnel et al., (1979).

3.4.6 EFFECT OF AGE AND MENOPAUSAL STATUS ON RECEPTOR LEVELS IN BREAST TUMOUR TISSUE

The receptor profiles of some breast tumour biopsies were reassessed according to the patient's menopausal status. Table 11 shows the distribution of receptor status in relation to menopausal status. It is clear that there is a slight variation in the distribution of patients between the first three classes of receptor status, but the most dramatic finding is the complete absence of tumours containing only nuclear receptor in premenopausal women. Since the premenopausal group concerned contains 68 patients, it is unlikely that this phenomenon represents an artefact produced by a statistically insignificant number of samples. The ability of receptors to "exist" in the nucleus when none are detected in the cytoplasm may represent some change brought about at the time of the menopause, or an alteration in the permeability of the cell membranes.

A comparison was drawn also between the mean cytoplasmic and nuclear receptor levels in pre- and post-menopausal, receptor-positive patients. Table 12 demonstrates the variation in these mean values. It shows that in the case of both cytoplasmic and nuclear receptors, levels are significantly higher in postmenopausal patients. This is also illustrated if a graph is constructed of cytoplasmic (Figure 22) or nuclear (Figure 23) receptor level against age of patient. In both fractions, a dramatic rise is seen in

Table 11 Comparison of the Distribution of Oestrogen Receptors Between the Cytoplasmic and Nuclear Fractions of Breast Tumour Tissue in Pre- and Post-menopausal Patients

The presence (+) or absence (o) of cytoplasmic (R_c) and nuclear (R_n) oestrogen receptors is summarised as in Table 5, but taking into consideration the menopausal status of each patient. The figures include biopsies of both primary and secondary lesions.

Receptor Content (R_c/R_n)	Premenopausal		Postmenopausal	
	No. of patients	Percentage	No. of patients	Percentage
+/+	22	32	69	36
o/o	34	50	81	42
+/o	12	18	26	13
o/+	0	0	17	9

Table 12 Variation in Mean Cytoplasmic and Nuclear Oestrogen Receptor Levels Between Pre- and Post-menopausal Patients

The mean value for cytoplasmic (expressed in fmol/mg cytosol protein) and nuclear (expressed in fmol/mg DNA) oestrogen receptors in breast tumour tissue was determined for pre- and post-menopausal patients. A student's T test was performed on each population to determine the difference between them.

Tissue Fraction	Mean Receptor Value		Significance
	Premenopausal	Postmenopausal	
Cytoplasmic	49	95	$p < 0.001$
Nuclear	1115	1750	$p < 0.001$

Figure 22 - Levels of Cytoplasmic Oestrogen Receptors in Breast Tumour Tissue as a Function of Age

Levels of cytoplasmic oestrogen receptor are summarised for 150 patients (chosen at random) as a function of age. Premenopausal patients are indicated by (O), perimenopausal by (▲) and postmenopausal by (●).

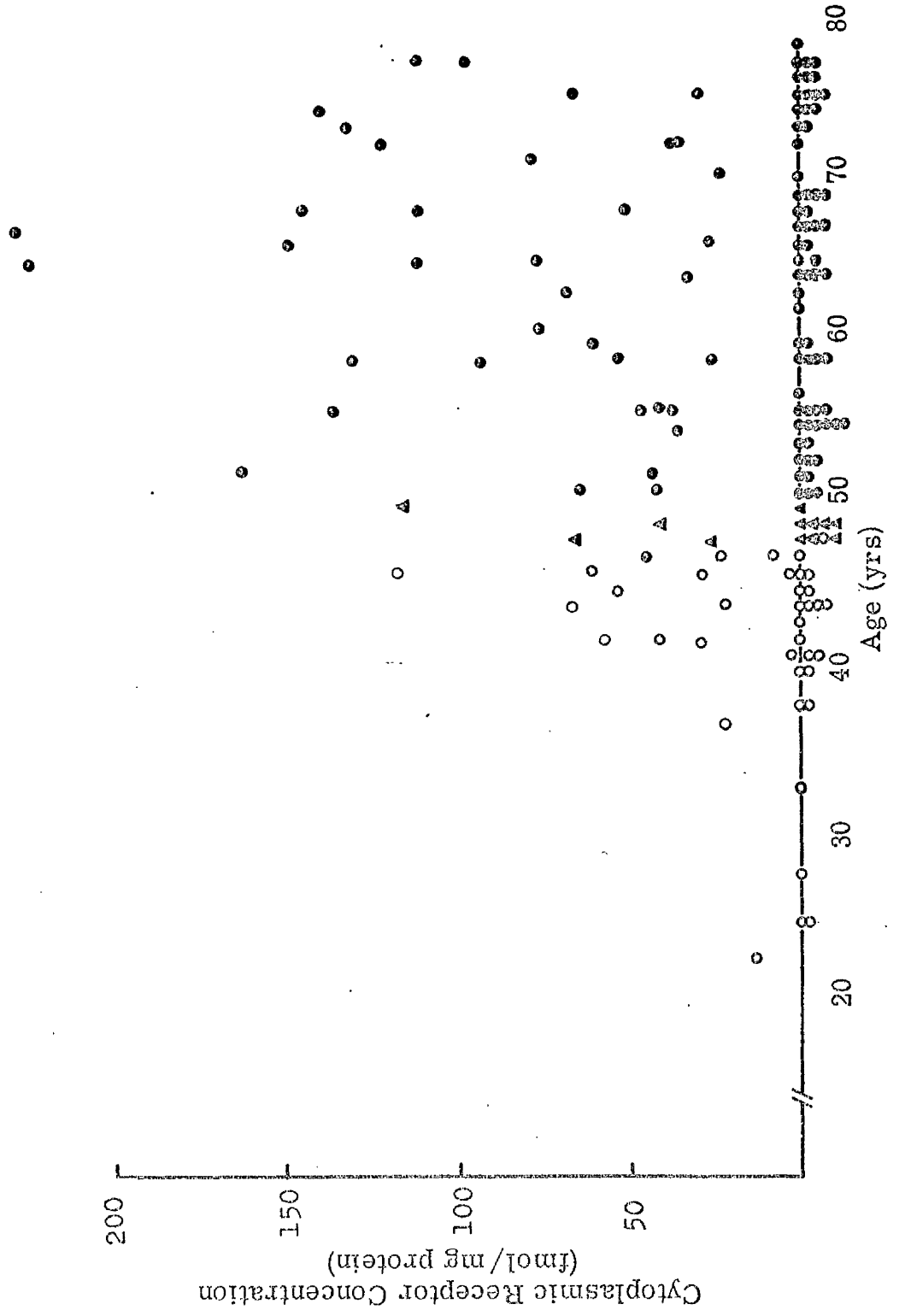
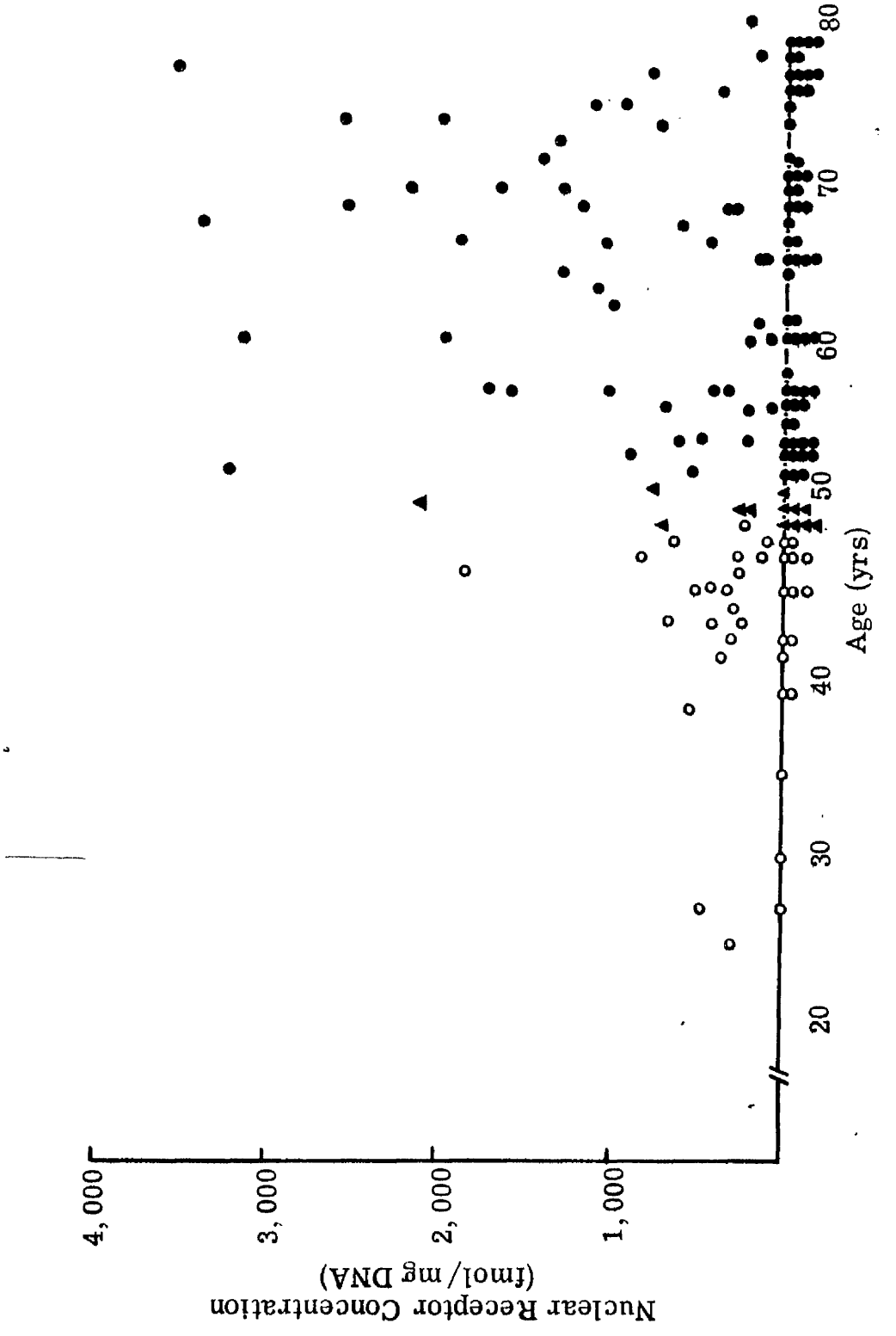

Cytoplasmic Receptor Concentration
(fmol/mg protein)
200
150
100
50
20
30
40
50
60
70
80
Age (yrs)

Figure 23 - Levels of Nuclear Oestrogen Receptors in Breast Tumour Tissue as a Function of Age

Levels of nuclear oestrogen receptor are summarised for 150 patients (chosen at random) as a function of age. Premenopausal patients are indicated by (O), perimenopausal by (▲) and postmenopausal by (●).


Nuclear Receptor Concentration
(fmol/mg DNA)
4,000
3,000
2,000
1,000
20
30
40
50
60
70
80
Age (yrs)

receptor levels around the age group 45-50 (the menopause region). This same age effect on cytoplasmic receptor has been noted by many workers, (e.g. Hilf & Wittliff 1974, King et al., 1979b). Allegra et al., (1979a) found no such correlation between cytoplasmic receptor levels and age once the menopausal effect was taken into account. This is in agreement with the findings illustrated here (Figure 22). The rise in receptor levels seen at the menopause age is a sudden, rather than a gradual, change. The latter effect would be noted if age had caused the rise rather than the menopause. The reason for the influence of the menopause on receptor levels is not clear. From the evidence accumulated in earlier sections, the assay being used here measures total receptor levels, so that there can be no suggestion of endogenous steroid masking receptors in premenopausal patients. Another proposed explanation has been that cytoplasmic receptor levels may be lower in premenopausal patients due to the large percentage residing in the nucleus as a result of higher circulating levels of oestradiol. The present finding that nuclear levels of receptor are significantly higher in postmenopausal patients tends to discount this theory. Further Maynard & Griffiths (1979), in a study of nuclear oestrogen, found no difference in levels between pre- and post-menopausal tissue. This was also a preliminary observation in the present study, but a difference became apparent as more data became available.

Whatever the real reason may be for the dramatic change in receptor levels after the menopause, it has been noted consistently, and Mobbs & Johnson (1976) suggest that different cut-off points should be set in pre- and post-menopausal situations to allow for it.

3.4.7 RECEPTOR LEVELS IN TISSUE OTHER THAN BREAST

Just as the determination of receptor status in breast tumours has proved an invaluable aid in the selection of therapy for such disease, it was postulated that the same test might be of value in the treatment of other forms of cancer, even those of tissues not normally regarded as responsive to steroids. One area which seemed appropriate for such a study was the gastro-intestinal tract. Colorectal tumours constitute the predominant form of cancer in the male in the West of Scotland, but no successful therapeutic regime has been discovered, as yet. Chemotherapy gives very unsatisfactory results, so hormonal therapy is a possible alternative, although its use at present would be merely tentative, in the absence of positive proof of hormonal involvement. There is some suggestion that the colon might be sensitive to hormonal manipulation. As mentioned in the introduction, cancer of the gastro-intestinal tract and hormone-dependent tumours have been linked by a common dependence on diet (Berg, 1975). Colorectal cancer is also one of the most common forms of cancer associated with breast cancer (Hermann, 1972). Water uptake in the colon is effected by changes in plasma oestradiol levels found in pregnancy and during the menstrual cycle, also suggesting some hormonal involvement. These arguments are all fairly tenuous but the presence of oestrogen receptors in colonic carcinoma tissue, even in only a percentage of cases, would suggest a firm basis for hormonal control in this area and, consequently, for a trial of hormonal therapy for this type of cancer.

With this aim in mind, oestrogen receptor levels were measured in colonic carcinoma tissue samples. Tissue was transported and stored, where necessary, as for breast tissue (see Methods, section 2.2.1). The method of fractionation of the tissue was again identical to that used for breast

tumour samples, except that use of an Ultra-turrax homogeniser was not necessary due to the much softer nature of the colonic tissue. Great care was required also to avoid contamination of the fractions with plasma, since this tissue was much bloodier than breast tumour tissue. Such contamination could lead to erroneous results due to binding of added steroid by plasma proteins, but this should be revealed by competition experiments using excess unlabelled DES, which will not, itself, interact with the major plasma binding proteins. Measurement of oestrogen receptor levels was carried out using basically the system described for breast tissue in Figure 19. Some variations in methodology were compared with this procedure to determine the viability of the assay system using the different tissue type. One of these was to assess the effectiveness of the thiol reagent dithiothreitol (DTT) in protecting the sulphydryl groups of colonic oestrogen receptor. As described in section 3.1.2, a comparison was made between DTT and monothioglycerol, another commonly used thiol reagent. No difference could be detected in the binding measured using either of these agents (see Figure 7). The temperature of incubation with ^{3}H-oestradiol was also varied to allow for the fact that the hormone/receptor complex in either fraction of colonic tissue might be more stable at low temperatures than the complex found in breast tissue preparations. Using incubations at 4^{o}C or 15^{o}C, either of which would be successful in breast tissue fractions, no binding could be detected.

Receptor assays were carried out on 25 samples of colonic carcinoma tissue from both male and female patients, all with advanced disease, and aged mainly over sixty. In no case, even with the variations noted above, could oestrogen receptor of the type found in breast tumour tissue be detected. In one case, a Scatchard plot was successfully constructed for only the

cytosol of a colonic sample, the nuclear fraction containing no evidence of binding. However, the binding was not competed out by DES, and so was deemed to reflect a non-cellular form. In a further two cases, a small level of specific binding could be detected by assay at a saturating concentration of ^{3}H-oestradiol (10^{-9}M) with and without excess unlabelled DES. However, in these cases, no satisfactory Scatchard plot could be constructed, and the origin of this binding could not be determined. Experiments were carried out to measure the uptake of ^{3}H-oestradiol into several colonic tumour samples. Tissue slices were incubated with the labelled steroid, washed, fractionated and the level of activity in each fraction measured immediately. The results from these studies indicated no evidence of steroid binding in any case.

These results, along with further results from this study have been published recently (Leake et al., 1980), and show that oestrogen receptor of the type found in breast tumour tissue cannot be detected in colonic carcinoma tissue, if binding is to satisfy the criteria set down in section 3.4. Response to the anti-oestrogen tamoxifen in such patients was also minimal. This is in contrast to the findings of McLendon et al., (1977) and Alford et al., (1979), both of whom found variable levels of receptor in 24-30% of colonic neoplasms. Alford et al., used very high levels of ^{3}H-oestradiol in their assay procedure, and used unlabelled oestradiol as competitor. As this would not rule out binding to non-cellular proteins, the binding they observe may not be attributable to a true oestrogen receptor, especially since the tissue type is more likely to be contaminated by other binding proteins than is breast tissue. Further work in this area may reveal the reason for the discrepancy between the findings from the present study and those in the other reports.

Apart from colonic carcinoma tissue, receptors were measured in a limited number of tumours from other sites. Assay of oestrogen binding in three samples of ovarian carcinoma and four biopsies from endometrial carcinoma tissue demonstrated not only that the assay system developed for breast tumour tissue was successful in determining receptor levels in these other tissues, but also that, as with breast, not all tumours from any particular target site in the body contain receptor. In addition, the viability of the assay system in these areas suggests that, if receptors are present in colon, they should be detected by the same method established for breast tissue.

3.5 PATIENT FOLLOW-UP

3.5.1 RESPONSE TO HORMONE THERAPY IN RELATION TO RECEPTOR STATUS

In order to assess the reliability of the assay system in providing an index of response to hormonal therapy by breast tumours, follow-up data were compiled on patients, the receptor content of whose tumours had been determined. The amount of this information which is of value in this respect is limited by a number of factors. Firstly, only patients with recurrent disease are given therapy to which the objective response can be measured and the findings may not be conclusive in all cases. Secondly, only patients receiving solely hormonal therapy can be included. The majority of hospitals are currently participating in various trials of mixed forms of therapy in order to elucidate the most successful therapeutic regimes. In these trials, the various combinations of therapy are allocated to patients at random. The augmentation of hormonal with another form of therapy, whether chemotherapy or radiotherapy, means that no assessment of

response to the hormonal part alone can be made, and so the patient must be excluded from this study. The last limiting factor is that, as a result of criteria established by the British Breast Group (1974), regression of a tumour must be observed for at least six months with no new lesions appearing during that time for a response to be recorded as having occurred. This means that six months must elapse, therefore, before any report relevant to this assessment can be made. If the tumour ceases to show regression at any time during the six month period, then a failure is reported. These criteria have been criticized as being too harsh by Stoll (1977), who found instances which he felt should be classed as responses, but which failed to satisfy the time limit specified. The British Breast Group rules also state that 50% decrease in tumour diameter should be noted, which, as pointed out by Stoll, means that the neoplasm must shrink to 1% of its original volume.

The strict nature of these criteria is emphasised when a report by Engelsman et al.,(1973) is considered. They found, using different rules for response, that 80% of receptor-positive tumours responded to therapy, although their numbers are very low and thus may not be entirely significant. Regression was required for only 3 months in their scheme instead of six. This figure is much higher than those of other workers who consider cytoplasmic levels of receptor as the sole index of responsiveness. In spite of these facts, the British Breast Group rules have been adhered to in the follow-up data presented here.

Response to hormone therapy was assessed in 124 tumours, biopsies of which had been assayed for the presence of oestrogen receptors. These results are summarized in Table 13. The hormonal therapy applied represents a

Table 13 Response of Breast Tumours to Hormone Therapy in Relation to their Oestrogen Receptor Content

The response of breast cancer patients to various forms of hormonal therapy, both additive and ablative, was determined and compared with the receptor status of their tumour. British Breast Group rules, as described in Section 3.5, were adhered to in determining "response". Only those exhibiting a complete response to a hormonal regime are included in the table.
(R_c - cytoplasmic oestrogen receptor,
R_n - nuclear oestrogen receptor).

Receptor Status (R_c/R_n)	Total No. of Patients	No. of Patients with complete response	Percentage Response
+/+	42	30	71
o/o	57	5	9
+/o	17	4	24
o/+	8	1	12

mixture of ablative and additive forms comprising mainly oophorectomy, stilboestrol and tamoxifen administration. It is shown that approximately 70% of patients who should, on the basis of containing both cytoplasmic and nuclear oestrogen receptors, respond to a hormonal form of therapy do, in fact, show a favourable reaction. Of those patients whose tumour tissue contained no measurable receptor in either fraction, only 10% responded to any form of hormonal therapy. These patients had principally received tamoxifen and presumably responded through some indirect pathway, such as the inhibition by tamoxifen, of prostaglandin synthesis (Tisdale, 1977). The tumours which had been found to contain cytoplasmic, but no nuclear, receptor, and thus would not be expected to display hormone-dependence, demonstrated a slightly better response to hormonal manipulation than those containing no receptor at all. In the last group, representing the interesting situation where receptors were found solely in the nuclear fraction of the tissue, response was no greater than that observed in tissue lacking receptor completely. The numbers in this group are low, and the percentage could change as more data are accumulated.

The finding that the last group of patients whose tumours lacked cytoplasmic receptor did not respond to hormone therapy is a guide to the functioning of these presumably unfilled nuclear receptors. It has not been possible up till now to determine whether these receptors are functional, although Zava et al., (1977) postulated that they did act to give oestrogen stimulation of growth in MCF7 cells, and moreover that such cells might not respond to ablative therapy but to antioestrogens. This is not completely reliable as a guide to this type of situation in vivo, since the receptors in MCF7 cells have been shown to be atypical of the normal cellular form. The results obtained in this study show conclusively that

the presence of the receptor exclusively in the nucleus does not suggest a favourable response to hormone therapy, ablative or additive. These abnormal receptors are thus not functional in stimulating tissue development in response to oestrogens.

The fact that the group containing only cytoplasmic receptor responded more favourably than the tumours containing no receptor is unexpected. The preliminary result shown in Figure 20 suggests that these receptors may be unable to enter the nucleus, and hence no oestrogen stimulation of growth should occur. Any benefit experienced as a result of hormonal therapy would then have occurred via an indirect pathway. Another possible reason for response would be that the biopsy assayed for receptor content had contained a very low level of nuclear receptors which were not detected by the assay method. This seems unlikely, however, due to the sensitivity of the procedure. It is possible that the percentage response obtained here, is, in fact, an artefact caused by the lower number of patients in this group. Further follow-up information may yield a percentage closer to that encountered for tumours with no receptor present.

The fact that only 70% of patients who should respond to hormone therapy actually do so, while 90% of those with no receptors in either fraction show no favourable response, confirms the idea that a receptor-negative result is of more predictive value than a receptor-positive one (Maass et al.,1972, 1975, Jensen, 1975, King, 1975, Lippman, 1976). However, the inclusion of a determination of nuclear receptor content has increased the success rate over that obtained when only cytosol receptors were measured. The principal benefit has come from the ability to identify false-positive tissue, containing only cytosol receptor. Some of these tumours do

respond to hormone therapy, as mentioned above, but this is only a fraction of the number of the genuinely receptor-positive cases which show a favourable response.

The nuclear assay has also proved useful in identifying the small percentage of tumours containing receptor at this site only. These tumours do not appear to respond to hormonal therapy either, and if no cytoplasmic determination of receptor content were carried out, then this group would be the "false-positives". A combination of both assays is thus essential for the maximum yield of predictive information on the behaviour of any tumour.

Initial studies on receptor content of colonic carcinoma tissue were carried out with the aim of establishing whether the same type of index for hormonal response could be prepared for these tumours. However, useful follow-up data were stictly limited. The advanced stage of the biopsies received meant that patient death occurred very soon after biopsy in the majority of cases, and hormonal treatment was not considered for others.

Subsequent expansion of this work has included the administration of the antioestrogen tamoxifen, and determination of response to this therapy relative to receptor content has been performed (Leake et al., 1980).

3.5.2 RELATIONSHIP BETWEEN DISEASE FREE INTERVAL AND RECEPTOR STATUS

A comparison was made between receptor status and the time which elapsed between the diagnosis of primary breast cancer and any recurrence. This could be made only where receptor status had been determined in the primary

tumour, the absence of this information leading to the exclusion of some cases. Figure 24 shows that the disease free interval is longer in receptor-containing tumours than in those containing no receptor at all, which was noted also by Maynard & Griffiths (1979). In the remaining two classes of tumour bearing receptor in one fraction only, too few recurrences have been noted (due to the lower incidences of these types of tumour) to draw any meaningful conclusions about relapse times. Preliminary data (Figure 25) show that these two groups of patients exhibit similar patterns of recurrence and that this is closer to that observed in receptor-negative than receptor-positive cases (Figure 24). From the findings of the response to hormone therapy by these tumour types, they appear to behave as receptor-negative, but this conclusion in respect of relapse rate awaits more follow-up data. A much higher proportion of receptor-negative patients relapse within a three-year period after initial diagnosis than do receptor-positive patients.

These findings are in agreement with those of Knight et al., (1977), Block et al., (1978) and Hähnel et al., (1979) that receptor-positive tissue displays a much longer disease free interval, and that the patient's prognosis was greatly improved over that of a patient bearing a receptor-negative tumour. This may be accounted for by the observation of Meyer et al., (1977) that receptor-negative tumours grow more aggressively than those possessing measurable levels of binding. The former would therefore spread to other parts of the body much more quickly. This may also form the basis of the suggestion by Byar et al., (1979) that the site of metastasis altered the prognosis of breast cancer patients and the observation in this study that receptor-positive tumours form mainly local metastases, receptor-negative ones spreading further in the body,

Figure 24 - Disease Free Interval in Breast Cancer Patients in Relation to the Oestrogen Receptor Status of the Tumour (+/+ and o/o Biopsies)

Oestrogen receptor content of both soluble and nuclear fractions of a biopsy of primary breast disease was determined at the time of simple mastectomy. Patients received no adjuvant therapy. Time and site of first clinical recurrence was noted. Percentage of patients remaining disease free was plotted against time for groups of 50 patients in the two categories: receptor-negative and fully receptor-positive.

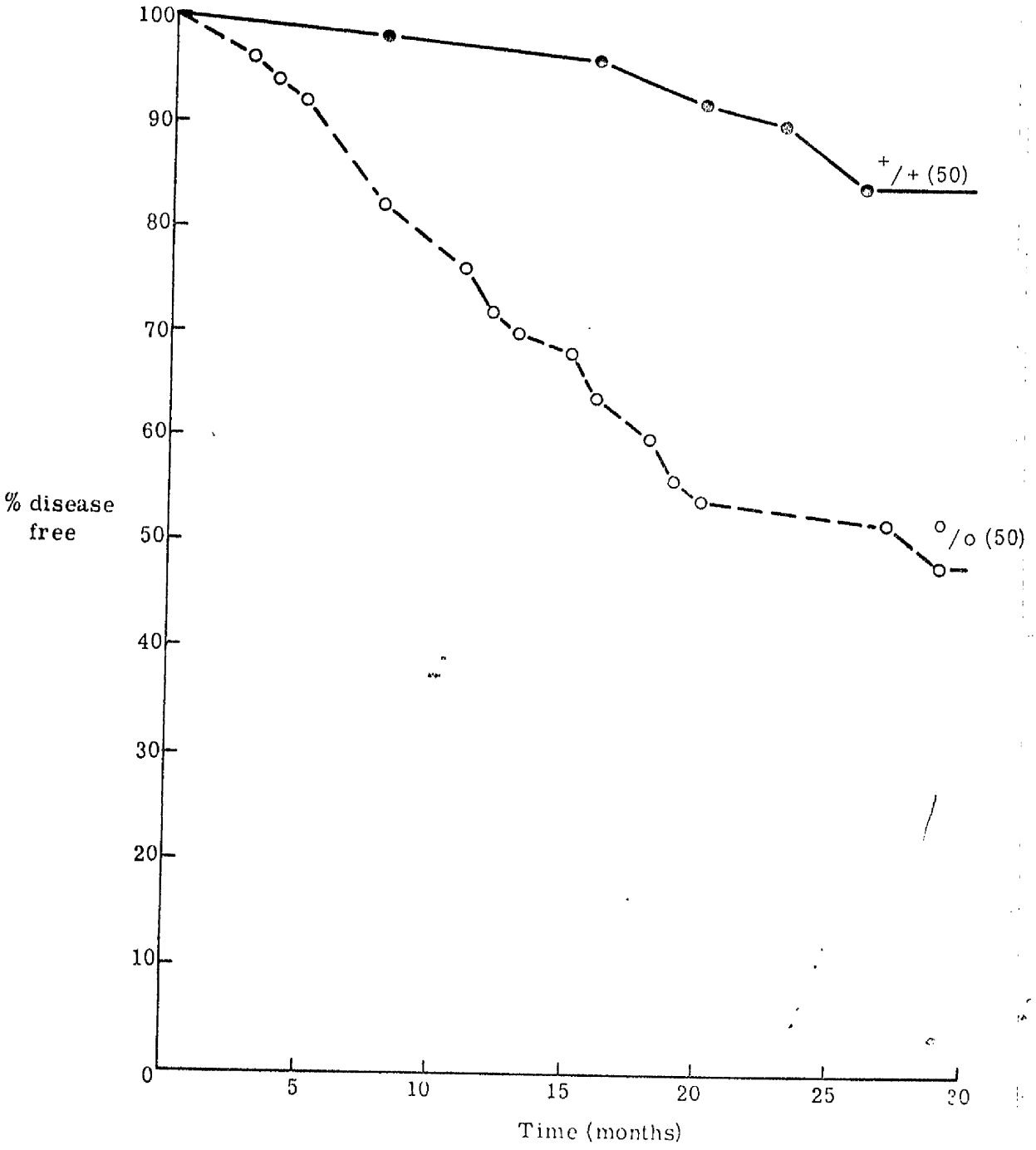
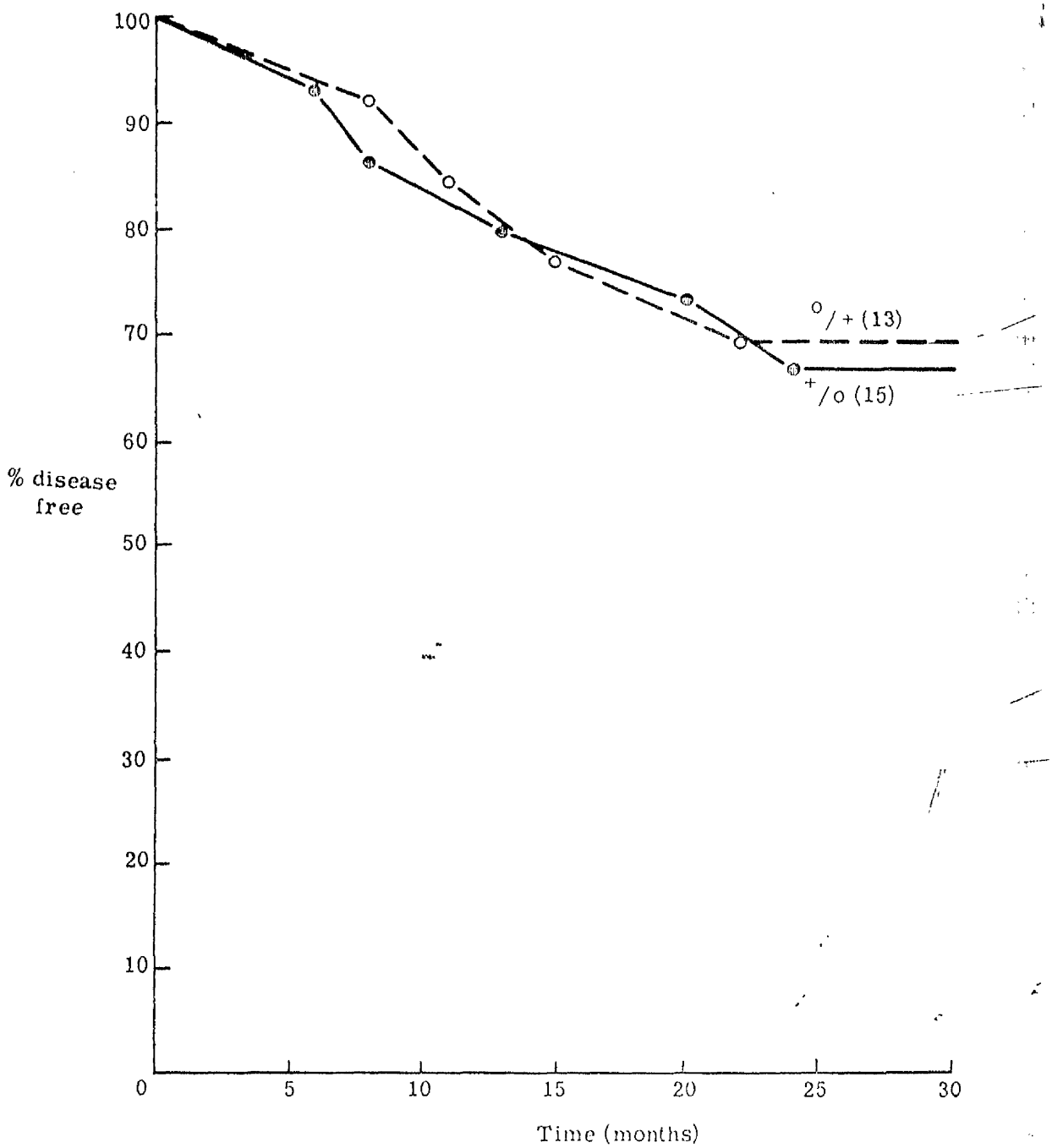

100
90
80
70
60
50
40
30
20
10
0
% disease free
+/+ (50)
o/o (50)
5
10
15
20
25
30
Time (months)

Figure 25 - Disease Free Interval in Breast Cancer Patients in Relation to the Oestrogen Receptor Status of the Tumour (+/o and o/+ Biopsies)

Patients in the two groups whose primary biopsies yielded abnormal oestrogen receptor were analysed in the same manner as those referred to in Figure 24.


100
90
80
70
60
50
40
30
20
10
0
% disease free
5
10
15
20
25
30
Time (months)
o/+ (13)
+/o (15)

which was also noted by Walt et al., (1976).

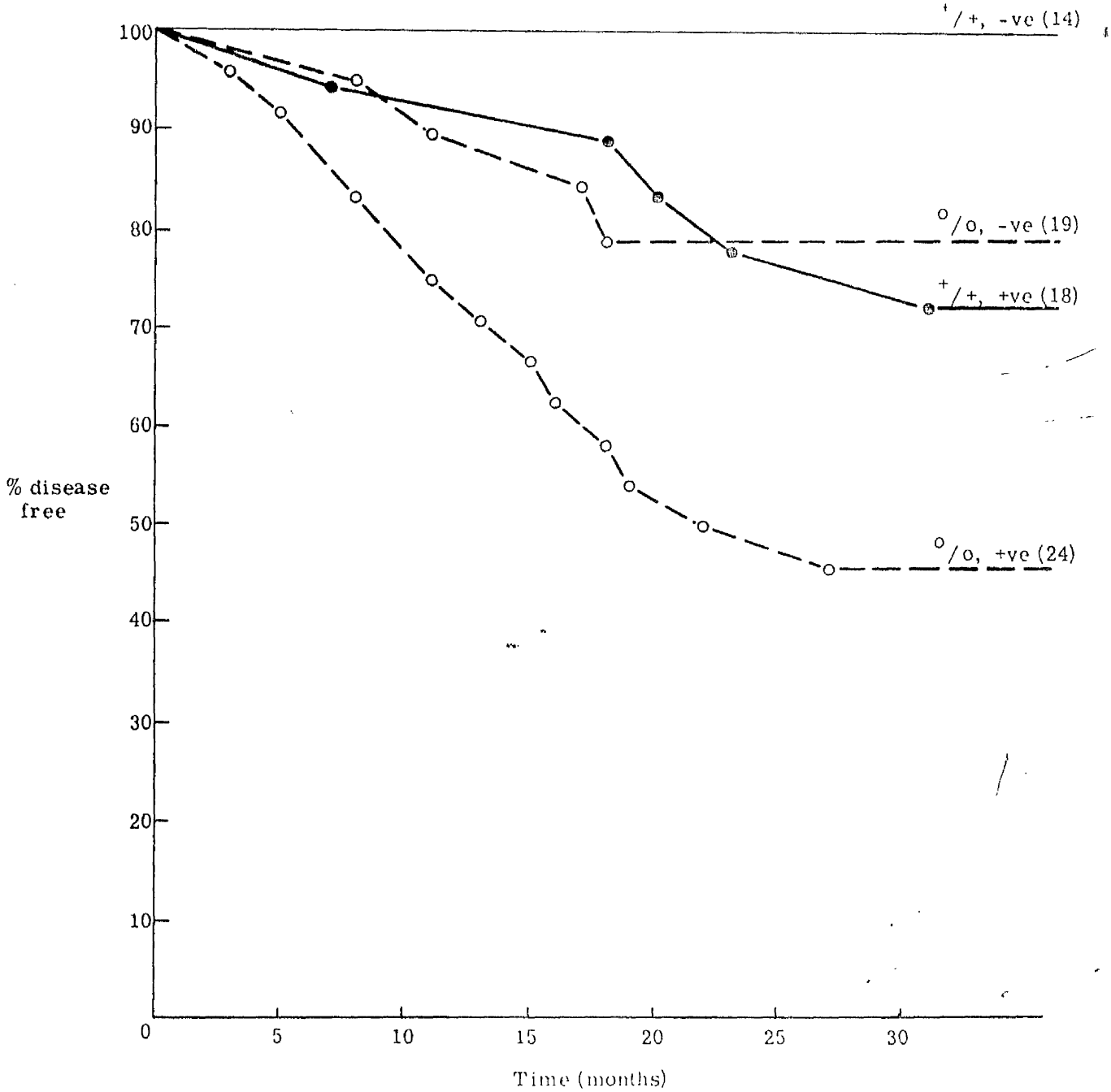
3.5.3 RELATIONSHIP BETWEEN NODAL STATUS, RECEPTOR STATUS AND DISEASE FREE INTERVAL

Nodal status, like receptor status, has been implicated in determining the length of the disease free interval in cases of breast cancer. Hähnel et al., (1979) discovered a considerably shorter disease free interval in tumours with lymph node involvement. When receptor status was considered also, receptor-negative tumours with nodal involvement were found to give the worst prognosis, although the effect of receptor content decreased as time progressed, possibly due to the effect of hormone-dependent tumours becoming autonomous. The influence of the nodal involvement did not lessen, however, this being the determining factor on prognosis at the end of their study.

The incidence of recurrences in tumours for which both receptor and nodal status had been determined in the present study was considered. The resultant graph is shown in Figure 26. Only tumours in the +/+ and o/o receptor groups are represented, since the relevant information was not available on a sufficient number of samples in the other two groups. The receptor-positive, node-negative group of tumours gave the best prognosis in Figure 26, receptor-negative, node-positive ones showing the highest recurrence rate. The other two classes (receptor-positive, node-negative and receptor-negative, node-positive) displayed a very similar pattern over the first 20 months considered. As time progressed, however, the effect of nodal status appeared to become predominant. This same effect was noted by Hähnel et al., (1979), but to a greater degree. Follow-up of these patients for a longer period of time may reveal whether receptor

Figure 26 - Disease Free Interval in Breast Cancer Patients in Relation to the Oestrogen Receptor Status and Nodal Involvement of the Tumour

Biopsies of primary breast cancer were assayed for oestrogen receptor status and corresponding nodal status determined by routine pathological section of the axillary tissue and histological examination of all identified nodes. Percentage of patients remaining disease free is plotted against time for both fully receptor-positive (●) and receptor-negative (O) in relation to presence or absence of nodal involvement (+ve or -ve). Figures in brackets indicate numbers of patients in each group.


+/+, -ve (14)
o/o, -ve (19)
+/+, +ve (18)
o/o, +ve (24)
% disease free
100
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
Time (months)

status or nodal status is the governing factor in the prognosis of breast cancer sufferers.

4. DISCUSSION

The mode of action of steroid hormones in normal, as opposed to transformed, target cells is presently accepted as basically the two-step mechanism simultaneously proposed by Gorski et al. and Jensen et al. in 1968. A greater understanding now exists of the intermediate processes which has led to suggested modifications of either certain steps (Linkie & Siiteri, 1978) or the concept as a whole (Sheridan et al., 1979), but, generally, a steroid enters the target cell, becomes associated with a specific receptor protein, enters the nucleus and elicits genetic responses. The hormone can enter the cell by passive diffusion. The receptor molecules available to it may be free in the cytoplasm, or loosely bound to the cytoplasmic membrane (Wittliff, 1975), but this is not certain as yet. Following the interaction between the hormone and receptor, there is a heat-dependent activation step, involving the addition of a further polypeptide subunit to the receptor (Notides & Nielsen, 1974, Leake, 1976). This was thought, initially, to occur within the cytoplasm, and constitutes a major requirement for entry of the hormone and receptor into the nucleus (Notides & Nielsen, 1974, Spelsberg, 1974). Linkie & Siiteri (1978), however, now propose that the activation process occurs within the nucleus itself, not representing an entry qualification. The actual site of activation is an important point in the understanding of receptor functioning. The concept that it occurs inside the nucleus would explain the discovery of unfilled receptors within the nucleus of some breast tumour material (Garola & McGuire, 1977a, Panko & MacLeod, 1978, Kato et al., 1978, Thorsen, 1979) and the proposal by Sheridan et al., (1979) that these are always present. Another reason for apparently empty nuclear receptors would be that receptor transport had been facilitated by binding to a weak ligand.

Following translocation of the hormone/receptor complex, whether this happens before or after activation, it binds to the chromatin within the nucleus at specific acceptor sites, presumably defined by their three-dimensional conformation (Leake, 1976). Binding at these sites may involve both non-histone proteins (Spelsberg et al., 1972, O'Malley et al., 1972, Charreau & Baldi, 1977) and DNA (King & Gordon, 1972), although their actual nature is still somewhat uncertain (Yamamoto & Alberts, 1976). As a result of these interactions, responses are seen within the cell, beginning after 15-30 minutes with an increase in RNA polymerase B activity, and continuing for many hours with more long-term effects. Specific products of oestrogenic stimulation of breast tissue cells include α-lactalbumin, casein, progesterone receptor and oestrogen receptor itself (Sarff & Gorski, 1971, Clark et al., 1977) a mixture of recycling and de novo synthesis of this latter protein occurring following release of the hormone/receptor complex from chromatin (Neithercut, 1977). Recently, even later protein markers of stimulation have been noted, suggesting further processing of the complex (Westley & Rochefort,1979).

The preceding paragraphs dealt with the situation encountered in a normal breast cell undergoing oestrogenic stimulation (e.g. during pregnancy and lactation). In the normal, non-lactating breast, oestrogen receptor-containing cells are present in very small numbers,although the potential to commence synthesis of more receptor and activate the processes described above does exist. In the course of malignant transformation of the cells the receptor-synthesising machinery may be either activated or abolished, so that, in the latter case, the tissue is no longer capable of hormonal response. In the situation where receptor synthesis is "switched on", tumour tissue growth will come under hormonal control. Thus, these

hormone-dependent tumours will be sensitive to hormonally-based therapies aimed at destroying the integrity of the stimulatory mechanisms within the cell.

Before the realisation that this process might occur during tumour formation, it was noted that not all breast cancer patients received positive relief from disease as a result of hormonal therapy, while some, as reported by Beatson (1896), benefitted greatly. Some prediction of which patients would respond was called for (Pearson & Ray, 1960, Baker et al., 1960). Finally, Jensen et al.,(1971) postulated that tumours which were hormone-dependent could be identified by their oestrogen receptor content. It was suggested that, if a tumour contained this receptor, then the machinery discussed earlier must have been activated, and the tissue would fall under hormonal control. Removing the source of oestrogens or impeding their action would thus arrest tumour growth. Absence of these receptor proteins would, then, indicate a lack of hormonal control, and suggest that hormonally-based therapeutic regimes would have no beneficial effect on that tumour.

Many methods have been described for the measurement of oestrogen receptors in breast tumour tissue during the last decade, following the introduction of the concept that this might aid in the treatment of breast cancer patients. Techniques have advanced greatly since the initial studies of Folca et al., (1961) on the uptake of tritiated-oestrogens into patients' breast tissue, relative to their response to adrenalectomy. Simple assessment of cytoplasmic receptor levels has never given absolute accuracy in the prediction of response of breast cancer patients to hormone therapy. Additional guides have been sought, with the idea that some further marker of oestrogenic

action would give a more reliable indication of hormone-dependence. Markers proposed have been selected on the basis that oestrogenic-stimulation is not a simple domino response arising from the binding of soluble receptor and so measurement of the latter does not preclude lesion(s) at a later stage(s). Some substances which have been proposed as indicators of oestrogenic stimulation are, for example, progesterone receptor (Asselin et al., 1977),casein mRNA (Rosen & Socher, 1977) α-lactalbumin (Ip & Dao, 1978) and peroxidase (De Sombre et al., 1975, Anderson et al., 1975, Lyttle & De Sombre, 1977a). Of these, the progesterone receptor has proved most popular, although doubt is now cast on whether measurement of both cytoplasmic oestrogen and progesterone receptor does, in fact, increase the success of predicting the response of breast cancer patients to hormonal manipulation (McGuire, 1978). Failure of tumours containing both species to react to hormonal stimuli (Ip et al., 1979) has led to the suggestion that further steps in the stimulatory pathway must exist, which may be blocked as a result of malignant trans-formation.

The assay system developed in this project considers the nuclear, as well as cytoplasmic oestrogen receptor concentration, on the basis that a major point of blockage in the oestrogenic pathway would exist at the entry of the receptor into the nucleus and its binding to the correct acceptor sites. Several properties of the receptor, and characteristics of breast tumours have also been explored in the course of designing and evaluating this procedure. Although the aim of the system was to demonstrate the presence of receptors in each cellular fraction, the nature of the process developed provides additional information about the binding observed. Such complete

analysis requires at least 150 mg of tissue. An amended method for receptor quantitation can be carried out on as little as 50 mg, but with a consequently decreased yield of information. In these cases, a "one-spot" assay, such as those used by Mobbs & Johnson (1976) and McGuire et al., (1977c) is employed.

A "one-spot" determination of receptor concentration compares the binding of ^{3}H-oestradiol-17β alone with that in the presence of excess unlabelled steroid. This serves to quantitate the receptor, and at the same time, reveal the degree of specificity of binding. The theory behind this is that specific sites are, by definition, saturable, while, at physiological levels of steroid at least, non-specific binding is unsaturable. When ^{3}H-oestradiol alone is added to a receptor-containing preparation, it will fill up specific receptors and any excess will bind to non-specific sites. Radioactivity measured would then represent both specific and non-specific binding, the proportion of one to the other being impossible to determine. In the presence of excess unlabelled steroid, however, this would be likely to fill the majority of the available specific sites, and a number of the non-specific sites. By far the majority of ^{3}H-oestradiol bound would then represent non-specific binding. A comparison of binding under these two conditions would, thus, determine the level of specific binding. A further point is that diethylstilboestrol (DES) is a better competitor than oestradiol itself, as the former does not interact with non-cellular binding proteins, such as SHBG (King & Mainwaring, 1974), which might contaminate the cellular fractions. A comparison of the efficiency of unlabelled DES or oestradiol in the role of competitor revealed no difference in the nuclear fraction, but a small difference in receptor levels detected

in the cytoplasmic fraction (see Results, section 3.1.3.3). This effect was not so great as to cause concern, but might be more evident in other tissue types, so that the use of unlabelled DES as competitor, in preference to oestradiol, is advised.

When an adequate amount of tissue (150 mg) is available for a more complete assay to be performed, not only the amount of receptor and its specificity, but also its affinity for oestradiol can be determined by the application of Scatchard analysis.

This requires measurement of ^{3}H-oestradiol binding at several different concentrations of steroid (seven are used in the present system). The assay developed by Hawkins et al., (1975) is very similar in form to the one described in this thesis. The authors, however, measure specificity of binding at every point on the Scatchard plot. In the course of this study, this procedure was found to be unnecessary on a routine basis (due to the relatively low - 15% - non-specific binding found in most breast tumours) and the tissue requirement is also kept to a minimum. Specificity was determined at only the maximum concentration of oestradiol (10^{-9}M), while the absolute level of receptors (specific and non-specific) and the dissociation constant of binding (Kd) are derived from the Scatchard plot (see Methods, section 2.2.4, for a full explanation). Only when the level of non-specific binding is high, would the absolute level of receptors determined from the plot vary significantly from the specific number. This would be detected using the "one-spot" test for specificity and, sample size permitting, the assay could be repeated with competition at each concentration. An assay of a similar form to the one reported here

was described by Feherty et al., (1971). That method required only 100 mg of tissue, but was more complicated in its operation. This fact would render it less suitable for application on a routine basis than the system described in this thesis.

In order to reduce the amount of material required to construct a Scatchard plot, Johnson et al., (1975) and Johnson & Nakamura (1977) have reported success in measuring receptors using a plot comprising only two points, as opposed to the seven deemed necessary by studies in this project. They were able to detect receptors in as little as 50 mg of tissue using their system. The potential errors which could result from such a method are very great, however, and a "one-spot" study would appear to be adequate in cases of a limited supply of tissue.

Although the use of Scatchard plot analysis greatly enhances the yield of information from a binding study, the theory behind its application and the calculations involved in its construction appear complex. Misunderstanding of the theoretical aspect can lead to errors in interpretation of results. This has been the subject of a review by Chamness & McGuire (1975), who concluded that misinterpretation of data caused delay in the publication of otherwise significant results, and they presented procedures for the correction of specific faults. The tedious nature of the calculations involved in processing the results of a Scatchard analysis can be alleviated by the application of a computer programme, such as that designed by Aitken & Lippman (1977).

In spite of problems which might be raised due to errors in construction or interpretation of these plots, they are, nevertheless, of great value

in providing useful information when properly applied. The fact that they can be used to determine the affinity of binding in addition to the amount of receptor means that it is possible to identify with greater certainty the nature of the receptor present. When used in combination with a competition study, therefore, three important parameters in connection with steroid binding can be determined using relatively little material (concentration of receptor, its affinity and its specificity). The use of a competition study on its own (a "one-spot" determination), although necessary in cases of minimal tissue availability, is not so satisfactory in its yield of information, as was demonstrated clearly in the course of quality control experiments, where "one-spot" assay results were shown to be misleading by comparison with results from the appropriate Scatchard plot (see Results, section 3.3).

An important observation made during the design of the present assay system was the instability of the hormone/receptor complex even at low temperatures. Katzenellenbogen et al., (1973),working with immature rat uterine material and Chamness et al., (1975) using mature rat uterus, found that exchange of added for endogenous steroid bound to the cytosol receptor did not occur to any measurable extent at $0^{o}C$, and determined that a temperature of $25^{o}C$ or $30^{o}C$ was necessary for any interaction to occur. Anderson et al., (1972), studying the nuclear receptor in the immature rat uterus, used incubation at $37^{o}C$ for 1 hour to achieve exchange. Studies carried out in this project revealed that exposure of nuclear receptor from postmenopausal breast tumour to these conditions would cause degradation, as demonstrated in Figure 9. However, stability of the premenopausal form of the receptor, or nuclear receptor from mature rat uterus appears to be maintained for a longer period of time at this elevated

temperature, as shown in Figures 10 and 8. This observation in pre-menopausal breast tumour tissue may not be reliable, however, since the study on this tissue type was performed only once due to lack of suitable material. It is possible that the result obtained represents an atypical form of the receptor.

Figures 8-10 also demonstrate the ability of the nuclear receptors in these systems to allow the uptake of steroid at lower temperatures. Since the majority of receptors in the nucleus should be filled, this uptake should represent exchange of added for endogenous steroid. There may also be some filling of empty receptor sites, as these have been demonstrated in the nuclei of breast tumour cells (Zava & McGuire, 1977, Kato et al., 1978, Thorsen, 1979) and form the basis of the mechanism of steroid action proposed by Sheridan et al., (1979). It is not possible to detect which of these situations prevails using the methods presented here.

The uptake of ^{3}H-oestradiol into breast tumour cytosol was also studied (Figure 11). The same pattern was noted as for the nuclear receptor. This again fails to distinguish whether exchange occurs since the majority, but not all, of cytoplasmic receptors should exist in the unbound state. The adverse effect of an elevated temperature (37^{o}C) on these receptors is also shown in Figure 11. This had been reported previously by Feherty et al., (1971).

In an attempt to clarify the situation further, studies were carried out on the dissociation of ^{3}H-oestradiol from nuclear and cytoplasmic fractions of postmenopausal breast tumour tissue in the presence of excess unlabelled steroid at 4^{o}C and 20^{o}C (see Figures 12 and 13). Preincubation of the

separate fractions with ^{3}H-oestradiol was carried out to ensure that all receptors would be filled with the steroid. Dissociation of labelled steroid in the presence of the unlabelled form in this system would, then, represent exchange since any possibility of heat degradation of receptor causing release of the labelled steroid is ruled out by studies carried out on the stability of binding at these temperatures (see Figures 9 and 11). Further reasons for destruction of receptor include action of agents such as proteases, but this can also be dismissed by the fact that labelled oestradiol can be taken up by the preparation to yield the original level of receptor after completion of the experiment (see Results, section 3.1.3.1).

The results of these studies were that exchange of added for endogenous oestradiol could occur up to a level of 80% at 4^{o}C over a period of 24 hours in either tissue fraction, the same level being reached at 20^{o}C after 2 hours. Studies in our laboratory on oestrogen receptor in endometrial tissue has produced the same ability to demonstrate exchange, but this can be achieved to a level of 98% using the conditions above. Anderson et al., (1972), Katzenellenbogen et al., (1973) and Chamness et al., (1975) studying receptor in rat uterus, all came to the conclusion that a higher temperature was required to give exchange of added for endogenous steroid, possibly reflecting the variation in properties of the receptor in the two species. The report by Anderson et al., (1972), however, does find a low level of exchange at 0-4^{o}C over a period of 1 hour. It may be that, had this study been continued for a longer time, exchange of the order observed in the present study would have been achieved. An experiment performed in this project on immature rat uterine nuclear receptors revealed that they could, indeed, bind ^{3}H-oestradiol at either 4^{o}C or 20^{o}C in the same way as could human breast tumour nuclear receptors (see Figure 15). This presumably

demonstrates the exchange properties of intact nuclear receptors even at low temperatures.

As stated earlier, it is not certain whether the difference noted between uptake of ^{3}H-oestradiol in pre- and post-menopausal breast tumour biopsies shown in Figures 9 and 10 is truly significant. This is because the experiment could be performed only once with premenopausal tissue, due to a lack of receptor-positive premenopausal samples of a suitable size (at least 600 mg) on which to perform such a study. In general, premenopausal samples tended to be smaller than those from postmenopausal patients, probably due to detection of the tumours in the younger patients at an earlier stage. If, however, the finding here were to be repeated in subsequent studies on premenopausal tissue, it might reflect an increased stability of the hormone/receptor complex in conditions of high circulating levels of hormone; an environment encountered also in the cycling rat.

Since it has been demonstrated that the assay system in operation here causes exchange of added oestradiol for endogenously bound steroid, it follows that what is measured in the course of the assay is unfilled receptors plus 80% of the filled ones. This is using the times and temperatures for incubation of 20^{o}C for 2 hours or 4^{o}C for 18 hours. This could be increased to 94% exchange using a 20^{o}C incubation for between 4 and 24 hours, but, in practice, the overnight incubation at the lower temperature is more convenient. What is important is that the time and temperature should be monitored carefully, so that conditions are as nearly as possible identical for all tumours assayed. If this is done, then what is measured in each tissue fraction can be regarded as total receptor, the amount remaining undetected being very small. These conclusions invalidate the proposals

that premenopausal tissue receptor levels are lower than postmenopausal ones due to endogenous steroid masking the majority of sites (Sakai & Saez, 1976), and that circulating levels of oestradiol should be considered when interpreting oestrogen receptor levels (Mobbs & Johnson, 1976, Nagai et al., 1979). Further evidence that circulating levels of oestrogens do not influence breast tumour receptor concentrations comes from the finding during the course of this study that there is no correlation between the two parameters (see Results, section 3.4.5.5).

Even though the receptor assay measures total receptor, it is still apparent that cytosol receptor levels in premenopausal patients are lower than those of postmenopausal patients (Figure 22). A similar significant difference is also found at the nuclear receptor level (Table 12). The transition from low to elevated cytosol receptor levels appears to be associated with menopausal status rather than age, since the increase shown in Figure 22 is a sudden one rather than a gradual rise, which would be noted if age were the determining factor. This was also the conclusion reached by Allegra et al., (1979a). Having ruled out the possibility that receptors may be undetectable because of endogenous steroid being bound, another possible cause of the decreased premenopausal level would be that receptor resides mainly in the nucleus of these patients, having undergone, translocation in the presence of circulating oestradiol. The finding that nuclear receptor levels are also lower in pre- than in post-menopausal patients (Table 12), tends to overrule that theory. It may be that there is some form of down regulation in operation in the premenopausal woman, caused by the high levels of circulating oestradiol. Anderson et al., (1972) suggested that there is a maximal level of nuclear receptors required to give a

physiological response in the rat uterus, reflecting a limited number of acceptor sites within the nucleus. The proposed down regulation would save unnecessary synthesis of cytoplasmic receptor, but this property might be lost at the time of the menopause, allowing synthesis of greater amounts of receptor.

Other methods and conditions of the assay system established in this thesis are in good agreement with those published elsewhere. A satisfactory procedure for the storage of tissue has been designed, which maintains receptor integrity for at least 3 months, but has potential for much longer periods. A different storage system, involving the lyophilization of tissue is currently under investigation (see Results, Section 3.3) to assess its effect on oestrogen receptors. A reliable means of storage is of great value where it is desirable to assess primarily the receptor content of the tissue, and subsequently perform investigations on various aspects of receptor mechanisms. An adequate storage system is also required where assays are carried out on a routine basis and for quality control purposes. It may be necessary to repeat a determination for one reason or another, and it must be certain that the receptor status can be maintained throughout the period of storage. Storage in liquid nitrogen is a popular form, but this may be harmful to nuclear receptors, as judged by our experience (see Results section 3.1).

The procedure used here for the preparation of the tissue fractions yields very crude forms of each one, but this has been found to cause no interference in the measurement of receptors. Even the use of the detergent Triton X-100 which would remove any non-nuclear material from the nuclear pellet was not

observed to cause a significant change in receptor levels. Other workers use very rigorous methods to purify the tissue fractions, but McGuire et al.,(1977c) agreed with the present finding that this was unnecessary.

In common with the findings of McGuire & De La Garza (1973b), it was noted that the thiol reagent dithiothreitol was successful in maintaining the binding properties of the receptor. This is required due to the presence of sulphydryl groups on the receptor, which are reported to participate in the binding of steroid (Jensen et al., 1967, Muldoon, 1971). Keightley et al.,(1978) found that the presence of 1 mM DTT did not enhance the level of cytoplasmic receptors found in fresh or stored human myometrium assayed by a method similar to that presented here. The reason for this is not clear, as the receptors in this tissue should have the same basic form as those of the mammary gland.

No requirement for protease inhibitors was found in the determination of oestrogen receptor levels in human breast tissue. This is in contrast to the findings of Garola & McGuire (1977b) that protease activity interfered with the detection of receptors in breast tumour nuclear fraction. However, the method being considered (Zava et al., 1976) measured salt extracted receptors, which may be more sensitive to attack by these enzymes than receptor associated with chromatin.

When the receptor assay method was evaluated using replicate assays of the same cytosol and nuclear preparations (see Table 3), or assay of different slices from the same piece of tissue (see Table 2), little variation was seen on a purely qualitative basis. However, when a quantitative assessment

of the variation in receptor levels across a tumour was made, a five-fold variation was observed. The variation within a bulk preparation of cytosol or nuclear fraction was relatively small (~±20%), so the difference in tumour slices reflects the heterogeneity of the tissue, rather than any fault in the assay system. Although Hawkins et al., (1977) proposed that this heterogeneity was so great that replicate tissue samples should be assayed, this does not appear to be necessary if a qualitative assessment of results is to be made. Engelsman et al., (1973) and Walt et al., (1976) found that response to hormone therapy was not effected by the absolute level of receptors in positive tissue. This suggests that the presence or absence of receptor in each fraction, along with a measure of its affinity and specificity, is all that is required to provide an index of potential response to hormone therapy. However, some groups have reported that a quantitative relationship may exist between receptor concentration and response rates.

By classing as "receptor-positive" only those biopsies containing receptor in both the nuclear and cytoplasmic fractions of the tissue, the occurrence of receptor-positivity is lowered relative to that found by workers measuring cytosol levels alone (e.g. McGuire et al., 1975a, Wittliff et al., 1976, Byar et al., 1979). Approximately 30% of the biopsies assayed in this study fell into the receptor-positive category using this criterion. A level of 50-60% or even greater has been reported on the basis of cytosol receptor measurement alone. This apparent discrepancy is attributable, at least in part, to the fact that the inclusion of a nuclear determination of receptors reveals a group of tissue samples which contain only cytosol receptor. A preliminary study was carried out to try to determine whether this receptor could be induced to translocate into a nucleus known to be

responsive to oestrogenic stimulation. Figure 20 shows results which suggest that this is not the case. The findings from this study are too limited to state with any certainty what defect, if any, prevents the translocation of receptors in these cases. As with other studies in this project, further investigation was impeded by a lack of suitable amounts of appropriate tissue. The frequency of samples bearing cytosol receptors alone is low (12% of biopsies bear this receptor status), and the requirement for 500-600 mg of such tissue further limits the supply of material for this study.

The fault in the translocation processes of these tumours containing solely cytosol receptor could lie with the receptor, which might have structural abnormalities, preventing its entry into the nucleus. The other possibility is that the defect is at a cellular level, the translocation mechanism being faulty. It is not clear as yet how the receptor or the hormone/receptor complex enters the nucleus, but if there were a facilitated entry process, this would be a likely site of blockage. In this case, a structurally normal oestrogen receptor would be prevented from entering the nucleus by such a defect. Investigations of the ability of cytoplasmic receptors from this abnormal tissue type to enter the nucleus of tissue known to permit translocation (see Results, section 3.4.1) could aid in the elucidation of the type of defect in operation. Consistent failure to observe translocation in such a system would suggest that the receptor was at fault. It is possible, however, that the abnormality is not the same in every case.

If there is, in fact, no defect in the cell or receptor, then another explanation for the occurrence of tissue bearing this abnormal receptor status is that no oestradiol is present <u>in vivo</u> to cause translocation of the receptors. This is unlikely, however, since oestradiol has been shown

to be necessary for the synthesis of its own receptors (Sarff & Gorski, 1971, Clark et al., 1977). The presence of cytoplasmic receptors thus, in itself, suggests the existence of circulatory steroid. Other evidence against this idea is the finding that plasma from patients bearing tumours with this receptor status has been demonstrated to contain oestradiol (see Results, section 3.4.5.5). A further explanation for the apparent absence of nuclear receptors would be that the nucleus does in fact contain a very low level of binding not detected by the assay system. The sensitivity of the assay tends to overrule this suggestion. Values as low as 25 fmol/mg DNA can be measured, although very few positive samples exhibit a level lower than 200 fmol/mg DNA. A final possibility for the absence of nuclear receptors in the presence of detectable levels of cytoplasmic binding is that some nuclear receptor may exist too tightly bound to chromatin to exchange with added steroid. This again would suggest a very low level of receptor, as it must all exist in this non-exchangeable form.

When patient follow-up data are analysed, only 24% of tumours with detectable receptor in the cytosol alone respond to hormone therapy. This suggests it is unlikely that functional receptor is present in the nucleus, but not detected because of some fault in the assay procedure. This in turn suggests that some translocation defect probably does exist within the cell, whether it be at the level of receptor structure or cellular functioning. The 20% of tumours in this class which do respond to hormone therapy may do so through an indirect pathway, such as the inhibition, by tamoxifen, of prostaglandin synthetase (Tisdale, 1977) or the repression, again by tamoxifen, of prolactin stimulation of the tissue (Jordan & Dowse, 1976), although this latter action of tamoxifen has been discounted by Moseson et al., (1978). A proportion of the responders may represent a few cases

where nuclear receptor levels are very low, or the receptor is non-exchangeable, although this appears unlikely, as discussed above. The total number of patients in this group for whom response to hormone therapy has been assessed is still relatively low. This means that the figure of 20% set for response rates of tumours containing only cytoplasmic receptor must be considered preliminary. Further follow-up information may reveal a figure closer to that encountered with receptor-negative patients, which would be predicted from a knowledge of receptor mechanisms. However, the fact that this percentage is higher than that for receptor-negative patients may prove to be significant, and indicate some other variable which should be considered.

Whatever the reason why 20% of tumours having only cytosol receptors respond to some form of hormone therapy, the vast majority do not. The ability to identify this group of patients is, thus, obviously of great value, since they are spared a regime of therapy, ineffectual for them, which may include the trauma of surgical procedures. However, modern endocrine therapy is additive and uses principally antioestrogens or chemical adrenalectomy by aminoglutethimide. Thus, the measurement of nuclear as well as cytoplasmic receptors is important, as it reduces the number of patients who would be predicted to respond to hormonal manipulation, but do not. This was also the conclusion of a study by Thorsen & Stoa (1979), although the number of patients they used (16) seemed to contain an unusually high incidence of cytosol-positive, nuclear-negative cases (25%), stressing the inadvisability of drawing conclusions from statistically insignificant numbers.

On the basis of the argument above, it might be concluded that the assay of

nuclear receptors alone would be adequate in estimating the responsiveness of tumours to hormone therapy. However, the existence of a percentage (6%) of tissue bearing only nuclear receptors invalidates this proposal since the majority of such tumours do not respond to hormonal manipulation. The situation where receptors are detected only in the nucleus has also been reported by Zava & McGuire (1977) and Zava et al., (1977) studying MCF7 cells, and by Panko & MacLeod (1978) using breast tumour tissue. They all report that these receptors exist in the nucleus in an uncharged state, but the method used in this report would not distinguish between filled and empty receptors, due to the exchange properties which have been demonstrated. It is not clear what situation exists in the cell in these cases, but it appears that receptor may have translocated into the nucleus in the absence of hormone, or perhaps bound to a weak ligand. It is physiologically possible for oestradiol to pass into the nucleus from the cytoplasm on its own, but whether this would subsequently bind to the empty receptor residing in the nucleus, form the activated complex with it and, further, elicit a genetic response is open to question. Perhaps the best guide to the situation is the response of such tumours to hormone therapy. When follow-up information was compiled on this group of patients, it was found that only 12% of them did respond to hormone therapy. This therapy includes ablative and additive forms and, thus, the failure to respond to either type contradicts the proposal by Zava et al., (1977) that antioestrogen, but not ablative, therapy should be effective in these cases. Their study was performed on MCF7 cells, however, and it is recognised that the receptors from this source may not be typical of those found in solid tumour deposits.

The numbers of these patients who have been successfully followed-up is low, and the percentage response may change as more information is collected.

At present, however, it appears that the empty nuclear receptor is not capable of stimulating growth of the tissue. Blocking the hormonal pathway is thus not effective in halting tumour development. These findings also suggest the presence of both the hormone and the receptor in combination in the nucleus is required in order to elicit a genetic response. It may be that the hormone directs the receptor to the acceptor sites on the chromatin. Since the receptor does not, on its own, cause growth, it may be able to interact with chromatin, but not at the appropriate active sites required to produce stimulation.

These ideas are valid only if the chromatin has remained undamáged throughout the process of malignant transformation. If any damage has occurred, then it is possible that it is the inability of the chromatin to bind the hormone/receptor complex at the correct sites which causes the tumour to be unresponsive to hormonal stimulation. In any case, the physiological regulation of receptor synthesis may be modified in these cells. It has been suggested that it is the disappearance of receptor from the nucleus, rather than its reappearance in the cytosol which may be important in causing oestrogenic stimulation (Koseki et al., 1977). Binding to the correct sites in chromatin may be required as part of this processing, and if processing is absent, receptor would be maintained in the nucleus, causing a block to any further stimulation.

When the receptor-positive tumours (i.e. those containing detectable levels of both nuclear and cytoplasmic receptor) are considered, 71% of those which would be predicted to respond do regress on hormone therapy.

"Response" has been defined by the British Breast Group (1974). Amongst the criteria for response are that the size of the lesion must shrink to 50% of its original, and this be noted over a period of six months, with no new lesions appearing. These rules have been criticized by Stoll (1977), as being too strict. He feels that the time period is too long, and that patients he would have regarded as showing a response cannot be classed as such due to their failure to complete the six month period. He also points out that to shrink in size by 50%, a tumour must decrease its volume to 1% of the original. This seems to call for a very great improvement. However, these rules have been adhered to in compiling the present follow-up data. Using less strict criteria, the success rate in predicting response to hormone therapy would be improved, but the figure of 71% based on both cytoplasmic and nuclear receptor status is still an improvement over that obtained using cytosol receptor status alone, and the same rules governing the definition of response.

Although the figure of 71% of potential responders who do respond might be improved upon using altered criteria, there are still a number of cases where the predicted response fails to occur. There are many explanations for this observation. One possible cause is that the tumour consists of a mixed cell population, a percentage of which are hormone-dependent and the rest autonomous. This could arise in two ways. If the tumour had been derived from one well-differentiated cell which could synthesise receptor, then all the daughter cells would be hormone-dependent initially. They could then slowly become autonomous, as observed by Kim & Depowski (1975), giving firstly a mixed cell population, and then a totally autonomous tumour. The other way in which a mixed cell population could arise, would be by the tumour being formed from transformation of two different

cells, one cell being well-differentiated and the other not. Autonomous cells appear to be more rapidly-growing than hormone-dependent ones, and the former would eventually become the predominant type. In either of these cases, sampling of the tumour might yield a receptor-positive result. However, when hormone therapy was applied, the hormone-dependent cells would respond, but the autonomous cells would continue to grow. The patient might, or might not, experience a short-lived relief from disease, depending on how great is the proportion of hormone-dependent cells. This fact might indicate that a combined therapy would be the best type in all cases of breast cancer. However, it is not now certain whether chemotherapy is of more benefit to autonomous or hormone-dependent tumours, since conflicting reports have appeared on the relationship between receptor status and response to chemotherapy (Kiang et al., 1978, Lippman et al., 1978). It must be assessed whether either of these reports is accurate, since both contain apparent errors in the assessment of patients. The discovery of what correlation, if any, exists between receptor status and response to chemotherapy will be an important step in the treatment of breast cancer, since chemotherapy has severe side effects associated with it, which makes it desirable to decide whether any benefit will be experienced as a result of therapy.

Another explanation for why some apparently hormone-dependent tumours do not respond to hormonal manipulation is that there may be some control point further along the chain of reactions to hormonal stimuli which can be blocked without effecting receptor levels. This has been suggested also by Jensen & De Sombre (1977), and was the reasoning behind the concept that some physiological product of oestrogen action should be sought to give a more accurate measure of hormonal stimulation. As

mentioned earlier, the most popular of these products has been the progesterone receptor. However, none of the markers which have been suggested as yet, including the progesterone receptor, has formed a completely successful guide to hormonal-dependence. A marker of a later response has been discovered recently by Westley & Rochefort (1979) in the form of a secreted protein, which is detected easily by one-dimensional polyacrylamide gel electrophoresis. This protein appears to be specifically produced by oestradiol, but the study was carried out on MCF 7 cells, and tests would have to be performed on different tissues before confirming this to be a true marker of oestrogenic stimulation.

Since only 9% of receptor-negative tumours respond to hormone therapy, it can be stated that in 91% of the cases, the correct prediction of response is given. This is compared to a predictive accuracy of 71% when considering receptor-positive tissue. This emphasises the conclusion reached by Maass et al., (1972, 1975), Jensen (1975), King (1975) and Lippman (1976) that a receptor-negative result is of more reliable predictive value than a receptor-positive finding. In the present study, overall prediction of response of all patients to hormonal manipulation is correct in approximately 85% of cases. The success rate in the other reports was lower, as only cytoplasmic oestrogen receptor was measured, but their observation is still applicable to the case being studied here. It was found in this study that a receptor-negative result was also more often reproduced after the transition from primary to secondary disease (see Table 6), or in comparison between one secondary deposit and another (see Table 7). In only 2 cases out of 32 did the receptor status change from being negative in the primary to some degree of positivity in the secondary. This phenomenon of gaining receptors has been reported by Korsten et al.,(1975)

and Liskowski & Rose (1976) also, but it is not readily explicable. One possible explanation could be that receptor synthesis was suppressed by some agent, e.g. tamoxifen (see Results, section 3.4.2) or cytotoxic drugs before the primary biopsy was taken. This practice is sometimes carried out to shrink a tumour in order to ease its removal. This was not the case, however, in either of the instances reported here. A further possibility would be that of heterogeneity of the tissue, discussed earlier in relation to false-positive results. If the piece assayed on either the first or second occasion were unrepresentative of the lesion as a whole, the consequence would be an atypical result. Studies carried out on the variation in receptor levels across a piece of tumour biopsy do not provide evidence of a difference of the magnitude required by this explanation, although heterogeneity may vary from one tumour to another.

The consistency of receptor-negativity is also noted when receptors are measured in more than one secondary deposit from the same patient. Here, in only one case out of 20 did a receptor-negative secondary lesion change to receptor-positive at a more advanced stage. It is not so easy, however, to assess the behaviour of receptor-positive biopsies on the same basis as has been used above. The smaller number of instances where repeat assays have been carried out on such lesions reflects the longer disease free interval and better overall prognosis observed in patients bearing hormonally-dependent tumours (see Results, section 3.5.2). If dedifferentiation of cells does occur, then all hormone-dependent tumours will become autonomous eventually, but this process may take a long time.

These findings of variation between primary and secondary tumour receptor levels and between those in different secondary lesions, although relatively

small, differ from the conclusions of King (1975) and Jensen et al., (1977) that receptor status at one site would consistently predict response at another. The numbers of patients in their studies were low, however, which could account for this apparent anomaly. On the basis of the present results, it is clear that the discovery of receptor in one fraction only means that there is very little chance of another lesion producing the same result (see Tables 6 and 7). However, in neither of these classes can a response to hormone therapy be expected on the evidence from follow-up information (see Table 13). Since the alterations seen in Tables 6 and 7 are mainly from one of these categories to the other, or into a state where no receptors are detectable in either fraction, the alterations in status do not generally reflect a change in predicted responsiveness. This fact, along with the reproducibility of receptor-negative results and low reappearance of receptor-positive tissue, means that routine assay of receptor content in primary breast tumour biopsies to aid in the choice of therapy for subsequent lesions, as proposed by Jensen (1975), is a viable concept. Variations may occur, but these should not, in general, alter the optimal therapeutic regime. Roberts et al., (1978) conclude that surgery, which might prove harmful to some patients, should not be performed with the sole object of providing a sample of tissue for receptor assay, because of inconsistencies between receptor levels and patient response. From the data presented here, this might be agreed, but a receptor assay should be carried out if surgery is being performed anyway.

The study of variation in receptor levels measured in serial biopsies has also yielded some interesting information on the mode of action of the antioestrogen tamoxifen. This is a very popular drug for the treatment

of breast cancer, but the reasons for its antioestrogenic actions are not fully understood. Clark et al., (1973) proposed that nafoxidine acted by detaining the receptor in the nucleus for an extended period of time (up to 19 days) but caused no cytosol receptor replenishment, thus blocking the action of further oestrogens. Competition for receptor sites and lack of replenishment were also suggested by Clark et al., (1974) and Katzenellenbogen & Ferguson (1975), although the repressed recycling of receptors was not observed by Nicholson et al., (1976) or Koseki et al., (1977), using levels comparable to those used in treatment of human breast cancer. Koseki et al., (1977) also demonstrated that the replenished cytoplasmic receptor could be translocated into the nucleus by oestradiol, and postulated that lack of replenishment was not the basis of anti-oestrogenic effects. They suggested that the slow clearance rate of tamoxifen from the nuclear fraction was more likely to be the cause of the inhibition of oestrogenic stimulation, the active loss of receptor from the nucleus being of greater importance than its reappearance in the cytoplasm. A new theory on the mode of action of the drug has been proposed recently by Fishman & Fishman (1979). They find that tamoxifen binds to only some of the receptors available in the cytoplasm, others being sensitive to oestradiol alone. They further postulate that oestrogenic stimulation requires the translocation of both of these forms into the nucleus, which does not occur in the presence of the drug.

The findings from this project were that tamoxifen appeared to abolish receptor completely for at least 3-4 weeks. Since the clearance rate of the drug itself is considerably less than this (Nicholson, 1979), the absence of detectable receptor is thought to be the result of repression of synthesis, possibly at a genetic level, rather than a blockage of oestradiol by

tamoxifen molecules already bound to the receptor. No receptor is found in either tissue fraction after tamoxifen treatment, so its effect must be on both receptor recycling and de novo synthesis of receptor, which is in agreement with the mode of action for anti oestrogens proposed by Clark et al., (1973).

When instances of bilateral breast cancer were encountered, it was found that the hormonal status of the disease in one breast was not always reflected in the other. The incidence of these cases was very low (4 cases out of 900 samples), but enough to demonstrate this fact. This may have great significance in the elucidation of the mechanisms involved in tumour formation. For a tumour to be hormone-dependent, activation of the genes employed in hormone receptor synthesis is required. This may or may not occur as a result of malignant transformation. The finding that two tumours of the same part of the body produced at different times may vary in hormone-dependence suggests that different stimuli may be involved in each case, given that either the cells initially transformed were both at the same stage of differentiation or that different stimuli react with different types of cell. The form, or forms, of malignant transformation not causing receptor synthesis activation is(are) more common, and also more lethal than the other type, as shown by the poorer prognosis of patients with receptor-negative tumours (see Results, section 3.5.2). The solution to why cells should react in different ways to these stimuli or whether some stimuli are specific for cells at a certain stage of differentiation may reveal their identity, and thus aid in the cure and, indeed, prevention of cancer.

The correlation between receptor status of the tumour and various other

factors linked with the disease were studied. As discussed previously, in common with many workers, a connection was found between menopausal status and cytoplasmic receptor levels. No correlation between receptor status and age was found. In accordance with a report by Allegra et al., (1979a), the effect seen on comparing cytosol receptor levels with age is solely due to the menopause. In agreement with Johansson et al., (1970), Rosen et al., (1975), Maynard et al., (1978) and Maynard & Griffiths (1979), no link could be found between any particular histological feature and receptor status (see Results, section 3.4.5.2). The latter two reports did find a connection between histological grade and receptor status, but only in post menopausal patients. This is another example of the effect of menopause on the tissue. Examination of clinical stage in relation to receptor status in the present study yielded no evidence of correlation when patients were considered without regard to their menopausal status (see Table 8), although the number of patients involved in some sub-groups was too small to allow definitive conclusions. Estimation of lactalbumin presence could not be correlated reproducibly either with receptor-positivity or-negativity (see section 3.4.5.3).

Another area studied was the level of oestrogen receptors in plasma of breast cancer patients. A report by Tisman & Wu (1976) suggested that the levels in the tumour were reflected by plasma levels. In common with Leclerq et al., (1973), no receptor activity could be detected in any plasma sample from patients with various forms of breast cancer (see Figure 21), ruling out the idea of using plasma instead of solid tissue to furnish an index of hormonal-dependence. Another study carried out on plasma compared the levels of various steroid hormones therein with the receptor status of the patients' tumours(see Results, section 3.4.5.5). This, again, showed no

correlation which could be of use in the treatment of breast cancer. These last two approaches could have proved very useful in the field of breast cancer treatment had they been successful, since a small amount of blood would have been the only requirement for the decision as to what type of therapy should be applied and could have been used to evaluate response to therapy. The use of blood would also provide a simple method whereby it could be decided whether any therapy at all should be prescribed. The prognosis of some patients is so poor, that it might be applicable, on an ethical basis, to save them further distress by surgical operations or complex therapeutic regimes which might serve only to cause more discomfort.

It appears from the results above as a whole, that there is no other parameter which can replace the measurement of solid tumour receptor status in giving an index of hormonal-dependence.

Another useful finding from the study of follow-up data is the relationship between receptor status and patient prognosis (see Results, section 3.5.2). Only tumours with positive or negative receptor levels in both tissue fractions could be assessed in this study, as the numbers involved in the other groups were so small. It was clearly demonstrated that receptor-negative tumours exhibited a much shorter disease free interval than receptor-positive ones. This has also been observed by many other workers (Walt et al., 1976, Knight et al.,1977, Block et al., 1978, Hähnel et al., 1979, Maynard & Griffiths, 1979) and is emphasised in Tables 6 and 7, where repeat assays have been carried out on many more receptor-negative than receptor-positive biopsies. Receptor-negative tumours were also noted to metastasise over a far wider area of the body, receptor-positive recurrences being limited to mainly local disease. This was also reported by Knight

et al., (1977) and Byar et al., (1979), and probably reflects the more virulent nature exhibited by receptor-negative cells, as noted by Meyer et al., (1977). Both of these observations indicate that the receptor-negative tumours are more aggressive, and prognosis of patients bearing such tumours is much worse than that for those with receptor-positive lesions.

Nodal status has also been related to receptor level of tumour tissue (Allegra et al., 1979a) and patient prognosis (Hähnel et al., 1979). The correlation between these factors was studied for the results presented here. Table 10 illustrates that no correlation could be found between receptor status and nodal status. When considering disease free interval also, data were analysed only for patients with either cytoplasmic and nuclear receptor-negative or cytoplasmic and nuclear receptor-positive status. Node-positive, receptor-negative patients had much the worst prognosis, as would have been predicted. Equally, node-negative, receptor-positive patients had the best prognosis (Figure 26). Intermediate groups behaved comparably until 20 months after diagnosis, beyond which nodal, rather than receptor, status appeared to act as the more reliable guide to prognosis. This conclusion is in agreement with that of Hähnel et al., (1979).

In addition to breast tumour biopsies, measurement of oestrogen receptor levels was carried out on biopsies of tumours from other parts of the body. The majority of these assays were performed on colorectal carcinoma samples. It was thought that the presence of receptors in this area might provide a useful aid to treatment selection, just as it had in cases of breast cancer. No oestrogen receptors of the type found in breast tissue could

be detected in colonic neoplasms, using the method successful with breast tumour biopsies. This is in contrast to the findings of McLendon et al., (1977) and Alford et al., (1979), although the levels found by them were sometimes relatively low. Binding was observed in a few cases in this study, but it did not satisfy the criteria established in Results, section 3.4 for true oestrogen receptor. The reasons why no receptors could be detected in colonic samples, whether from male or female patients, is not clear, since much circumstantial evidence, such as a link between colo-rectal and breast cancer (Hermann, 1972), a connection of hormone-dependent tumours with diet (Berg, 1975) and the influence of the menstrual cycle and pregnancy on water uptake of the intestine, suggests potential hormonal influence in this area. One possible explanation is that all the biopsies obtained were from patients with well-advanced disease, which might have lost any hormonal-dependence due to dedifferentiation. Further work in this area, including studies of less advanced disease, and patient response to hormonally-based therapies, may yield further, relevant information.

Another tissue source in which oestrogen receptor levels were measured was breast tissue of either normal or benign tumour origin. Normal tissue was obtained from patients undergoing mammaplastic reduction. The majority of these patients were post menopausal and the normality of such tissue might be questioned. Care was taken, when measuring receptor levels in this tissue, to minimise adipose tissue contamination, since this could lead to erroneous results. In spite of the findings of Feherty et al., (1971) and Hawkins et al., (1975) that receptor could be detected in benign tumour tissue (although in very low levels), no binding was found in any such sample examined in the present study and no receptor

could be detected in any normal tissue samples either. This is in agreement with the theory that in the normal, non-lactating mammary gland, oestrogen receptor levels are so low as to be virtually non-existent, and the finding of Block et al., (1975) that normal breast exhibited only a very low level of oestradiol uptake.

The studies reported in this thesis could contribute much towards the treatment of breast cancer. By developing a reliable method of determining both cytoplasmic and nuclear levels of oestrogen receptor protein from breast cancer biopsies, it has been possible to increase the value of receptor measurements in providing an index of response to hormone therapy. Follow-up studies show that this is so, although there exists still a proportion of patients who should respond to hormone therapy, but do not. It is hoped that the in vitro approach discussed in Part II may reduce the size of this percentage as well as aiding in the selection of patients for chemotherapeutic regimes. The reproducibility of the assay system has shown that, in the majority of cases, receptor status is carried from one disease site to another. This knowledge is particularly valuable in the treatment of inoperable, secondary deposits often associated with breast cancer. This has been suggested before, but the evidence reported here represents many more patients. Although other assay systems have been developed to determine the hormonal-dependence of breast tumours, the one discussed here appears to be more successful in terms of potential responders who do experience relief from hormone therapy of any kind.

The valuable information furnished about oestrogen receptors by this work includes the data on the stability of the hormone/receptor complex at low

temperatures. Whereas it was thought that high temperatures were required to achieve the displacement of the hormone from this complex in either tissue fraction, it has been shown that exchange can occur at temperatures as low as 4°C or 0°C. Studies on the receptor content of both the cytoplasmic and nuclear fractions of breast tumour tissue have also revealed defects which may exist in steroid mechanisms of action. The most interesting of these is the ability of the receptor to exist, unfilled, in the nucleus without causing oestrogenic stimulation. Many interesting facts may emerge from the knowledge of the role of this species in the nucleus - whether it will combine with hormone *in situ*, or whether it passes into the cytoplasm before this action takes place. The possibility of translocation induced by weak ligands has also to be considered. Several plasma steroids could potentially promote translocation of oestrogen receptor especially in post menopausal women. The ability of anti-oestrogen/receptor complex to induce some but not all oestrogenic responses raises the possibility of interaction with only a proportion of the acceptor sites.

Although the present detection method does not distinguish between empty and filled receptors, the ability to establish whether the empty form is present in the nucleus at all times would help to clarify the question of whether the classical two-step mechanism of action of steroid hormones holds, or whether the new theory proposed by Sheridan *et al.*, (1979), involving receptor freely passing from one tissue fraction to the other might pertain. The nature of the regulation of translocation should be clarified after further examination of the receptor in biopsies of the cytosol-positive, nuclear-negative type.

PART II

in vitro studies

1. INTRODUCTION

1.1 DEVELOPMENT OF CULTURE TECHNIQUES

The establishment of a system whereby cells and tissues could be isolated in artificial surroundings away from the controlling and modifying influences of other parts of the body has long been recognised as a useful step in the understanding of the metabolic regulation of an individual tissue. The first recorded instance of such a system was in 1885 when Wilhelm Roux studied the actions of the medullary plate from a chick embryo in warm saline solution. With this model, he was able to prove that closure of the medullary tubes was a function of the constituent cells rather than the action of pressure from surrounding tissues. This report was quickly followed in 1887 by one from Arnold, who noted migration and survival of leucocytes formed after transplantation of alder pith into frogs. He also used warm saline as the bathing medium. Following these first demonstrations that cells in fact could be maintained for several days outside the body and without links with others, many reports appeared of similar findings with different types of tissue. These systems utilised different kinds of medium. Ljunggren in 1898 bathed skin in ascitic fluid and maintained its survival for some time. Agar was used by Loeb (1902) to grow epithelial cells from guinea pig epidermis. The cultivation of isolated plant cells was first attempted by Haberlandt in 1902, but his initial system failed, although subsequent models were more successful (Paul, 1975). Jolly in 1903 actually observed cell division in salamander leucocytes isolated by the hanging drop technique (see Willmer, 1965). Defibrinated blood was the medium used by Beebe and Ewing (1906) in their attempts to grow a canine infectious lymphosarcoma.

All these systems contributed towards an understanding of the requirements for growing isolated cells or tissues, but in 1907, a major breakthrough was made in this field by Ross Harrison. He grew nerve cells from the spinal cord of a tadpole in clotted lymph from a frog. This system not only proved a successful culture method but also furnished the answer to a very controversial question at that time; namely whether nerve fibres grow from nerve cells or are secreted by the tissues through which they pass. Harrison's work proved conclusively that the cell alone is responsible for the formation of the axone.

The method of using a clot as the culture medium was further developed by Burrows, who substituted plasma for the lymph clot. Together with Alexis Carrel, he developed routine methods for the culture of normal and neoplastic animal cells (Carrel & Burrows, 1911a,b). Unfortunately, their method could not be used for human tumour cells, as these tended to liquefy the plasma clot.

One of the main problems which impeded a more widespread application of tissue culture techniques at this time was that of bacterial contamination. In the absence of antibiotics, which were not used in tissue culture until around 1940, aseptic conditions were very difficult to achieve. Alexis Carrel used his surgical experience (Carrel, 1923) to attain a sterile environment, but, unfortunately, his tedious and complicated methods proved discouraging to many potential tissue culturists. However, using these techniques, Carrel (1912, 1914) did succeed in maintaining some connective tissue cells in an active state for thirty-four years (Parker, 1950).

Other innovations which served to make cell culture a more feasible system included the development of suitable culture vessels. Carrel introduced the method

of cultivating cells in flasks, and Gey (1933) developed the roller tube which held more medium, thus permitting the growth of more cells. Following this, from the 1940's onwards, the potential of an in vitro system, applied to cancer research in particular, was being appreciated, and much effort was expended in the development of better media. The early workers in tissue culture had used saline as their medium, but it was realised that all requirements of the cells should be considered when preparing a growth medium. As reported by Fell (1965) and Willmer (1965), many investigators worked to develop the forms of media available today, their efforts being complicated by the different requirements of different cell types.

The term "tissue culture" is commonly used to encompass three different systems. The first of these, which was the type of system used in the early experiments discussed above, was literally tissue culture in that small fragments of tissue were used, and encouraged to grow in isolation, whilst continuing normal functions. Another method developed later was cell culture, in which the organisation of the original tissue is disrupted. The preparation of single cells can be achieved by digestion of the tissue in trypsin. The action of trypsin was first noted by Claude Bernard in 1856 who was interested in why the stomach and intestine are digested by their own juices after death. Fermi in 1910 then reported that cells can withstand prolonged treatment with trypsin which has since been disproved by Ceriani et al., (1978). Cells can endure brief exposure to the enzyme, however, and a method of using trypsin as a means of dispersing viable cells from their parent tissues was developed by Moscona & Moscona (1952) and by Dulbecco (1952). Sanford et al., (1948) had demonstrated the cloning of single cells of one strain isolated from others before this. This group also developed a method of preparing single cells after growing

tissue in suspension (Evans et al., 1951). They, however, used a mechanical rather than enzymic method, passing the cells through a metal mesh.

The other culture method now used is organ culture. In this case, maintenance of the normal functions of the tissue is the chief aim, and the tissue is kept in an intact state, as close as possible to the conditions found in vivo.

1.2 MAMMARY TUMOUR CELL CULTURE

1.2.1 THE APPLICATION OF AN IN VITRO SYSTEM TO BREAST CANCER THERAPY

As discussed in Part I, there does not exist a reliable guide to aid in the selection of the optimal therapeutic regime for a patient with breast cancer. It may be that a tumour will respond well to hormone therapy, chemotherapy or, indeed, a combination of the two, and that the identity of the actual agent(s) is critical. Clinical features, such as nodal infiltration or disease free interval can be useful predictive tools as can the presence or absence of oestrogen receptor proteins in the tumour, but there always exists a proportion of patients expected to respond to a form of therapy who do not. A routine method of quickly assessing the form which will give optimal benefit to the patient by observing the effect on the actual tumour cells in vitro would be of great value.

The establishment of a successful system for the cultivation of mammary epithelial cells from solid tumour would allow determination of hormone-dependence, and thus whether the tumour would respond to some form of hormonal therapy. The advantage of an in vitro assay system over the measurement of steroid receptors, or any of the products of hormonal stimulation, is that any

physiological defects which are not made apparent by the latter systems might be made evident by the long-term behaviour of the cells in culture. Most forms of hormone therapy have been proposed on a purely empirical basis, but the establishment of an *in vitro* assay studying cell survival or proliferation would provide not only the information of whether a tumour should respond to hormonal manipulation, but also which particular form would be most beneficial to the tumour in question. This could be applied also to cytotoxic and combination forms of therapy, and several types of therapy could be tested at a single time. As mentioned in Part I, section 1.3.2.1, the form which the therapy takes is as important as the general type of therapy itself. The establishment of such a system was the aim of Burstein *et al.*, (1971), Wellings & Jentoft (1972) and Aspegren *et al.*, (1975), all of whom used organ culture and tested the short-term effects of various hormones. Due to problems in maintaining the viability of the cells, these tests could last for only a few hours, and so long-term observations could not be made.

Another possible advantage of the *in vitro* system derives from the suggestion that hormonal-dependence would give an indication of prognosis in cases of primary breast cancer (Barker & Richmond, 1971, Block *et al.*, 1978). In addition to predicting the most effective therapeutic regime for any particular tumour, the ability to grow neoplastic cells may give an insight into the mechanisms by which mammary tumours in general survive and take over the body so successfully.

1.2.2 THE GROWTH OF MAMMARY TUMOUR EPITHELIAL CELLS

Despite the fact that the growth of mammary tumour cells could prove to be an invaluable aid in the choice of therapy for breast cancer patients, it has

been found to be a very difficult procedure. Whitescarver (1974) even published a review of the types of problems encountered. However, cultivation of normal mammary epithelium and other types of tumour tissue had been achieved (Lasfargues, 1953, Ebner et al., 1961). Many reasons have been proposed to explain why normal, but not neoplastic breast tissue was successful in culture. One of the main reasons proposed was that the tumour tissue contains a large proportion of dead cells due to previous therapy, necrosis or calcification (Whitescarver, 1974), and so the yield of viable cells is much smaller than from the same amount of normal tissue. Other suggestions have been that tumour epithelial cells grow more slowly and thus are not so successful in establishing a hold over the stromal cells (Buehring & Williams, 1976), or that neoplastic transformation may cause a loss of junctional communication and, thus, organization between the cells (Fentiman & Taylor-Papadimitriou, 1977).

The first report of successful culture of breast carcinoma cells came in 1937 when Cameron & Chambers observed the pattern of growth from a tissue explant on a coverslip. Coman (1942) and Royle (1946) adapted the roller tube culture method developed by Gey (1933) to the growth of mammary tumour epithelium. These studies all examined the cultural characteristics of the cells moving out from the tissue explant. Coman noted differences between normal and malignant epithelium; in particular, that some isolated malignant cells broke away and displayed amoeboid movement, which may be related to invasive behaviour in vivo. One important common finding was that, prior to the appearance of the epithelial cells from the explanted pieces of tissue, stromal cells emerged. The presence of these fibroblasts is one of the major problems in the successful growth of mammary tumour epithelium. The mammary tumour is a solid one and contains a high proportion

of fibrous stroma, giving rise to a high ratio of fibroblasts to epithelial cells (Foley & Aftonomos, 1965). It was thought at one time that epithelium might require the presence of fibroblasts for growth due to exchange of ions or other growth factors, but this may not be the case. (Fentiman et al., 1976). Fibroblasts replicate much more actively than epithelial cells (Owens et al., 1976), and, under normal tissue culture conditions, it has been observed that they quickly encircle epithelial colonies (Stoker et al., 1978), investing the epithelial cells with a coating of collagen which the stromal cells exude (Lasfargues, 1973). This coating renders the epithelial cells inaccessible to trypsin, which is normally used to subculture colonies of cells. Thus, epithelial colonies die out after only a very short period of time (Ozzello, 1977). Of all the various difficulties encountered when attempting the culture of breast tumour tissue, the persistent appearance of fibroblasts has always been the major one.

The fragility of epithelial cells when isolated from their natural environment presents another considerable problem. It has been reported that even agitation of the culture vessel can cause damage to these cells (Wiepjes & Prop, 1970). Certainly, the method of dissecting tissue in order to release cells must be very gentle. This is complicated by the fact that tumour tissue can be so hard due to varying stages of calcification. Lasfargues & Ozzello (1958) described a method whereby cells were "spilled" from tissue during slicing with a scalpel, which appeared to be a very successful procedure, but great care had to be taken during slicing not to damage the cells by undue pressure. Using this method, they managed to establish the first successful cell line of mammary tumour epithelium (BT20) from a primary duct cell carcinoma. This was serially cultivated for at least 20 months (Ozzello et al., 1960).

Since there were dangers of damaging cells by this mechanical technique, the search for a more satisfactory procedure of breaking up tumour tissue continued. The alternative was an enzymic method, but the proteolytic enzyme trypsin was not successful as it could not digest the stroma, and also tended to cause cellular damage. Eventually, Lasfargues & Moore (1971) reported that collagenase digestion of tissue gave a high yield of epithelial cells. They claimed, moreover, that intermittent treatment with this enzyme (0.5 mg/ml) for 3 days every 3-4 weeks kept the culture almost free of fibroblasts. Even exposure to the enzyme for up to one week does not appear to harm epithelial cells (see Section 3.2.1.2), despite the presence of proteolytic factors in crude preparations of collagenase.

Collagenase was thought at one time to be a cytotoxic enzyme, and its application in this role had been discounted. However, collagenase is now known to act by attacking the dermal-epidermal junction (Kahl & Pearson, 1967), although some less pure grades of commercial preparation contain proteolytic activities also. Collagenase has certain advantages over trypsin, the other digestive enzyme used in the isolation of single cells. Unlike trypsin, collagenase retains its full activity in the presence of calcium and magnesium ions and is not rendered inactive by serum (Lasfargues & Moore, 1971). Thus, tissue can be exposed to collagenase for a long period of time in the presence of a complete medium, which maintains the viability of the cells. It has been reported that viable cells can be recovered even after a long exposure of up to 5 days to collagenase (Freshney, 1972). Another advantage of collagenase is that stromal cells are dissociated first and, by careful timing of the digestion of the tissue by the enzyme, a stage can be reached where there

are clumps of resistant epithelial cells which can be separated by sedimentation from the dispersed fibroblasts. The main point in favour of collagenase is that cellular damage is kept to a minimum, whereas sustained exposure to trypsin can cause epithelial cells much harm by stripping off the cell surface proteins (Ceriani et al., 1978).

1.2.3 SELECTION OF EPITHELIAL CELLS

The major aim of any approach has always been to discourage the growth of the stromal cells, and thus encourage the development of the epithelial cells. One of the most successful methods of achieving this was to cover the surface of the culture vessel with a coating, thereby preventing fibroblastic attachment - a property essential to stromal cell survival. The first type of covering to be used was reconstituted rat-tail collagen (Ehrmann & Gey, 1956). This was fairly successful, but had limitations, because the cells growing on the collagen coating caused it to tear, exposing areas of surface to which the stromal cells could then attach (Whitescarver et al., 1968). The same theory has been used more recently by Katiyar et al., (1978) who have used floating collagen gels to study mouse mammary epithelium.

Another method of covering the culture vessel surface which has been widely used is to grow a layer of different cells on it until they reach confluence, then arrest their division either by irradiation or mitomycin C treatment. The desired epithelial cells can then be innoculated on to this "feeder layer". The feeder layer cells are now incapable of division, but it is not absolutely certain whether they support the growth of sparse viable cells by conditioning the medium or by direct contact. They also act to discourage the growth of fibroblasts and this alone promotes epithelial

cell growth. The first report of the use of a feeder layer came from Puck & Marcus (1955) who used cells irradiated by X-rays or ultraviolet radiation to enhance the growth of HeLa cells. They considered that the feeder layer acted by conditioning the growth medium. Studies of Stoker & Sussman (1965), however, showed that the feeder effect is not due to a release of some factor into the medium. They used petri dishes half-coated with feeder layer of X-irradiated mouse fibroblasts and half left bare. Enhancement of growth of BHK21 cells occurred only on the coated half of the dish, demonstrating that the feeder effect is not diffusible. Rheinwald & Green (1975a,b) further studied the action of X-irradiated 3T3 cells as feeder layer, and noted that keratinocytes settled on the monolayer and pushed it aside, fixing to the surface of the culture vessel, while fibroblasts appeared unable to do this. However, a recent report from Armstrong & Rosenau (1978) concludes that enhancement of growth of neoplastic mammary epithelium on a feeder layer of embryonic mesenchyme may be due to a growth factor released into the medium, so the question of exactly how feeder layers exert their effect still remains unanswered. It is now generally accepted that feeder layers enhance epithelial cell growth, 3T3 cells being popular for this purpose, but other fibroblastic cells and even foam cells from human milk (Taylor-Papadimitriou et al., 1977b) having proved successful.

Other methods of selection for epithelial growth in preference to that of stromal cells have involved the use of some agent or mechanical process which specifically inhibits either the growth or action of the fibroblastic cells. Puck et al., (1958) used the fact that fibroblasts have more stringent growth requirements than epithelial cells, eliminating the former by omitting foetal serum from the medium. Another method utilised the fact

that fibroblasts do not contain D-amino acid oxidase (Gilbert & Migeon, 1975), which is found in epithelial cells. The fibroblasts could not, therefore, convert D-valine into L-valine - the form required by the cell. Sykes et al., (1970) reported a new method of actually separating fibroblasts from epithelial cells using a discontinuous Ficoll density gradient, and separation was also achieved by Owens (1974), using a carefully timed exposure to trypsin, which was enough to loosen the fibroblasts, but not the epithelial cells from the culture vessel. This last method would require great care as epithelial cells might also be lost. However, the use of another proteolytic enzyme, dispase, was introduced by Matsumura et al., (1975), which gave a greater yield of epithelium. Fibroblast growth has been selectively inhibited by preventing attachment to the culture vessel (Braaten et al., 1974, Steele et al., 1978). Cis-hydroxyproline added to the medium (Kao & Prockop, 1977, Liotta et al., 1978) also interfered with the functioning of the stromal cells, this time by preventing collagen formation and thus inhibiting the smothering effect of the fibroblasts on epithelial cells.

1.2.4 STIMULATION OF EPITHELIAL CELL GROWTH

The methods used to select for epithelial cell growth, although fairly efficient, are not always completely successful in eliminating fibroblasts, which may reappear after a few passages. An alternative approach to the problem is to stimulate the epithelial cell development by the addition of some agent to the medium. One such agent is epidermal growth factor (EGF), which is widely used for this purpose.

The presence of EGF was first noted by Cohen (1962), who reported the discovery of a protein of the mouse submaxillary gland, which caused premature eyelid opening in the new-born animal. Further studies (Cohen & Elliot, 1963) showed that this effect was due to a stimulation of keratinization by the protein. Turkington (1969) used EGF in the course of experiments on mammary gland development *in vitro*, and found that it specifically stimulated epithelial cell growth as determined by increased DNA and RNA synthesis. However, receptors for this factor, identified as glycoproteins in human placenta (Hock *et al.*, 1979) were detected also in fibroblasts (Hollenberg & Cuatrecasas, 1973, Cohen *et al.*, 1975). Moreover, EGF has been shown to stimulate uptake of RNA precursors (Hollenberg & Cuatrecasas, 1973), DNA synthesis (Armelin, 1973) and increased cell proliferation (Armelin, 1973, Cohen *et al.*, 1975) in fibroblasts. It can also cut the serum requirement of fibroblasts down to only 1% (Carpenter & Cohen, 1979), all of which establishes that EGF stimulation is not limited to epithelial cells.

The stimulation of growth seen with EGF is enhanced by the presence of serum (Hollenberg & Cuatrecasas, 1973). At one time it was thought that the polypeptide might be used in place of serum, but it has been found that it cannot replace serum completely (Stoker *et al.*, 1976). It also increases the stimulation of epithelial cell growth and colony formation noted in the presence of feeder layers (Rheinwald & Green, 1977). Further to this finding, Taylor-Papadimitriou *et al.*, (1977a) found that EGF was effective in increasing human mammary epithelial cell growth only in the presence of feeder layers. This was presumably due to the dual action of selection of epithelial cells by the feeder layer and their stimulation by EGF.

Another group of agents commonly used to stimulate mammary epithelial cell growth are hormones, and in particular the peptide hormone insulin. As

discussed in the introduction to Part I, the development of the mammary gland is controlled by a milieu of hormones which interact with one another (Topper, 1970). The maintenance of breast epithelial cell growth has also been shown to depend on this milieu (Young et al., 1978). The essential role of insulin in mammary tumours has been demonstrated in vivo by making tumour-bearing rats or mice alloxan- or streptozotocin-diabetic, which causes regression of the majority of tumours(Heuson & Legros, 1972, Puckett & Shingleton, 1972, Cohen & Hilf, 1974). The stimulatory effect of insulin was also noted when its administration reversed regression of mammary tumours in rats following hypophysectomy (Heuson et al., 1972).

It is not fully understood how insulin achieves its action, although it has been timed as a post-transcriptional event (Osborne et al., 1976). In contrast to the stimulatory effect of glucose, insulin does not act by increasing collagen synthesis, which would stimulate fibroblastic growth, but there is instead an increase in total cell protein (Villee & Powers, 1977). It was thought that the protein synthesised might be EGF or that insulin might be itself mimicking this growth factor, but these modes of action have both been discounted(Turkington, 1969, Hollenberg & Cuatrecasas, 1973). Insulin action on casein synthesis (Turkington & Topper, 1966) and cell division (Voytovich & Topper, 1967) have also been reported. Studies on binding and degradation of insulin by the cell have been carried out in an attempt to elucidate the action of the hormone. These investigations, using labelled hormone, have shown that the concentration used is very important in determining how much stimulation will be achieved, and that brief receptor interaction appears to be sufficient to elicit a long-term response (Osborne et al., 1978). The question of exactly how insulin acts still remains unanswered.

The actions of other hormones on the growth of mammary epithelial cells have been studied. Aldosterone and corticosterone were found to be involved in differentiation of the mammary gland (Turkington et al., 1967b). Prolactin, insulin and hydrocortisone were found to be required in combination to cause stimulation of various mammary gland functions (Turkington & Topper, 1966, Turkington et al., 1968, Turkington, 1970). Turkington et al., (1967a) found that prolactin alone induced casein synthesis, and prolactin in combination with hydrocortisone was noted as a requirement for differentiation (Voytovich & Topper, 1967). A quantitative role of prolactin in casein secretion has been reported also by Katiyar et al., (1978). Barker & Richmond (1971), however, found that prolactin and hydrocortisone had no effect on mammary epithelial cells at all. They also discovered that stilboestrol actually inhibited cell growth, an effect also noted in the treatment of breast cancer with high doses of this steroid. The way in which mammary cultured epithelial cells will respond to any hormone will depend on the integrity of the receptor for that hormone.

Other stimulatory conditions have been considered through the years. Ozzello et al.,(1960) used acid mucopolysaccharides, especially hyaluronate, to maintain the growth of their mammary carcinoma cell line BT20. They had previously used umbilical cord extract for the same purpose. It was considered that these substances conditioned the environment of the cells, making it very similar to that encountered naturally. However, it is not clear whether the mucopolysaccharides would be of value in general, or only for this one line of cells. Following the report by Puck et al., (1958) that epithelial cells had no requirement for foetal serum, Foley & Aftonomos (1965) added adult serum as well as foetal serum and found enhanced cell growth. Stoker et al.,(1976) and Taylor-Papadimitriou et al., (1977a) further observed

that human serum was more effective than bovine serum. This effect may be due to some factor which is missing from foetal calf serum. A similar effect was noted by Kaighn (1977), who found that dialyzed foetal bovine serum could not maintain human prostatic epithelial cells as efficiently as the untreated form.

1.2.5 MAMMARY CARCINOMA CELL LINES FROM SOLID TUMOURS

In spite of all the associated problems, some cell lines from solid breast tumours have been successfully established. As mentioned earlier, the first of these to be reported was BT20, isolated by Lasfargues from a duct cell carcinoma. Since then, some other lines have been described. However, it is essential when a cell line is set up to characterize the cells. Some cell lines have been used to try to find suitable markers for this purpose (Bassin et al., 1972, Plata et al., 1973). More recently, Lasfargues et al., (1978) reported the isolation of another two cell lines and used these to investigate the growth requirements of mammary epithelial cells. Although some cell lines have emerged from all the research carried out in this field, the successes represent only a very small fraction of the number of tumours selected for culture. Cailleau et al., (1974) reported attempts to culture 200 breast tumours with no success at all. A routine method whereby every mammary tumour can be grown in culture relatively quickly is required if the cancer patient is to receive any benefit from this approach to the selection of therapy.

1.2.6 CELL LINES DERIVED FROM PLEURAL EFFUSIONS

Since the main problem connected with the culture of solid breast tumours is the presence of fibroblasts derived from the large proportion of stromal

tissue, it was decided that the culture of cells from pleural effusions of patients suffering from breast cancer might be an alternative method of studying the various aspects of the disease. Pleural effusions have very little if any fibroblastic content. The culture of cells from this source has now been attempted by many groups. The first report of success came from Soule et al., (1973), who established a cell line - MCF7-from a pleural effusion. This cell line has proved a useful tool to many workers wishing to study steroid hormone action as well as those interested in cancer mechanisms as the cells are derived from a hormone-responsive tumour and have been shown to contain oestrogen (Brooks et al., 1973) and progesterone receptors (Horwitz et al., 1975b) although these are not of the normal cellular forms. Rose & McGrath (1975) characterized the MCF7 cells and were satisfied that they were mammary and epithelial due to their capacity to synthesise α-lactalbumin, although not all mammary tumours contain the compound (see Part I, section 3.4.5.3).

Amongst the investigations in which MCF7 cells have been utilised are the elucidation of oestrogen receptor mechanisms (Zava et al., 1977, Zava & McGuire, 1978), the action of androgens on oestrogen receptors (Zava & McGuire, 1978), studies on oestrogen metabolism by mammary tumour cells (Lippman & Bolan, 1975, Brooks et al., 1978, Jozan et al., 1979), and the functioning of the antioestrogen tamoxifen, which is widely used in the treatment of breast cancer (Horwitz & McGuire, 1978). MCF7 cells have also been found to contain, in addition to oestrogen and progesterone receptors, receptors for glucocorticoids, androgens and thyroid hormones (Horwitz et al., 1975b, Burke & McGuire, 1978). This may implicate a role for these other hormones in breast cancer.

Despite the fact that this cell line has proved very useful in the field of hormone research, the use of this and any other cell lines from pleural effusions such as those of Allegra & Lippman (1978), Cailleau et al., (1978) and Keydar et al., (1979) in cancer research may be limited. Pleural effusions do have the advantage that they are rich in tumour cells and correspondingly contain very few stromal cells (Cailleau et al., 1974). Also, the cells can be easily isolated (Illiger et al., 1975),and sequential samples can be obtained if desired. However, as pointed out by Kirkland et al.,(1979),pleural effusions are not common, and may represent only a very small, highly selected class of cells and type of disease. This fact must limit the usefulness of this type of cell in the study of cancer.

1.3 IDENTIFICATION OF CELL TYPE

Even when cells have been isolated successfully from solid tumour tissue and encouraged to grow by any of the means previously described, they must be characterized fully before relevant conclusions can be drawn from their behaviour. It is quite possible that during all the procedures involved in processing the tissue to give viable single cells, they may have undergone some form of dedifferentiation. When considering human mammary tumour epithelial cells, three points must be proved. The cells must firstly be of human mammary origin, secondly display malignancy and lastly show epithelial characteristics.

1.3.1 MARKERS OF HUMAN MAMMARY CELLS

Proof of mammary origin of cells may take the form of either some ultrastructural characteristic or the measurement of a specific product of the mammary gland.

Buehring & Hackett (1974), using the electron microscope, noted intracytoplasmic ductlike vacuoles which are suggestive of mammary epithelium. Rose & McGrath (1975), instead measured levels of α-lactalbumin, a specific product of the mammary gland. They found that its synthesis was often retained during transformation, so that its presence acted as a marker of either normal or neoplastic mammary cells. The presence of another specific product, casein, may not be such an efficient guide, since a smaller proportion of mammary carcinomas synthesise this (Hurlimann et al., 1976). Another marker, used by Ceriani et al.,(1977), was an antibody raised against the milk fat globule.

Chromosomal examination can be performed to determine the human origin of cells. At the same time, specific chromosomal markers associated with HeLa cells should be looked for (Nelson-Rees et al., 1974). These HeLa cells, derived from a cervical carcinoma, are a major source of cross-contamination. In addition to characteristic chromosomal banding patterns, HeLa cells can also be recognised by the presence of type A glucose-6-phosphate dehydrogenase (Ozzello, 1977). Several cell lines which were thought to have originated from different tissues have since proved to be HeLa (Buehring & Hackett, 1974, Nelson-Rees et al., 1974) upon being subjected to such investigations.

1.3.2 MARKERS OF MALIGNANCY

Since no correlation exists between structural differentiation and malignancy except that tumour cells display polyploidy which normal breast cells rarely do (Macpherson, 1970), other kinds of tests must be employed. Daniel & De Ome (1965) suggested that the neoplastic cells might be returned to their site of origin, where they would be distinguished easily from the normal cells.

This idea was carried further to produce the concept of introducing the supposedly malignant cells into thymectomized mice, which could not reject the cells due to their immunological incompetence. This method can only prove that cells are malignant if they form a tumour in one of these mice. If no tumour appears, it is not a positive indication that the cells are not malignant, and so this parameter is not really a reliable guide to tumourigenicity (Plata et al., 1973, Marshall et al., 1977). The in vitro capacity of the cells to grow in soft agar can be used as supplementary evidence (Shin et al., 1975).

Das et al., (1974) examined the growth patterns of normal and neoplastic mammary epithelium in an attempt to find some easily recognisable difference between the two types of cell. However, they found that any difference must be very subtle. Any property specific to tumour cells would suffice. One such property associated with mammary carcinoma cells is that of resorbing bone without the need for osteoclasts (Eilon & Mundy, 1978). This property is probably associated with the ability of breast cancer to form secondaries in the bone. It is not yet known if this is a general marker of malignancy, or restricted to breast cancer cells alone. Another property found to be associated with malignant cells was that they permitted invasion by fibroblasts more easily than do normal cells (Stoker et al., 1978). As this property is comparitive in nature, it might prove difficult to assess.

Murine mammary tumour virus-related antigen is a glycoprotein (Yang et al., 1978) which has been found to be excreted by mammary tumour epithelial cells (McGrath & Blair, 1970, McClelland, 1979) and has been discovered associated with the MCF7 cell line (Yang et al., 1977). Cathepsin B, a proteinase has been found in elevated levels in malignant cells (Poole et al., 1978).

As this enzyme can degrade both collagen and proteoglycan, it could be involved in metastasis formation. It may or may not prove a useful marker for tumour cells as again it is the comparison of the level of the proteinase in neoplastic cells relative to that in normal cells which is important.

A substance produced by cells which has become popular as an indicator of neoplastic growth is plasminogen activator. This is probably the factor responsible for the problems encountered by Orr & McSwain (1954, 1955) when trying to grow human tumour cells in plasma clots. They noted that the tumour cells liquefied the clot, whereas normal cells did not. Unkeless et al., (1973) demonstrated this activity in virus-transformed fibroblasts, and purified and characterized the factor (Unkeless et al., 1974). They found that it was a serine protease of molecular weight 39,000 with arginine specificity, which preferentially cleaves one fraction of plasminogen. It is found in high concentration during the latent period between carcinogen administration and tumour formation, and so some role in tumour growth has been suggested (Hince & Roscoe, 1978). Its release has been found to be inhibited by interferon (Schroder et al., 1978). In addition to its activity in cleaving plasminogen, the protease has also been implicated in destroying large external transformation-sensitive protein which is found associated with normal, but not malignant epithelial cells (Pearlstein et al., 1976, Marshall et al., 1977).

Jones et al., (1975) developed a technique for the measurement of plasminogen breakdown by the activator using a fibrin overlay. Using this method, much work was carried out on the presence and identity of this factor in different cells. Nagy et al., (1977) found it in breast and cervical tumour cells, but not in normal cells of the same origin. Tucker et al., (1978) further

investigated the similarities between plasminogen activating factors produced by different tissues. The finding of these studies was that the factor produced by brain tumours was different from that produced by all other tumours. This fact may have some significance in the mechanism of brain tumour formation.

Although plasminogen activator has been found associated with a wide range of tumours (Pearlstein et al., 1976, Howett et al., 1978), San et al., (1977) found no correlation between its production and malignancy in rat liver. This finding may limit the usefulness of the protease as a marker of neoplastic cells.

1.3.3 MARKERS OF EPITHELIAL CELLS

In contrast to properties of malignancy, epithelial nature can be distinguished easily by growth pattern (Bassin et al., 1972) and morphological characteristics. Electron microscopy is a very good method of proving the epithelioid nature of cells. Identifying features include the presence of desmosomal junctions, tonofilaments, microvilli and large, irregular nuclei, and these have all been used repeatedly to define the epithelial content of cultures (Owens et al., 1976, Cailleau et al., 1978, Kanoza et al., 1978, Young et al., 1978). Another property of epithelial cells, made use of by Kanoza et al., (1978), is their ability to cover a collagen gel, and thus protect it from the degradative action of the enzyme collagenase.

A potential problem in the identification of mammary tumour epithelial cells is that it has been reported that the mammary gland contains two types of easily-distinguishable epithelial cells - E cells and E' cells (Hallowes et al., 1977). It was found by Hallowes et al. that tumour tissue contains both

types of epithelial cells, normal or hyperplastic tissue containing E cells only. However, Kirkland et al., (1979) could not corroborate this finding, with both kinds of epithelial cell being present in any mammary tissue.

1.4 OBJECTIVES

The aim of this in vitro part of my thesis work was to establish a successful method by which breast tumour cells could be introduced routinely into culture. This system, once in operation, could be of great value both in the determination of the most potentially effective therapy regime in any particular case of breast disease and in analysis of steroid receptor function. The immediate aim, therefore, was to determine the optimal conditions for the routine cloning of neoplastic mammary cells.

2. MATERIALS AND METHODS

2.1 TISSUE CULTURE MATERIALS

2.1.1 MEDIA

Hams F10 (10X) and Hams SF12 (10X), a modification of Hams F12, were obtained from Flow Laboratories, Irvine, Scotland.

2.1.2 ANTIBIOTICS were obtained from the following:-

"Crystapen" benzylpenicillin (sodium)BP	Glaxo
Streptomycin sulphate BP	"
"KANASYN" Kanamycin sulphate BPC	Sigma, London
Mitomycin C	" "
Gentamicin	Flow Laboratories, Irvine, Scotland
Fungizone (amphotericin β)	Gibco Bio-Cult, Paisley, Scotland

2.1.3 FINE CHEMICALS were obtained as follows:-

Foetal Calf Serum	Gibco Bio-Cult, Paisley, Scotland
Glutamine	" " "
N-2-hydroxypiperazine-N'-2- ethane sulphonic acid (HEPES) (1M)	" " "
Non-essential amino acids (100X)	" " "

Sodium Bicarbonate (7.5%)	Flow Laboratories, Irvine, Scotland
Hanks balanced salt solution (BSS)	" " "
Giemsa's stain (0.68% solution in methanol/glycerol)	BDH, Poole, Dorset
Solvent methanol (microscopical reagent)	" " "

2.1.4 HORMONES were obtained as follows:-

Oestradiol-17β	Sigma, London
Insulin	" "

2.1.5 ENZYMES were obtained as follows:-

Collagenase (type 1)	Sigma, London
Trypsin (2.5% solution)	Flow Laboratories, Irvine, Scotland
Protease, neutral (Dispase II)	The Boehringer Corporation (London) Ltd

2.1.6 MISCELLANEOUS

Tissue culture flasks were obtained from Falcon, Division of Becton, Dickinson & Co., Oxnard, California, USA and Corning, New York.

Nalgene Filter Units were obtained from Nalge Co., Division of Sybron Corporation, Rochester, New York, USA.

Millex Filtration Units were obtained from Millipore, London

Disposable plasticware was obtained from Sterilin, Teddington, England.

2.1.7 CELLS

Foetal intestinal cells (FHS 74 Int) were kindly supplied by Drs. Adelene Hackett and W.A. Nelson-Rees, Naval Biosciences Laboratory, Naval Supply Center, Oakland, California, USA.

2.1.8 HUMAN TISSUE

Breast tumour tissue was kindly supplied by hospitals of the Greater Glasgow Health Board.

Normal breast tissue was kindly supplied by Canniesburn Hospital, Bearsden, Glasgow.

Human foetal intestinal tissue and umbilical cord was kindly supplied by the Queen Mother's Hospital, Glasgow.

2.2 TISSUE CULTURE SOLUTIONS

2.2.1 COMPOSITION OF MEDIA

(i) Working media.

Both Hams F10 (10X) and Hams SF12 (10X) were treated the same way before use:-

Hams F10 (10X)/Hams SF12 (10X)	45 ml
deionised distilled water	400 ml
non essential amino acids (100X)	5 ml

HEPES buffer (1M)	9 ml
sodium bicarbonate (7.5%)	2.5 ml
sodium hydroxide (1N) added to pH 7.2	
foetal calf serum	100 ml
glutamine	5 ml
penicillin (10,000 units/ml)	2.5 ml

These media were then known as F10/20 and SF12/20. Although the majority of culture work was carried out in 20% foetal calf serum, recent data suggest that 10% may be an adequate level.

(ii) Collection medium (F10/PSFK or SF12/PSFK)

The working medium was made up without foetal calf serum, penicillin or glutamine. To this was added the following:-

penicillin	250 units/ml
streptomycin	250 µg/ml
kanamycin	100 µg/ml
or	
gentamicin	50 µg/ml
amphotericin β(fungizone)	2.5 µg/ml

(iii) Digestion medium (F10/20/PSK or SF12/20/PSK)

Made up as for "working medium" with the following additions:-

kanamycin (1000 µg/ml)	5 ml
streptomycin (10,000 µg/ml)	5 ml

(iv) Dissection BSS

Hanks balanced salt solution (BSS)	400 ml
penicillin (10,000 units/ml)	20 ml
kanamycin (1,000 µg/ml)	8 ml
streptomycin (10,000 µg/ml)	4 ml
amphotericin β(fungizone)	3.5 ml

2.2.2 ANTIBIOTICS

Penicillin, kanamycin and streptomycin were all prepared at the desired concentration in Hanks BSS.

Mitomycin C was made up in "working medium" to the required concentration.

2.2.3 ENZYMES

(i) Collagenase - made up in Hanks BSS at a concentration of 2,000 units/ml.

(ii) Trypsin - 2.5% solution diluted 1:10 in the following diluent before use:-

sodium chloride 6.0 g
trisodium citrate 2.96 g
tricine 1.79 g
phenol red 0.005 g

} in 700 ml of distilled water

pH of the diluent was adjusted to 7.8, and distilled water added until the osmolarity was equal to 290 m osmol before addition to trypsin.

(iii) Protease, neutral (Dispase II) - 2.5% solution made up in Hanks BSS.

2.2.4 HORMONES

All hormones were made up in Hanks BSS at an arbitrary strength and diluted with working medium to the required concentration.

Sterilization of solutions in sections 2.2.2 - 2.2.4 was carried out using either a Nalgene or Millex filtration unit, depending on the volume involved.

2.3 TISSUE CULTURE METHODS

2.3.1 PREPARATION OF PRIMARY CELL CULTURES FROM SOLID TISSUE

2.3.1.1 Tissue Collection

Tissue, whether normal or neoplastic, was collected fresh from the operating theatre and transported to the laboratory in a sterile container kept on ice. Where it was not possible to process the tissue immediately, collection medium was added and it was then kept refrigerated until use. Viable cells could be recovered from tissue stored in this way for up to 3 days.

2.3.1.2 Tissue Dissection

The tissue was transferred to a 9 cm diameter petri dish containing 25 ml of dissection BSS. Any obvious pieces of fatty, fibrous or necrotic material

were removed at this stage. The remaining tissue was then transferred to a fresh petri dish containing dissection BSS as before. The tissue was chopped into pieces of about 2 mm cube using apposed scalpel blades. Care was necessary at this point not to damage the tissue by the use of excessive pressure, and, for this reason, the scalpel blades had to be kept sharp, and the chopping completed as quickly as possible. The pieces were then transferred by pipette to a sterile universal container. The pieces were allowed to settle by gravity and the supernatant aspirated off. Any remaining fatty tissue which floated on top of the supernatant was also removed at this stage. Fresh dissection BSS was added to the pieces, and again removed after settling out of the material had occurred. This washing procedure was repeated three times with dissection BSS and the pieces finally resuspended in a medium containing high antibiotic concentrations (SF12/20/PSK or F10/20/PSK). The suspension was transferred by pipette to 25 cm^2 culture flasks and collagenase added at a final concentration of 200 units/ml. The flasks were then incubated at 37^{o}C for a period from 1-7 days until disaggregation of the tissue was apparent by the formation of smaller fragments and free cells on shaking the flask. The timing of this incubation in collagenase was varied (see Results, Section 3.2.1.2), as it appeared that epithelial cells could survive collagenase exposure better than fibroblastic cells. The type of collagenase used contains some protease and peptidase activity, so it cannot be stated with certainty what causes the release of epithelial cells.

After incubation, the free cells and clumps of cells were spun out of collagenase by transferring the suspension to a sterile universal container and centrifuging at 200 g for 5 minutes on an MSE bench centrifuge. The pellet was then resuspended in SF12/20 or F10/20 medium (10 ml) and transferred

to fresh 25 cm^2 culture flasks. These flasks were maintained at 37°C to allow growth of cells and medium renewed regularly as indicated necessary by a change in colour of the phenol red. This methodology is summarised in Figure 27.

2.3.2 SUBCULTURE

2.3.2.1 Trypsinisation

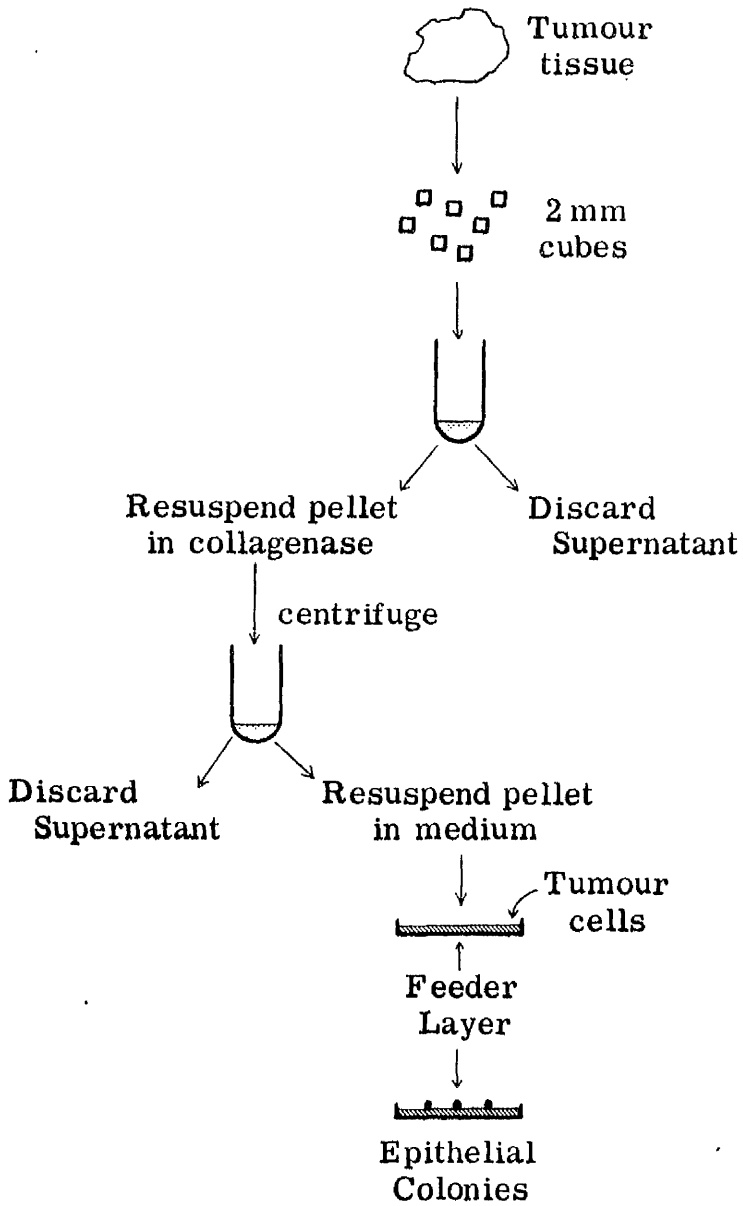
Trypsin treatment was used to remove adhering cells from a flask prior to counting or transferring them to other flasks. This was done at regular intervals to prevent cells from becoming overcrowded.

The medium was removed from the cells to be trypsinised, and they were washed with phosphate-buffered EDTA. Trypsin solution was then added to cover the layer of cells (approximately 5 ml for a 25 cm^2 flask). The cells were kept exposed to the enzyme for 30 seconds before its removal. The flask was placed at 37°C and the behaviour of the cells noted. When the cells were released from the surface of the flask, fresh medium was added to resuspend the cells and stop the action of trypsin. The suspension could then be counted and innoculated into different flasks as desired.

2.3.2.2 Dispase Treatment

Dispase was used as a more gentle method of subculture than trypsinisation, where it was desired to separate one cell type from another. Medium was removed from the cells to be treated. Dispase solution was added diluted 1:9 with working medium. The flask of cells was then incubated at 37°C until the desired ones were observed to have become detached from the surface

Figure 27 - Procedure for the Dissociation of Breast Tissue into Cells


Tumour tissue
2 mm cubes
Resuspend pellet in collagenase
Discard Supernatant
centrifuge
Discard Supernatant
Resuspend pellet in medium
Tumour cells
Feeder Layer
Epithelial Colonies

of the flask. The medium was then removed, and the cells sedimented by centrifugation at 200 g for 5 minutes, followed by washing twice in medium to avoid any contamination by residual dispase. The desired cells could then be resuspended in working medium, counted and used as required.

2.3.3 INHIBITION OF CELL DIVISION

This was achieved by the action of mitomycin C, a safer and cheaper technique than X-irradiation (MacPherson & Bryden, 1971). Cells from one flask were trypsinised, as described in Section 2.3.2.1, and counted. This gave the approximate number of cells in each of the other flasks set up in the same way. Mitomycin C prepared in working medium was added to the cells at a concentration of 2 $\mu g/10^6$ cells. The volume of medium in the flasks was adjusted, if necessary, to the normal level (10 ml for a 25 cm^2 flask), and exposure to mitomycin C was maintained at 37°C for 24 hours. At the end of this time, the medium containing mitomycin C was removed and fresh medium used to wash the cells twice. Fresh medium was again added and the flasks of treated cells replaced in an environment of 37°C.

2.3.4 PREPARATION OF CELLS FOR USE AS FEEDER LAYER MATERIAL

2.3.4.1 Human Foetal Intestine

Tissue was collected as in section 2.3.1.1. Several different methods were then used in an attempt to isolate feeder cells:-

(i) Flushing method

A long piece of intestine (50-60 cm) was cut into smaller pieces of 7-8 cm for ease of handling. These were flushed out with phosphate-buffered EDTA using a large syringe. One end of each piece was then sealed off using a suture, and the middle of the intestine filled with either collagenase or trypsin solution. The other end was then also sealed, and the piece of intestine left at 37°C for 30 minutes in the case of trypsin digestion, or 2 hours in the case of collagenase digestion. After incubation, the end of the piece of intestine was opened, and the trypsin or collagenase digest collected. This was then centrifuged at 200 g for 5 minutes, and the resulting pellet of cells resuspended in a medium containing high concentrations of antibiotics, (SF12/20/PSK or F10/20/PSK). These suspensions were innoculated into 25 cm^2 culture flasks and incubated at 37°C.

(ii) Chopping method

This method was identical to that described in Section 2.3.1.2 for use with solid tissue. The inside of the intestine was washed out first of all with phosphate-buffered EDTA as described above. It was then chopped into small pieces in dissection BSS and the pieces suspended after washing, in medium containing collagenase at a concentration of 200 units/ml. This was then incubated at 37°C for four days to allow collagenase digestion to take place, after which time the cells were spun out of collagenase and resuspended in fresh medium before innoculation into 25 cm^2 culture flasks at 37°C.

(iii) Scraping method

In this method, the intestine was washed out as previously described using phosphate-buffered EDTA. It was then slit longitudinally and spread out in a petri dish. The cells from the inside of the intestine were then gently

scraped off using a scalpel blade. The resultant cells were suspended in medium and innoculated into a 25 cm^2 culture flask, which was then incubated at 37°C.

(iv) Evertion Method

A long piece of intestine was cut into pieces of approximately 5 cm in length. Using fine forceps, the pieces were everted. The pieces were then tied off at each end using sutures, and digested either in a concentrated collagenase solution (2,000 units/ml) at 37°C for 30 minutes, a more dilute solution of collagenase (1,000 units/ml) at 37°C for 2 hours, or a trypsin solution at 37°C for 30 minutes. Chopped pieces of the everted material were treated by dilute collagenase (200 units/ml) at 37°C for 3 hours. In all cases, at the end of the digestion period, the suspension was centrifuged at 200 g for 5 minutes, and the resuspended material set up in 25 cm^2 culture flasks which were incubated at 37°C.

2.3.4.2 Human Umbilical Cord

Umbilical cord was collected fresh and transported to the laboratory on ice. It was always used fresh, and so it was not necessary to attempt storage. In an attempt to prepare cells from the inside of the cord, the following methods were used:-

(i) Flushing method

The piece of cord was suspended from a hypodermic syringe needle held in a clamp, in a manner such that the lower end of the tissue hung into a sterile universal container. It was then flushed through with phosphate-buffered

saline to clean it out. A fresh universal was placed below the cord, and now collagenase at 2000 units/ml was flushed through, followed by some more phosphate-buffered saline. The effluent was then centrifuged at 200 g for 5 minutes, and a small pellet of cells resulted. This was suspended in medium and transferred to a 25 cm^2 culture flask in which it was incubated at 37°C.

(ii) Digestion method

The cord was set up as in (i) above. It was again cleaned out using phosphate-buffered saline, but this time, one end was closed off using a suture and the cord filled with collagenase at 2000 units/ml. This was incubated at 37°C for 30 minutes and opened up to allow the collagenase digest to escape. The digest was spun at 200 g for 5 minutes and the resulting pellet of cells treated as in (i) above.

2.3.5 CELL STAINING

Medium was removed from cells to be stained. Hanks balanced salt solution (BSS) was then added to cover the cells and remove any remaining medium. This was poured off, and approximately half the volume of BSS reapplied, along with an equal volume of methanol. After this was discarded, methanol alone was added to cover the cells, and exposure to this maintained for 10 minutes. The cells were now fixed. 1 ml of Giemsa's stain was added and washed over the cells, followed by 9 ml of distilled water. This was left covering the cells for 10 minutes and then discarded. The cells were then washed with distilled water until no more unbound stain could be detected, and the flasks left to dry. This whole procedure was carried out at room temperature.

2.3.6 CELL COUNTING

The flask of cells to be counted was trypsinised, as described in Section 2.3.2.1. The cells were suspended in an arbitrary volume of medium and 20 ml of counting fluid added to 0.4 ml of cell suspension. This was then counted on a Coulter Counter at a setting best suited to the cell type being studied.

3. RESULTS

3.1 FEEDER CELL CULTIVATION

A popular and successful method of selecting for epithelial, in preference to fibroblastic, growth is to cover the surface of the culture vessel with a layer of non-dividing cells. These have been found to act as a satisfactory support for epithelial cell growth. This concept has been applied to the growth of mammary epithelium in this project. Initial experiments were carried out using as feeder layer a strain of cells (FHS 74 Int) derived from human foetal intestine. These cells appear to be epithelioid in nature. An essential feature of a feeder layer is that the cells should completely cover the entire growth surface of the culture vessel, in order to prevent attachment by stromal cells. FHS 74 Int cells achieve this by contact inhibition, which means that they stop growing when a complete monolayer is formed. Other types of cell may not exhibit this ability, and chemical (mitomycin C treatment) or mechanical (X-ray irradiation) means are required to arrest their growth at the correct time. If the cells grow beyond confluence, they will "pile up" on top of each other and then become dislodged from the culture vessel, leaving a space for fibroblastic attachment.

FHS 74 Int cells proved very convenient as a feeder layer because of their property of contact inhibition and also their characteristic staining, which made them easily distinguishable from other cells which were growing on top of them. However, since the FHS strain supply was limited, and the cells did not remain viable beyond approximately 30 passages, it proved desirable to grow other cell strains which would be equally effective in the formation of feeder layers.

3.1.1 UMBILICAL CORD

Before the successful establishment of the FHI strain of cells from foetal intestine (see Section 3.1.2), the possibility that umbilical cord could be used as a substitute source of feeder cells was explored. The advantage of using umbilical cord instead of foetal intestine is that the former is much more readily available. The methods used to attempt the preparation of feeder cells from umbilical cord tissue were very similar to those used with foetal intestine (see Methods, Section 2.3.4.2).

The procedures detailed in the methods section were carried out on four samples of umbilical cord, but in no case were any viable cells of any kind produced. This may reflect the instability of umbilical cells in the presence of collagenase, of their increased sensitivity to handling. The use of umbilical cord tissue as a source of feeder cells was discarded on the basis of these findings.

3.1.2 FOETAL INTESTINE

Since FHS 74 Int cells were originally derived from human foetal intestinal tissue, the preparation of cells from this type of tissue was attempted. Accordingly, several techniques were applied to the process of forming viable feeder cells from this type of tissue. These procedures are detailed in Section 2.3.4.

The most successful results were produced by the enzyme collagenase and the evertion technique. Both incubating the everted material in collagenase (1000 units/ml) at 37^{o}C for 2 hours followed by spinning down the cells at

200 g for 5 minutes and chopping of everted tissue followed by the same digestion treatment used for solid tumour tissue (see Section 2.3.1.2) produced viable cells similar in appearance to FHS 74 Int cells. Flushing out the middle of the intestine with collagenase (200 units/ml), as in Section 2.3.4.1, also produced cells, but these were mainly fibroblastic.

Using the combination of collagenase (1000 units/ml) and the evertion technique, a cell strain (FHI) has been prepared, which behaves in a manner very similar to FHS 74 Int, except that complete contact inhibition is not always exhibited. This incomplete contact inhibition means that, if long-term experiments are to be performed using the feeder layer, cell division may have to be arrested by mitomycin C treatment.

The new cell strain is similar in appearance to FHS 74 Int (see Plates 1 and 2), and, more importantly, has proved successful in sustaining breast tumour epithelial cell growth (see Section 3.2.1.1).

3.1.3 STROMAL CELLS

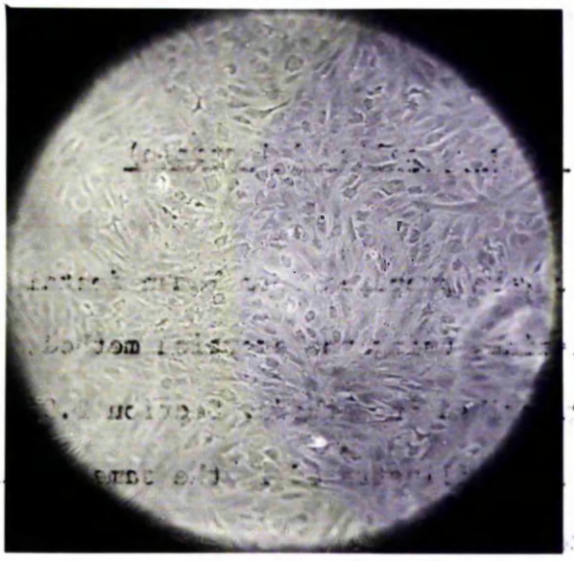
The situation found in vivo is that fibroblasts and epithelial cells coexist, with possible exchange of material between the two cell types. For this reason, it was thought that they might be essential to each other for the maintenance of growth. Using a stromal feeder layer, therefore, conditions close to the natural environment of the epithelial cells could be established.

Fibroblasts were obtained from normal and neoplastic mammary tissue prepared as in Methods, Section 2.3.1.2, making no attempt to select for epithelial growth. The cells were then allowed to grow almost to confluence before

Plate 1 Feeder layer cells (FHI strain)

Cells were prepared from human foetal intestine, using the evertion method, as described in Methods, Section 2.3.4.1. The scale illustrated is the same for all plates.

Plate 2 Feeder layer cells (FHS 74 Int strain)



treating them with mitomycin C (2 μg/10^6 cells) in order to arrest their division. The timing of this treatment was very important as cells undergo one or two divisions after such treatment, and so, if carried out too late, the cells would still overgrow and detach from the culture vessel. Unfortunately neither normal nor neoplastic mammary fibroblasts proved successful in sustaining epithelial cell growth.

3.1.4 EMBRYONIC GLIA

Another source of feeder cells explored was glial cells from chick embryos. Since the brain constitutes a common site of metastatic breast disease, the cells from this region could have specific properties in maintaining breast tumour epithelium in a viable state. It was thought that embryonic cells might also have special properties in this role after the success of FHS 74 Int cells. Chick embryo was chosen as it was easily obtained and no evidence existed that it should be less capable of supporting epithelial growth than material of human origin. The glial cells were prepared by collagenase (200 units/ml) digestion of 15-day old chick embryo brain at 37°C for 3 days. The cells were plated out and treated with mitomycin C (2 μg/10^6 cells) before use. Unfortunately, attempts to arrest their growth were not as successful as with fibroblasts, and the cells became too thick to be able to distinguish epithelial development on top of them.

3.2 CULTIVATION OF MAMMARY TUMOUR EPITHELIAL CELLS

3.2.1 TECHNIQUES FOR EPITHELIAL SELECTION

3.2.1.1 Application of Feeder Layer

All the forms of feeder layer described in the previous section, with the exception of umbilical cord cells, have been applied to the culture of mammary tumour epithelium. As mentioned in the course of section 3.1, these have met with varying degrees of success. Even the successful forms (FHI and FHS 74 Int) do not display consistent enhancement of epithelial growth.

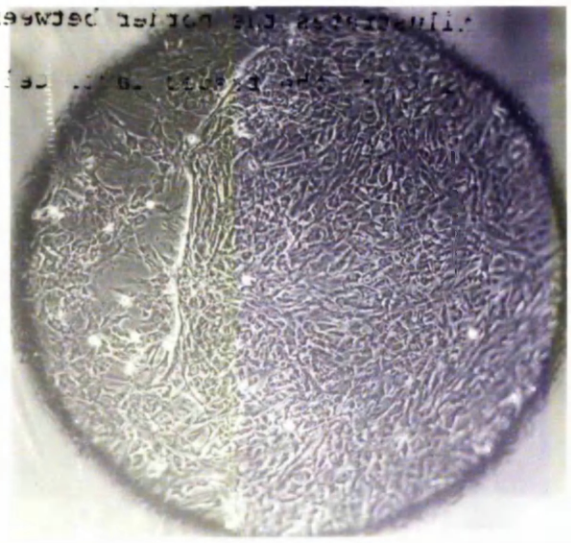
Fibroblasts from both normal and neoplastic mammary tissue gave no enhancement of epithelial cell growth in any of five experiments. Nothing could be concluded from the limited experience with chick embryo glial cells as feeder layer due to an insufficient number of studies. In fact, the only successful forms of feeder layer were those derived from human embryonic intestinal cells (FHS 74 Int or FHI strain). They support the development of epithelial-like colonies of the type shown in Plates 3 and 4. In both of these plates, it is clear how easily distinguishable is the feeder layer (in this case FHS 74 Int) from the other cells. Plate 4 in particular demonstrates the distinct border between the epithelial and feeder layer cells.

Apart from epithelial colonies with the expected characteristics, other cell types have been observed growing on this form of feeder layer. One of these was found to display proteolytic-type properties. As illustrated in Plates 5 and 6, bare patches appear in the area of feeder layer adjacent to colonies of these cells, apparently due to some form of digestion of the feeder cells. In these cases, the colonies with proteolytic properties and the customary epithelial colonies coexisted in the same flasks in approximately equal numbers and, otherwise, showed the same growth characteristics. However, no further characterization of the "proteolytic" colonies was made.

Plates 3 & 4 — Human Mammary Tumour Epithelial-like Colony Cultivated on Foetal Intestinal Feeder Layer (FHS 74 Int strain)

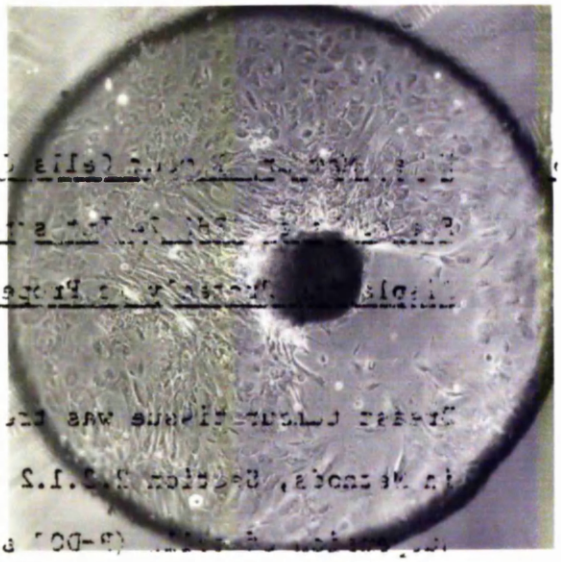
Breast tumour tissue was treated as described in Methods, Section 2.3.1.2 to give a cell suspension (B-MAB strain). Some of this was innoculated on to feeder layer cells of the FHS 74 Int strain. Plate 3 shows a colony of epithelial-like cells which developed from this tissue.

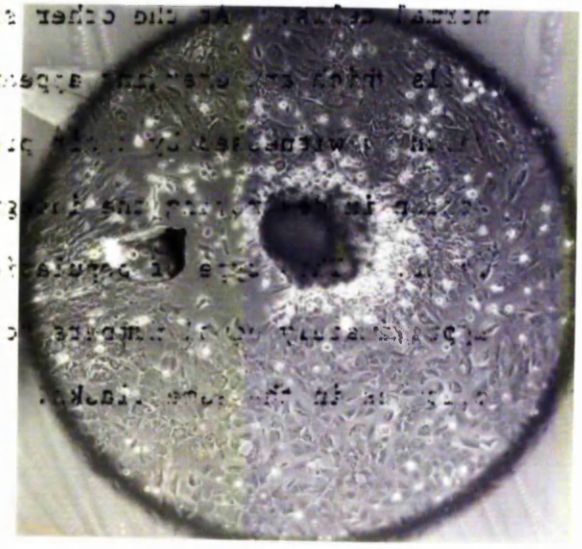
Plate 4 shows the same cell strain but illustrates the border between the epithelial-like and the feeder layer cells.



Plates 5 & 6 Human Mammary Tumour Cells Cultivated on Feeder Layer (FHS 74 Int strain) and Displaying Proteolytic Properties

Breast tumour tissue was treated as described in Methods, Section 2.3.1.2 to give a suspension of cells (B-DOS strain). Some of this was innoculated on to FHS 74 Int feeder layer. These plates show clumps of tissue which have settled on the feeder layer. From one side of each clump are growing out apparently normal cells. At the other side, however, the cells which are emerging appear to be different, which is witnessed by their proteolytic-like action in destroying the integrity of the feeder layer. This type of population existed in approximately equal numbers to epithelial-like colonies in the same flasks.





Unfortunately, although epithelial colonies could be supported in 6 out of 9 cases by these intestinal feeder layers, their presence could not be sustained for more than 3-4 weeks. After this length of time, cultures which had been completely free of fibroblasts could become overgrown within the space of 48 hours. These stromal cells had presumably been transferred with the primary culture, and perhaps remained dormant until some unidentified change within the environment activated their development.

In contrast to the cases where foetal intestinal cells promoted mammary tumour epithelial cell development, feeder layer inhibition of epithelial cells and apparent promotion of fibroblastic growth also occurred. In one extreme case 5-10 times as many epithelial colonies developed in the control flask as in that containing the feeder layer. Stimulation of growth by oestradiol was seen in both control and feeder layer flasks.

In another instance, feeder layer (in this case FHI strain) failed to support any epithelial colony development at all, although some colonies were observed in the control flasks.

It appears, therefore, that although definite conclusions can be drawn about the inability of stromal and umbilical cord cells to act as feeder layers for mammary tumour epithelium, no conclusive judgement can be made in the case of the foetal intestinal cells. It is clear that they have potential in this role, but perhaps other factors could augment their actions, the most important one being the elimination of fibroblasts from the early stages in the isolation of cells from solid tissue.

3.2.1.2 Collagenase Digestion

In the procedure for preparing cells from solid tissue (see Methods, section 2.3.1.2), pieces of chopped tissue were normally incubated in whole medium containing collagenase (200 units/ml) for 3-5 days. During the preparation of cells from one mammary tumour, this time period was not sufficient to digest some of the chopped tissue pieces, which were allowed to digest for a further two days, while the pieces which had digested were removed from collagenase and plated out in fresh medium at day 5. During the course of the subsequent experiment, it became evident that the portion of tissue which had experienced the longer collagenase digestion yielded a much higher ratio of epithelial cells to fibroblasts, suggesting that fibroblasts are much less able to withstand prolonged exposure to the enzyme. This could prove a very valuable finding if it were reproducible, since it would be advantageous to minimise the level of fibroblasts from the earliest point possible.

3.2.1.3 Dispase Action

The use of dispase as a substitute for trypsin in carrying out subculture of cells may be advantageous due to its gentler mode of action. When used for the first time on a cell strain, it was found to remove selectively epithelial colonies even from a feeder layer, leaving fibroblasts and feeder cells behind. This was not found to be the case in subsequent treatment of the same cell strain, however, when fibroblasts and feeder layer were released from the culture vessel along with the epithelial cells, this effect being noted in another cell strain also. This method may be of value in the selection of epithelial cells, but perhaps some modification is required to render it reproducible.

3.2.2 HORMONAL STIMULATION

One way to enhance further the growth of mammary epithelium in general would be to provide the cells with hormones additional to those already contained in the foetal calf serum component of the bathing medium. As described in the introductions to parts I and II of this thesis, the mammary gland is responsive to a wide range of hormones. It was of interest to establish whether any combination of these hormones had growth promoting effects.

3.2.2.1 Insulin

An initial experiment carried out on the effects of various concentrations of insulin showed that insulin was active in promoting epithelial colony formation. This effect was seen both in the presence and absence of feeder layer. An insulin concentration of 5 µg/ml was found to be more effective than 10 µg/ml. Insulin at 5 µg/ml was subsequently added to culture medium used for the growth of all mammary epithelial cells.

3.2.2.2 Oestradiol

Oestradiol-17β was added in a range of concentrations from 10^{-9}M - 10^{-5}M to flasks containing mammary tumour cells. An initial experiment showed that stimulation of epithelial growth was greatest when 10^{-7}M oestradiol was added, regardless of the presence or absence of feeder layer. Unfortunately, the number of colonies being compared here was very small, the maximum being 14. Accordingly, no valuable quantitative assessment could be made, but no further enhancement of growth appeared to occur at concentrations of hormone in excess of 10^{-7}M.

This study was repeated using a higher concentration of mammary cells and a narrower range of oestradiol concentrations. Table 14 shows the findings of this second experiment. With no feeder layer present, increasing the concentration of oestradiol increased the number of epithelial colonies formed. In combination with feeder layer, however, no such correlation could be drawn. These findings require more thorough investigation before any conclusion as to the worth of oestradiol as a stimulant of mammary epithelial growth can be made.

3.3 CULTIVATION OF NORMAL MAMMARY TISSUE EPITHELIAL CELLS

An important step in the cultivation of any type of cell is the characterization of that cell strain to establish its identity beyond any doubt. In the present study, human mammary origin, malignancy and epithelial nature must be demonstrated. Consideration has been given to only one of these points - that of malignancy. A promising method of establishing malignancy which was explored was that the property of establishing colonies of epithelial cells on a feeder layer might be restricted to cells of neoplastic origin (Aaronson & Todaro, 1968).

To examine this possibility, "normal" mammary tissue was obtained from mammaplastic reduction material. This had the obvious disadvantage that the tissue was very fatty, but the procedure detailed in Methods, section 2.3.1.2 was applied to prepare cells, removing as much of the fat as possible at each stage. After collagenase digestion, the cells were spun down, resuspended in medium, and innoculated on to flasks containing feeder layer as with mammary tumour cells. The feeder layers used in these experiments were the foetal intestinal strains (FHS 74 Int and FHI).

Table 14 Effect of Varying Concentrations of Oestradiol-17β on Mammary Tumour Epithelial Colony Formation in the Presence and Absence of Feeder Layer

Breast tumour tissue was treated as described in Section 2.3.1. After removal from collagenase, equal amounts of the cells (BEH strain) were innoculated either into empty 25 cm^2 plastic flasks, or flasks containing a confluent monolayer of feeder cells derived from human foetal intestine. Each set of flasks was fed with medium containing 0, 10^{-9}, 10^{-8} or 10^{-7}M oestradiol-17β, which was changed at regular intervals for 3 weeks. The cells were then stained, and the epithelial colonies counted. The number of colonies formed in the absence of both feeder layer and steroid could not be assessed due to contamination.

Oestradiol conc. (M)	No. of Colonies	
0	N/A	NO FEEDER LAYER
10^{-9}	98	
10^{-8}	101	
10^{-7}	181	
0	13	GROWN ON FEEDER LAYER
10^{-9}	18	
10^{-8}	7	
10^{-7}	12	

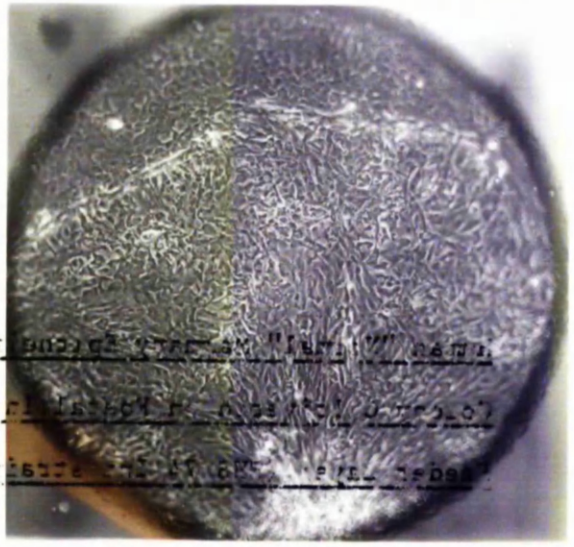
Preliminary findings from these studies were in agreement with the concept that normal epithelial cells could not establish colonies on feeder layers. However, exceptions did occur where epithelial colonies derived from the "normal" mammary tissue appeared on the foetal intestinal feeder layer in two experiments out of the nine performed. Plates 7 and 8 show epithelial colonies from one of these cell strains (N-CAR) growing on FHS feeder layer. Colonies also grew in the absence of feeder layer, but were surrounded by stromal cells, as shown in Plate 9.

The yield of epithelial colonies growing on the feeder layer was great enough to conduct an experiment to determine whether they could be cloned on fresh feeder layer. One flask containing a mixture of epithelial and feeder cells yielded 1.7×10^6 cells, the epithelial component of which was originally derived from approximately 250 mg of normal mammary tissue. Aliquots of these cells were innoculated into flasks with and without confluent feeder layer. The results of this experiment are shown in Table 15. Unfortunately, a cloning efficiency cannot be determined in this experiment since the cells innoculated into the flasks represented a mixed population of epithelial and feeder cells. The Table shows that epithelial cells can only be passaged onto a feeder layer. Thus, epithelial cells will occasionally grow in the absence of feeder layer (Plate 9) but passage of these colonies is enhanced in the presence of feeder layer.

On the basis of the observations with these two normal breast samples, growth on feeder layers could not be used to satisfy the criterion of malignancy in breast epithelium. The possibility remains, however, that these samples, although apparently normal, might have been preneoplastic. Further studies should be carried out to determine whether these results are reproducible, or

Plates 7 & 8 — Human "Normal" Mammary Epithelial-like Colony Cultivated on Foetal Intestinal Feeder Layer (FHS 74 Int strain)

"Normal" mammary tissue from a patient undergoing anaplastic reduction was treated as described in Methods, Section 2.3.1.2, to yield a cell suspension (N-CAR strain). Some of this was innoculated on to FHS 74 Int feeder layer. These plates show epithelial-like colonies which developed in two flasks set up in this way. As in Plate 4, the border between the epithelial colony and the feeder layer cells is clearly illustrated.



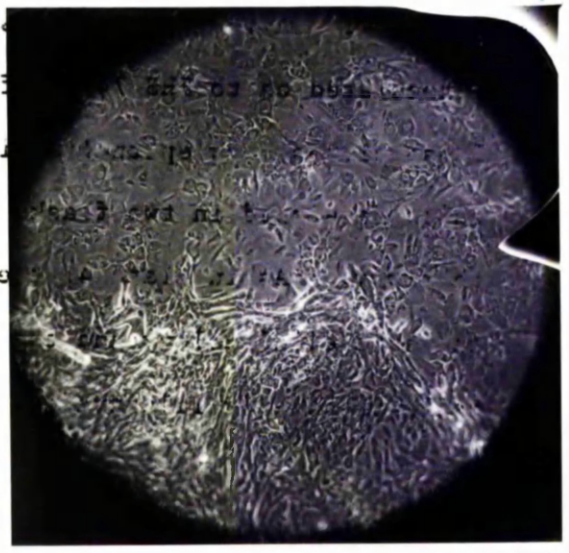


Plate 9 Human "Normal" Epithelial - like Colony Cultivated in the Absence of Feeder Layer

This shows an epithelial-like colony from the N-CAR strain of cells as in Plates 7 and 8, but cultivated in the absence of feeder layer. Although the colony developed without the support of a feeder layer, it can be seen that it is surrounded by stromal cells, which eventually overgrew the entire flask.

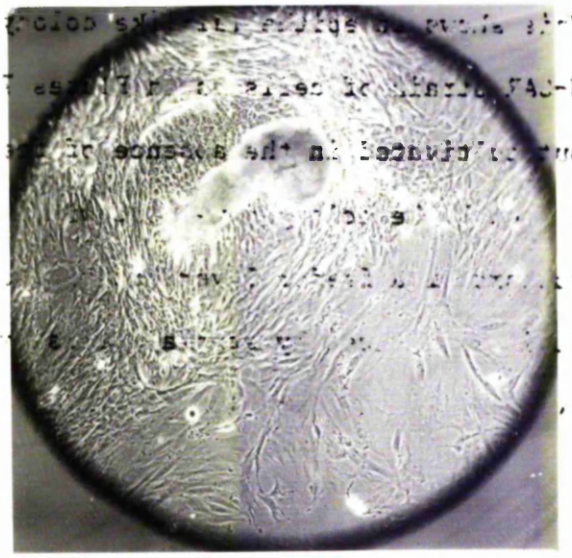


Table 15 Growth of "Normal" Breast Epithelial Cells in the Presence and Absence of Feeder Layer

"Normal" breast tissue from a patient undergoing mammaplastic reduction was dissociated as described in Section 2.3.1. After removal from collagenase, the cells (N-CAR strain) were divided equally between flasks containing a confluent monolayer of feeder cells derived from human foetal intestine and empty flasks. All flasks were fed with working medium, and one containing both mammary and feeder cells trypsinized, counted and aliquots of the mixed cell suspension innoculated into fresh flasks with or without feeder layer as before. The medium in these flasks was changed regularly, and the cells stained after three weeks. The epithelial colonies in each flask were then counted.

No. of cells innoculated	No. of Colonies formed	
10^3	39	GROWN ON FEEDER LAYER
5×10^3	62	
10^4	106	
5×10^4	confluent epithelium	
10^5	"	
10^3	1	NO FEEDER LAYER
10^4	0	
10^5	1	

whether feeder layer does act as an inhibitor of growth in truly normal mammary epithelium.

4. DISCUSSION

Observations in this study are limited by the fact that although tumour cells are of epithelial origin, any biopsy contains also a high proportion of stromal cells. A large tumour biopsy, therefore, yields only a small number of epithelial cells. A high yield of purely epithelial cells is required if a particular investigation is to be carried out with all tests and controls considered. This has not proved possible in many cases in this study (3 tumour samples out of 20 were too small to provide cells of any type). It is likely that the lack of sizeable tumour biopsies is due, in turn, to the increased awareness on the part of the general public that early action in cases of cancer can increase the chances of survival, and thus breast tumours go to surgery at a much earlier, and smaller, stage. This problem illustrates the advantages of working with pleural effusion material from breast cancer patients to establish optimum growth requirements of mammary cells. This source gives a high yield of cells (Cailleau et al., 1974), which appear to be easily cultivated, and strains such as the much-quoted MCF7 (Soule et al., 1973) have proved very useful in the elucidation of different aspects of both tissue culture and endocrine mechanisms (e.g. Lippman & Bolan, 1975, Zava et al., 1977, Horwitz & McGuire, 1978, Strobl & Lippman, 1979). The disadvantages of using cells of this origin are that pleural effusions are not common and, as stated by Kirkland et al., (1979), may represent a highly selected class of cells. Thus, although pleural effusions yield material potentially useful for application to the growth requirements of mammary cells in general, findings must be confirmed at some stage with solid tumours. Studies from pleural effusion cells cannot readily be applied to the therapy of solid tumours. The establishment of

the methods for routine cultivation of this more common form of malignant breast disease is necessary if investigations into the best therapy for each tumour are to be carried out.

The present findings from studies on hormonal requirements of the cells agree with those reported by other workers. An enhancement of epithelial colony formation by insulin at a concentration of 5 µg/ml was found and administered routinely to all cultures. This is in keeping with the findings of Puckett & Shingleton (1972), Heuson et al., (1972), Heuson & Legros (1972) and Cohen & Hilf (1974) in studies on DMBA-induced mammary tumours in rats and mice, Osborne et al., (1976) using MCF7 cells and Allegra & Lippman (1978) using another mammary cell line (ZR-75-1). Campbell & Craig (1979) reported a general requirement for insulin by all cells in culture. The requirement of mammary cells in particular for this hormone is explained by its activation of casein and lactalbumin synthesis (Turkington & Topper, 1966, Turkington et al., 1968), RNA synthesis and general cell division (Voytovich & Topper, 1967, Turkington, 1970). These studies were carried out using mouse mammary gland explants. Since these effects are noted in both normal and neoplastic mammary tissue, a requirement for insulin is not generally a property which is lost during malignant transformation.

The other possible hormonal requirement discovered was one for oestradiol-17β, which appeared to be most effective at 10^{-7}M, not giving any further stimulation of growth at concentrations greater than this in the preliminary experiments performed. This was also the conclusion reached by Lippman & Bolan (1975), who further found that concentrations above 10^{-7}M were actually inhibitory to mammary cell growth, a conclusion reached also by Stormshak et al., (1976), studying rat uterus. This was not observed, however, in

this study. Oestrogen receptors have been detected in mammary cells of the MCF7 cell line (Brooks et al., 1973, Strobl & Lippman, 1979), so it appears that the machinery for processing steroid hormones can endure culture procedures, and thus responses to these hormones can be expected. Strobl & Lippman (1979) also report that oestrogen retention by the cells for over 48 hours can be taken as an indication of receptor presence, since this property was exhibited specifically by the oestrogen receptor-containing MCF7 cell line, but not by receptor-deficient control lines. This could not be used as conclusive evidence of receptor presence, however, as binding might be due to an atypical cellular protein.

The effects of other oestrogens such as oestrone, which has been reported to inhibit mammary cell growth when administered for only a short time (Jozan et al., 1979), were not studied in this project.

Feeder layer cells were originally used in the cultivation of HeLa cells (Puck & Marcus, 1955, Fisher & Puck, 1956). They were later found to be successful in the enhancement of growth in kidney, (Stoker & Sussman, 1965, MacPherson & Bryden, 1971), keratinizing (Rheinwald & Green, 1975a,b) and mammary epithelial cells (Katiyar et al., 1978, Armstrong & Rosenau, 1978, Kirkland et al., 1979). In the present study, feeder layers have been found to be of positive value in encouraging mammary tumour epithelial growth in approximately 65% of cases, but, in some cases, evidence of actual inhibition by feeder cells was found. It was thought from preliminary experiments that the great value of feeder layers might be that they would exclude normal mammary epithelial growth, thereby establishing the malignancy of cells which did form colonies on feeder layers. Normal mammary cells have been used as feeder layers for malignant ones (Taylor-Papadimitriou et al., 1977a,b),

but no published evidence of normal mammary growth on any type of feeder layer exists.

In the course of studies on the ability of feeder cells to inhibit selectively "normal" mammary epithelial cell growth, on two occasions epithelial cells did grow well and established colonies on the feeder layer. This, therefore, discounts the possibility of using growth on a feeder layer as a marker of malignancy. The source of the normal mammary cells was tissue from patients undergoing mammaplastic reduction. Due to the nature of the condition necessitating such surgery, it could be argued that this tissue is not in fact normal. However, no abnormalities appeared in the histology of the tissue, although the possibility exists that the cells which did grow on feeder layer may have been derived from preneoplastic tissue. Other workers wishing to isolate normal mammary cells used expressed milk as their source (Taylor-Papadimitriou et al., 1977a,b), but the advantage of using the surgical specimens is that they are readily available in great quantity, such that none of the problems arising from lack of tissue mentioned earlier are encountered.

One important area which was not explored in the course of this study was the characterization of the cells which were successfully cultivated. It is realised that the establishment of the human mammary, malignant and epithelial characteristics in the cells is essential if any conclusive results are to be obtained from work on a particular system. It was thought, as described above, that a test for malignancy was available, but this did not prove reliable. The epithelial nature of the cells was assumed by their growth pattern, being in circular rather than the parallel array displayed by fibroblasts. Cells of this same appearance resulted from both normal and neoplastic breast tissue.

The overall conclusions from this study on the *in vitro* growth of mammary tumour epithelial cells are that the inclusion of the peptide hormone insulin in the growth medium gives a definite enhancement of growth, while that of the steroid hormone oestradiol may benefit the cells also. The use of a feeder layer composed of human foetal intestinal cells of epithelial nature will also encourage mammary epithelial cell development in a percentage of cases. No other types of cell used in the composition of a feeder layer had any success in this direction. In spite of these measures, however, it has not proved possible to sustain the growth of the epithelial cells in culture for longer than 3-4 weeks, as found also by Whitescarver *et al.*, (1968), Stoker *et al.*, (1976) and Ozzello (1977), while fibroblasts seem capable of growing for much greater periods than this. A longer period of growth by the epithelial cells is necessary if the effects of various stimuli and inhibitors are to be observed and made use of in the treatment of malignant breast disease.

Even when extended growth of epithelial cells on feeder layer is achieved, extensive characterization of the cells must be performed. The malignant, mammary and epithelial nature of the cells must be established. For example, determination of the presence of plasminogen activating factor, as mentioned in the Introduction, would seem to be an appropriate and readily applicable test for malignancy of cultured mammary epithelium. Then, before studies can be carried out on the effects of various growth regulators on these cells, the toxicity of such treatments towards feeder layer cells will first have to be determined. It is possible that some of the therapeutic regimes applied to breast tumours, especially chemotherapeutic forms, might prove more toxic to the feeder layer than to the breast cells. In this case, mammary epithelial growth would be arrested by an indirect action of the agent under study.

Once all these unknown variables have been determined, the *in vitro* system should prove the most effective way to predict the optimal form of therapy for breast cancer patients. Studies on the regulation of oestrogen and progesterone receptor synthesis, and on the nature and functionality of receptor in +/o and o/+ cells would benefit also from an *in vitro* approach.

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