THE MOLECULAR BASIS OF STEROID HORMONE PROMOTED GROWTH

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This thesis is dedicated to my parents.

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ABBREVIATIONS

The standard abbreviations, as recommended in the "Biochemical Journal, Policy of the Journal and Instructions to Authors, 1981", are used throughout this thesis with the following exceptions:-

BSA	-	bovine serum albumin
BSS	-	balanced salt solution
^{o}c	-	degrees centigrade
cAMP	-	cyclic adenosine monophosphate
CBG	-	cortisol binding globulin
cGMP	-	cyclic guanosine monophosphate
CPM	-	counts per minute
DCC	-	dextran coated charcoal
DTT	-	dithiothreitol
E_2	-	oestradiol 17β
EGF	-	epidermal growth factor
ER	-	oestrogen receptor
FHI-4	-	foetal human intestine, epithelial cell line
FHI-F	-	foetal human inestine, fibroblast cell line
HAP	-	hydroxylap atite
HEPES	-	N - 2 - hydroxy - piperazine - N' - 2 ethane sulphonic acid
hn RNP	-	heterogeneous ribonucleoprotein
IgG	-	immunoglobulin G
POPOP	-	1, 4 di - (2 - (5 - phenyloxazolyl)) - benzene
PPO	-	2, 5 - diphenyloxazole
SBG	-	steroid binding globulin

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SUMMARY

1. The literature concerning measurement and stability of the oestrogen nuclear receptor has been reviewed in relation to the use of receptor assays for management of breast cancer.

2. The influence of temperature and ionic strength on the stability of the human nuclear oestrogen receptor complex was studied in relation to the major methods used to assay for nuclear receptor. The exchange rate of (^{3}H) - oestradiol from the nuclear receptor was measured as a decrease in specifically bound labelled oestradiol during an incubation with an excess of unlabelled oestradiol.

3. When the nuclear oestrogen receptor complex was bound to intact nuclei, the rate of exchange increased with temperature but a significant exchange could be measured over 24 hours at 4^{o}c. The use of protease inhibitors together with re-labelling of nuclear receptor after incubation confirmed that the observations represented exchange of hormone rather than degradation of the hormone receptor complex.

4. Measurement of the on-rate of (^{3}H) - oestradiol to the oestrogen nuclear receptor, prefilled with unlabelled oestradiol, also demonstrated that there was a significant rate of exchange of hormone at 4^{o}c.

5. The exchange rate from salt extracted, hydroxylappatite precipitated receptor also increased with temperature. However, this extracted complex was more stable and no exchange could be detected at 4^{o}c.

6. Only 50% of total detectable human nuclear oestrogen receptor was solubilised by the standard method of salt extraction in 0.6M KCl. As the salt concentration was raised (0 - 0.6M KCl) an increase in stripping of oestradiol from the hormone receptor complex was observed. This would create a false impression of the proportion of "empty nuclear receptor".

7. These results were compared with other reports of the stability of the hormone receptor complex. The consequences of these results were discussed in relation to current nuclear oestrogen receptor assays and their use in stratifying therapy for patients with breast cancer.

8. The literature concerning current in vitro models of human breast cancer has been reviewed and the merits of these models have been compared.

9. A method has been developed for the routine primary culture of cells from breast cancer biopsies. This method involved digestion with collagenase followed by growth on a feeder layer. Approximately 85% of biopsies gave rise to viable primary cultures.

10. Growth of these cells was in the form of ring colonies which could be maintained in culture for up to 3 months. Cells were characterised as epithelial by electron microscopy and immunofluorescence and tests were carried out to demonstrate the retention of malignant potential.

11. The effect of hormones and growth factors on the growth of cells in the ring colonies has been investigated. In particular cortisol

and oestradiol were found to act synergistically to stimulate growth. Cholera toxin, prolactin and cortisol were found to stimulate growth or plating efficiency to a lesser extent. Epidermal growth factor did not stimulate growth of breast epithelial cells. The effect of these hormones was compared with published reports of other in vitro systems.

12. Differences between primary tumours (such as oestrogen receptor status or growth rate) were reflected by the growth and the response to hormonal stimulation of the ring colonies.

13. A method was developed, using autoradiography, to measure the effect of hormones and growth factors on (^{3}H) thymidine incorporation in a small number of cultured breast epithelial cells. The possible applications of this system have been discussed.

PART I

The stability of the human nuclear oestrogen receptor.

1. INTRODUCTION

1.1 CONTROL MECHANISMS

Both the functions of the body and its response to environmental fluctuations are regulated by two major control systems: the nervous system and the hormonal or endocrine system. There is interaction between neural and endocrine control at several levels but, in general, the nervous system controls the rapid activities of the body, such as muscular contractions, by the transmission of electrical impulses along a network of nerve fibres. On the other hand, the endocrine system is concerned principally with the different metabolic functions of the body, including aspects of cellular metabolism, growth and differentiation. The effects of a hormone can take place in seconds or over a period of many years.

A hormone is a chemical substance that is secreted into the body fluids by one cell, or a group of cells, and then exerts a physiological control effect on other cells of the body, classically at a distant site. In general, a hormone is secreted by a specific endocrine gland and is transported in the blood to cause physiological actions at distant, so called target tissues. Hormones can affect either one or more target tissues and a given tissue can be responsive to more than one hormone. The classical concept of a distant target tissue may have to be modified to include prostaglandins, interferon and some of the hormones such as secretin released in the gastrointestinal tract and growth hormone release inhibiting factor produced in the D cells of the pancreas, which are able to exert their effects on cells

in the same tissue. The specificity of a hormone is determined by the presence of a chemical receptor (the entity with which the hormone interacts in order to initiate a response) on or in the target cell.

Chemically, hormones can be divided into three classes -

a) the amino acid derivatives, e.g. the catecholamine, adrenaline
b) the peptide hormones, e.g. insulin
c) the steroid hormones, e.g. testosterone

Studies on the molecular mechanism of hormones led to the division of hormones into two groups depending on whether the hormones acted at the cell surface or entered the cell. This separation has recently become blurred (Kolata, 1978; Kahn et al, 1981; Posner et al, 1981) because of evidence of internalization of plasma membrane hormone/receptor complexes.

1.2 STRUCTURE OF STEROID HORMONES

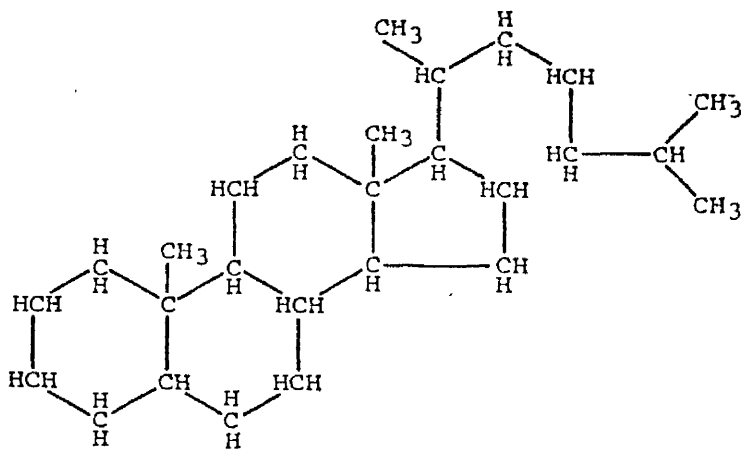
All steroids are derived from cholesterol which is a constituent of virtually every animal tissue and occurs partly as a free alcohol and partly esterified with higher fatty acids. The cyclohexane rings adopt the most stable 'chair' conformation resulting in the three dimensional structure of steroid hormones (figure 1.1). A set of rules for the systematic nomenclature of all steroids has been defined by the International Union of Pure and Applied Chemistry (IUPAC - IUB, 1969; 1971). Virtually all steroids can be defined with reference to cholestane by removal of all or part of the carbon side chain (figure 1.2).

Figure 1.1 - The Structure of Cholestane

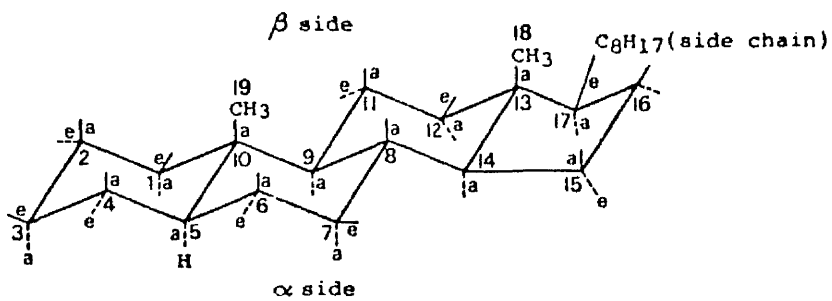
A. Two-dimensional representation of the structure of Cholestane.

B. Representation of the three-dimensional structure of Cholestane.

A.



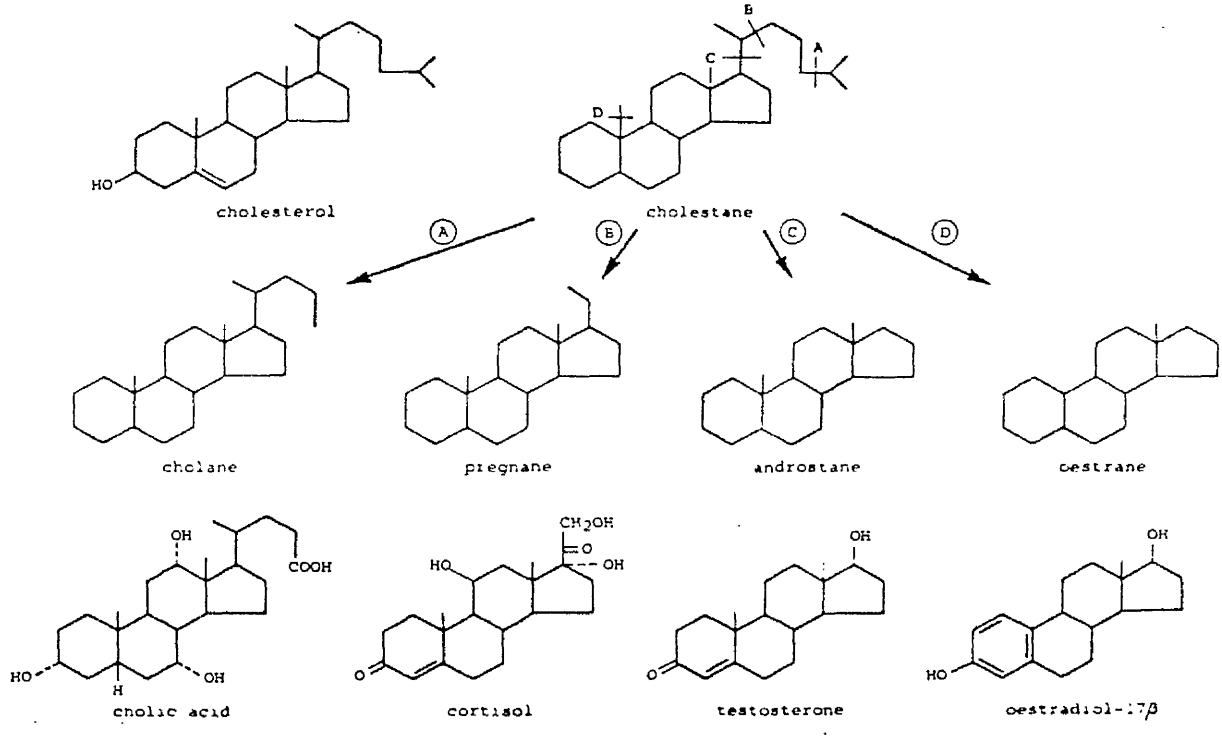
B.



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

Figure 1.2 - Structural Relationship between Cholestane and Cholane, Pregnane, Androstane and Oestrane.

Examples of the biologically important steroids related to each parent hydrocarbon are also shown.


HO
cholesterol
A
B
C
D
cholestane
A
B
C
D
cholane
pregnane
androstane
oestrane
OH
COOH
HO
OH
H
cholic acid
CH2OH
O
HO
OH
O
cortisol
OH
O
testosterone
OH
HO
oestradiol-17β

1.3 SYNTHESIS OF OESTROGENS

The ovaries are the main source of oestrogens in premenopausal women. During the early stages of the menstrual cycle oestrogens are synthesised in the graafian follicle by aromatization, in the granulosa cells, of androgens produced by theca cells. After ovulation oestrogens are produced in the resulting corpus luteum. Oestrogens both stimulate the growth and development of the female reproductive organs and induce the secondary sex characteristics. The growth responses include proliferation of the epithelium of the fallopian tubes, endometrium, cervix and vagina. Oestrogens in concert with pituitary hormones, progestins and glucocorticoids, stimulate mammary growth though the precise balance of hormones required for mammary development varies among species.

In the ovaries, cholesterol is converted to oestrogen, via the androgens. Oestrogens are also produced from other sources, e.g. the testes are able to produce oestrogens and the adrenals can synthesise small quantities of oestrone. Other recent work has revealed that peripheral tissues contain an aromatase, associated particularly with adipose tissue, which has the ability to utilize plasma -4- androstenedione and testosterone to synthesise oestrogen both in men and women. In postmenopausal women most of the circulating oestrogens come from plasma -4- androstenedione (Jensen, 1979).

In 1960 Jensen and Jacobson prepared tritiated oestradiol of high radiospecific activity and demonstrated the uterus and the vagina as target tissues. Later the hypothalamus, anterior pituitary

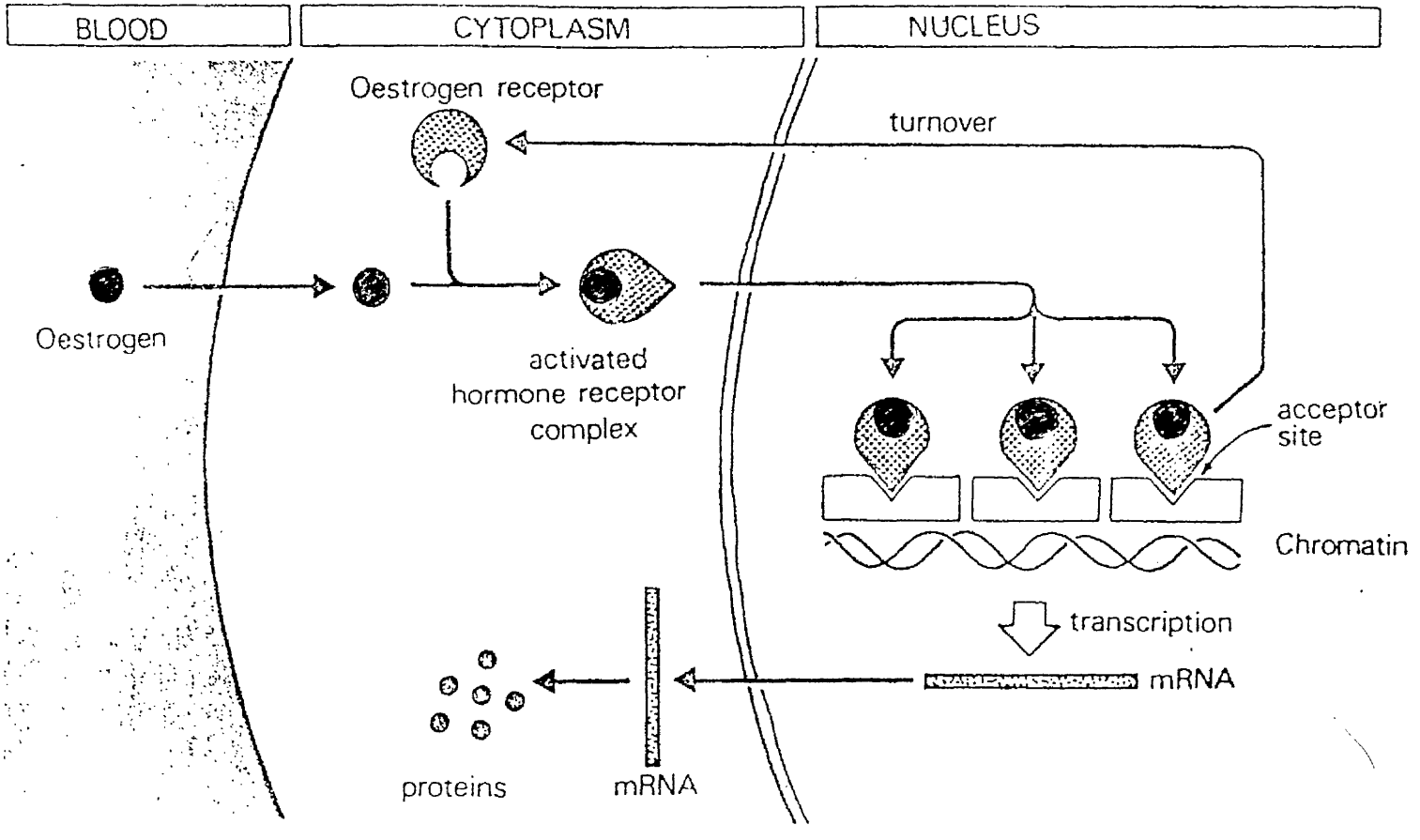
(Eisenfeld & Axelrod, 1966) and the breast (Sander, 1968) were also shown, through retention of steroid, to be target tissues.

The most biologically active of the natural oestrogens is oestradiol - 17β, but both oestrone and oestriol can be important under certain conditions. Oestrone activity was thought to result from its conversion to oestradiol - 17β (Williams - Ashman & Reddi, 1971). It was later shown to be effective at higher concentrations than were required for oestradiol 17β (Ruh et al, 1973) but acted as an antagonist when both were injected simultaneously. This was due to the lower affinity of the oestrogen receptor for oestrone (Lövgren et al, 1978) so that it did not maintain receptor in the nucleus for an adequate period (Lan & Katzenellenbogen, 1976). When a pellet of oestrone was implanted to maintain the plasma concentration, and so re-cycle receptor, it was found to be a potent oestrogen. A similar argument explains the biological activity of oestriol (Clark & Peck, 1979).

1.4 MECHANISM OF ACTION OF STEROID HORMONES

Steroid hormones are normally found in the plasma at levels less than 10^{-8}M although the actual levels vary from species to species. They are transported bound to serum proteins to (1) protect them from liver metabolism (2) overcome their relative insolubility and (3) provide an immediate store of steroid. Some steroid hormones bind largely to one plasma protein, i.e. cortisol binds principally to cortisol binding globulin (CBG) while others bind to several with relatively low affinity, e.g. aldosterone. At normal blood levels (in humans $\sim 10^{-10}$M) most oestrogen is bound to steroid binding globulin (SBG), with weak binding

Figure 1.3 - Mechanism of Action of Steroid Hormones


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

to every plasma fraction (Sandberg et al, 1957). At higher concentrations, such as during pregnancy, binding to albumin and α_2-globulin predominates (King & Mainwairing, 1974).

Despite the proposal, on several occasions, of a transport mechanism for cellular uptake of oestrogen (Milgrom et al, 1973) or corticosteroids (Harrison et al, 1974; Svyemisto & Terayama, 1975), it is now generally accepted that entry into the cell is by passive diffusion (Gorski & Gannon, 1976; Giorgi & Stein, 1981). Shortly after an injection of radioactively labelled steroid hormone, it can be detected in all tissues (Jensen & Jacobson, 1960; 1962). The possession of a relatively small number (10 - 20,000/cell) of highly specific receptor molecules is responsible for the retention and, possibly, concentration of hormone within a cell and the presence of such receptors defines a target tissue (Folca, et al 1961; Clark & Gorski, 1969; Clark & Peck, 1979; Leake, 1981a). The unfilled receptor molecule is thought to be free in the cytosol or loosely associated with the membrane of the cell and the endoplasmic reticulum (Wittliff, 1975; Pietras & Szego, 1979; Muller et al, 1979).

In 1968 the two groups of Jensen and Gorski separately proposed a general two step mechanism for oestrogen action (figure 1.3) (Gorski et al, 1968; Jensen et al, 1968). The research that led to these theories, and much of the subsequent research on oestrogen action, involved studying its effects on the immature rat uterus (Jensen et al, 1974) and, later, on chick oviduct (O'Malley et al, 1976). The two step mechanism has also been demonstrated for progesterone (Schwartz et al, 1976), androgens (Mainwairing & Peterken, 1971), glucocorticoids

(Rosseau et al, 1973), mineralocorticoids (Edelman et al, 1968), and vitamin D (Brumbaugh & Haussler, 1974).

After binding to the receptor in the cytosol, the hormone - receptor complex is thought to be activated by the addition of a polypeptide (Notides & Nielson, 1974; Leake, 1976; Thampan & Clark, 1981). The site of activation is unknown but the process is reported to be heat dependent (Jensen et al, 1974) and to permit translocation of the hormone receptor complex across the nuclear membrane into the nucleus. A positive role for the nucleus has, however, been discounted (Linkie & Siiteri, 1978). Once in the nucleus the complex binds to specific acceptor sites on the chromatin (Spelsberg et al, 1975; De Boer et al, 1977) where it regulates gene expression. The acceptor site (see section 1.9.2) is thought to be defined by some combination of non histone protein and DNA (Spelsberg, 1974; Clark & Peck, 1979; Leake, 1981a).

The first effects occur rapidly after hormone administration and include an increase in hnRNA and mRNA synthesis (Spelsberg, 1976; Aziz & Knowler, 1978; 1980). The hormone receptor complex is released from the chromatin after retention for a specific time (Clark & Peck, 1976; 1979). Horwitz & McGuire (1978a;b ; 1980) have recently suggested that processing of the nuclear receptor complex must occur before induction of protein synthesis.

In general the steroid hormone is released from the cell unmetabolised (Puca & Bresciani, 1968). Exceptions are the androgens, which are metabolised to their active form, dihydro testosterone, in the target

cell prior to action, and some breast cancers which are able to metabolise steroids (King et al, 1965; Castagnetta et al, 1980; Miller et al, 1981). The level of receptor in the cytoplasm is replenished by a combination of de novo synthesis and recycling (Clark & Peck, 1979). After release from the cell, steroid hormones are removed from the bloodstream by the liver and then excreted, primarily as conjugates of glucuronic acid and sulphuric acid.

1.5 KINETICS OF OESTRADIOL - INDUCED RESPONSES IN TARGET TISSUES

In the immature rat uterus the effects of oestrogen are characterized by hypertrophy followed by hyperplasia. The earliest detectable response is an increase, starting after 15 mintues, in RNA polymerase II activity. This peaks at 30 minutes and returns to control values within 2 hours (Glasser et al, 1972; Borthwick & Smellie, 1975). By 30 minutes there is a detectable stimulation in the synthesis of hn RNA (Knowler & Smellie, 1971 ; 1973), with an increased content of sequences complimentary to mRNA (Aziz & Knowler, 1978; 1980). This is followed by the appearance of hnRNP particles (Knowler, 1976) and the aggregation of existing ribosomes into polysomes containing newly made mRNA (Merryweather & Knowler, 1980).

One hour after oestradiol administration synthesis of rRNA is stimulated and this peaks at 2 - 4 hours (Knowler & Smellie, 1973) although accumulation of new rRNA into ribosomes does not reach a peak until 12 hours. Inhibitor studies suggest that the stimulation of rRNA is dependent on prior stimulation of hnRNA synthesis and protein synthesis (Gorski, 1964; Borthwick & Smellie, 1975). A similar result was

obtained for glucocorticoid stimulation (Chen & Feigelson, 1979) suggesting that early protein synthesis is a prerequisite for many of the later steroid hormone-induced genetically-mediated responses. Other early effects that have been reported are increased fluid retention, increase in nucleotide precursor uptake and changes in cyclic nucleotide levels (cAMP$\downarrow$ cGMP$\uparrow$) (Kuehl et al, 1974; Nicol et al, 1974). The increase in cGMP is inhibited by actinomycin D (Nicol & Goldberg, 1976; Flandroy & Galand, 1979) suggesting the increase occurs subsequent to a transcriptional event. The increase in fluid retention is, however, thought to occur independently of transcription as a result of eosinophil migration to the uterus (Tchernitichin, 1979; 1982). Other oestrogen induced but non-receptor mediated responses, include an increase in glycogen, and induction of several glycogenolytic enzymes and a cervicovaginal antigen (Tchernitichin, et al, 1975; 1977). Jensen & De Sombre (1972) reported an early rise in mRNA, phospholipid and glycogen synthesis followed (6 - 12 h) by a rise in total protein, RNA and DNA. The first specific protein to be induced is IP which has been recently characterized as 'brain type' creatine kinase (Kaye, 1979; 1982).

Late responses to oestrogen include a sustained high RNA polymerase I activity, a second rise in polymerase II (Glasser et al, 1972; Borthwick & Smellie, 1975) and a sustained increase in glucose metabolism (Gorski & Raker, 1973). There is a general growth and division of cells, and the receptor must remain in the nucleus for 6 - 12 hours to elicit these 'late' responses (Clark & Peck, 1976; 1979).

Since RNA polymerase activities were measured in isolated nuclei, it is

possible that they reflect changes in template activity rather than enzyme activities. When Borthwick & Smellie (1975) extracted and fractioned uterine RNA polymerase I and II, no oestrogen induced effects were observed. Tsai et al, (1975) studying chick oviduct found an increase in initiation sites available to bind RNA polymerase after oestrogen stimulation. A similar level of increase in initiation sites was found for oestrogen in rat uterus (Markaverich et al, 1978), for progesterone in chick oviduct (O'Malley et al, 1976) and for androgen in rat prostrate (Thomas et al, 1977). However, there have been doubts expressed (Parker & Roeder, 1977) over the validity of the rifampicin challenge assay used to quantify initiation sites (Tsai, et al, 1975) since it uses a bacterial RNA polymerase though the study of Thomas et al (1977) used an animal polymerase. Palmiter & Lee (1980) have concluded that the increase in RNA synthesis detected in chick oviduct is an artefact produced by transcription of contaminating ovalbumin mRNA. Nevertheless, most authors are in agreement that hormone treatment does increase the number of initiation sites. In the chick oviduct the secretion of egg white proteins ovalbumin and conalbumin are under the control of oestrogen and progesterone. Hybridisation techniques with cDNA were used to show an increase in ovalbumin mRNA production from zero to 48,000 molecules per cell which, after withdrawal of oestrogen treatment, decreased to zero (Harris et al, 1975; 1976). The differential control of oviduct genes by steroid hormones was studied by Palmiter et al (1976; 1981) who found that conalbumin gene transcription was linearly related to receptor levels while ovalbumin gene transcription was related to receptor levels in a manner indicative of co-operative interactions among receptors during induction. This result, in combination with the effect of simultaneous

progesterone administration on the kinetics of synthesis of the two proteins, has led to the conclusion that a single receptor binding site is involved in conalbumin gene regulation and multiple sites are involved in ovalbumin gene regulation (Compere et al, 1981; Palmiter et al, 1981). This led Palmiter et al (1981) to propose a model based on multiple regulatory sites and intermediary proteins to explain both the synthesis of ovalbumin and conalbumin and the apparent interaction between the androgen, progesterone and oestrogen nuclear receptors in controlling their synthesis.

1.6 OESTROGEN RECEPTOR

The oestrogen receptor was first characterized in immature rat uterus by Toft & Gorski (1966) as a protein of molecular weight 80,000. More recently the oestrogen receptor from calf uterus has been purified to homogeneity by affinity chromatography (Sica & Bresciani, 1979). The dissociated form has a molecular weight of 70,000 by SDS gel electrophoresis comparing with 76,200 for the rat uterus receptor (Jensen & De Sombre, 1972) and 50,000 for the receptor from human breast cancer cells (Greene et al, 1980). The similarities between different species are demonstrated in the cross reaction of monoclonal antibodies across a wide variety of species (Greene et al, 1980; Greene & Jensen, 1982). There is still uncertainty regarding the molecular weight of, and number of subunits in, the native functional form of the protein (Sica & Bresciani, 1979).

The oestrogen receptor protein is heat labile (McGuire & De La Garza, 1973) and has iso-electric points at pH 5.9 and 6.7 corresponding to the two

molecular forms (Gustafsson et al, 1978). It has a sulphydryl group which is important both to maintain the conformation and for the steroid/receptor interaction (Jensen et al, 1967; King et al, 1979b). The receptor is destroyed by repeated freezing and thawing (King et al, 1979b; Sica & Bresciani, 1979). Both the cytoplasmic and nuclear forms of the receptor demonstrate a marked tendency to aggregate (Stancel et al, 1973; Schneider & Gschwendt, 1980) and this probably accounts for many of the problems encountered in trying to determine molecular weights. The concentration of receptors present in normal tissue depends on circulating levels of oestrogen, progesterone and prolactin (Sarff & Gorski, 1971; Asselin et al, 1977). This is demonstrated by the cyclic variation of uterine receptors during the ovarian cycle in both rats and humans (Feherty et al, 1970; Pollow et al, 1975; Soutter et al, 1979).

1.7 CYTOPLASMIC BINDING

There are two types of cytoplasmic binding found in oestrogen target tissues. The high affinity site (type I) has a dissociation constant (Kd) of 10^{-10}M in human tissue (McGuire & Julian, 1971) and is the classical oestrogen receptor which undergoes translocation to the nucleus and mediates cellular activity (Feherty et al, 1971; King, 1975; Clark et al, 1978). Type I binding sites are thought to be able to concentrate oestrogen within the target cell (Clark & Peck, 1979; Leake, 1981b).

The lower affinity binding sites (type II) have a dissociation constant (Kd) in humans of 3×10^{-9}M, do not undergo translocation and have an

ill-defined cellular function (Clark et al, 1978; Eriksson et al, 1978). Type II binding sites seem to be a general phenomenon. They are found in rat uterus (Clark et al, 1978), chick oviduct (Smith et al, 1979) and human breast tumours (Panko et al, 1981) for oestrogen, for glucocorticoids (Barlow et al, 1979; Do et al, 1979) and aldosterone (Farman et al, 1978). The situation has been further confused by the report of a third oestrogen binding site in chick oviduct (Smith & Taylor, 1981) which can translocate, but which induces only some of the oestrogenic responses. This may be related to the report of Fishman & Fishman (1979) that the antioestrogen, tamoxifen, only binds to a proportion of soluble oestrogen receptors in human target cells.

Pietras and Szego (1977 ; 1979) have reported that the major portion of the cytoplasmic receptor is found in association with the plasma membrane, with lesser amounts in the lysosomal and mitochondrial fractions and with very little actually in the supernatant. However Muller et al (1979) claim that the receptor detected represents only a small portion contaminating the plasma membrane since mixing cytoplasmic receptor and rat diaphragm membrane demonstrates that the association is not tissue specific. But both Pietras and Szego (1979) and Parikh et al (1980) strongly dispute these claims.

Estimates of the size of the cytoplasmic form of the classical oestrogen receptor have varied from 7-10S (Toft & Gorski, 1966; McGuire & Julian, 1971; Wittlif & Savlov, 1978). In low ionic strength buffers it is now generally accepted to be, and is referred to as, 8S (Kute et al, 1978; Hawkins et al, 1980). In solutions of high ionic strength (0.4 - 0.6M KCl) the predominant form is 4S. It has been proposed

that the 4S is the functional receptor while the 8S, an aggregate of the 4S, serves to maintain the pool of 4S (Powell - Jones et al, 1975). In contrast, Wittlif has repeatedly proposed that the 8S receptor is the active form, and only breast tumours containing this form can be oestrogen dependent (Wittlif & Savlov, 1975 ; 1978; Wittlif et al, 1976; Savlov et al, 1977) though most groups have failed to find any correlation between the molecular form of the oestrogen receptor and hormone dependence (Jensen et al, 1975; Dao & Nemoto, 1980; Freedman & Hawkins, 1980). Many of the problems regarding the size, and distribution of sizes, appear to indicate that the form of the receptor isolated is very dependent on the methodology used. Additionally, because of salt and protein dependent aggregation phenomena, it is not possible to clearly define the size(s) of soluble receptor in vivo.

The presence of the oestrogen receptor is used to indicate that a breast tumour is dependent on oestrogen for growth (Section 1.11). The standard method for measurement of cytoplasmic or soluble oestrogen receptor is to occupy unfilled receptors and to exchange endogenously bound hormone for radioactive hormone and then absorb unbound hormone onto dextran coated charcoal (Schrader & O'Malley, 1972; Peck & Clark, 1977; King et al, 1979) or absorb the labelled receptor to glass pellets (Clark & Gorski, 1969) hydroxylap atite (Erdos et al, 1970) or DEAE filters (Santi et al, 1973). More recently attempts have been made to develop an oestrogen receptor assay suitable for small amounts of tissue, such as needle biopsies (Section 5.6) and one of the most promising utilizes isoelectric focusing (Gustafsson et al, 1978; Lloyd et al, 1982). Other possibilities suggested have been monoclonol antibodies (Greene & Jensen, 1982) and cyto- and histo- chemical methods

(Chamness et al, 1980) although there is much debate about the reliability of these methods (see Section 5.6).

1.8 TRANSLOCATION / ACTIVATION

The two step mechanism proposes that the binding of oestrogen to the receptor allows the translocation of the hormone receptor complex into the nucleus. The temperature sensitive 'activation' (Williams & Gorski, 1971) or addition of an extra 50,000 molecular weight polypeptide (Notides & Nielson, 1974; 1975; Thampan & Clark, 1981) was thought to change the shape of the hormone receptor complex and allow its passage into the nucleus. This activation causes a change in the sedimentation coefficient from 4S to 5S in salt containing sucrose density gradients (Notides & Nielson, 1974; 1975; Weichman & Notides, 1980). The activated or 5S form can be distinguished from the 4S by use of bovine serum albumin (BSA), which sediments between the two forms on sucrose density gradients. Activation of the hormone receptor complex increases its affinity for DNA or DNA cellulose (Fleischmann & Beato, 1979; Gschwendt & Kittstein, 1980). Activation of steroid receptors can be achieved in a number of ways, by increasing the ionic strength (Gschwendt & Kittstein, 1980; Weichman & Notides, 1980) or increasing the pH (Bailly et al, 1978) or by dilution, filtration or dialysis to remove a low molecular weight inhibitor (Sato et al, 1980; McDonald & Leavitt, 1982).

Originally Williams & Gorski (1971) reported that activation was temperature sensitive, though more recently Pavlik et al (1979) and Traish et al (1977) have reported activation at low temperature.

There is presently disagreement on whether activation takes place in the cytosol (Jensen & De Sombre, 1972; O'Malley & Means, 1974) or in both the nucleus and cytoplasm (Linkie & Siiteri, 1978). This latter suggestion compliments the revised model of steroid hormone action proposed by Martin & Sheridan (1982). This disagreement is further complicated by reports that the two processes 1) activation (increased affinity for DNA) and 2) transformation (change from 4S to 5S)actually occur as independent steps (Bailly et al, 1980; Gschwendt & Kittstein, 1980).

1.9 NUCLEAR BINDING

1.9.1 THE NUCLEAR OESTROGEN RECEPTOR

The concentration of receptor-bound steroid by the nucleus was one of the first observations in the study of steroid-receptor interaction (Noteboom & Gorski, 1963; King & Gordon, 1966; Jensen et al, 1968). The binding of oestrogen receptor complex to the appropriate nuclear sites triggers the genetically mediated biological response. The hormone/receptor complex must be retained in the nucleus for periods longer than 6 hours to induce a full oestrogenic response (Clark & Peck, 1976; 1979). It is widely believed that the binding of the nuclear receptor complex causes a change in the gene structure permitting access (at the initiation sites) to the enzymes responsible for transcription. This is demonstrated by the appearance of puffs, indicating gene transcription, on the Drosophila polytene chromosomes of larval salivary glands resulting from ecdysterone binding (Groenmeyer & Pongs, 1980) and by the increased sensitivity of vitellogenin genes to nuclease digestion, after oestrogen treatment (Gerber - Huber et al, 1981).

Horwitz & McGuire (1978 a ; b) first reported the rapid loss, subsequent to translocation, of 70% of the total cellular receptor in MCF-7 cells. This rapid loss of nuclear receptor, now termed nuclear receptor processing, was shown to correlate quantitatively with the induction of the progesterone receptor. Actinomycin D, and other drugs which intercalate between G-C base pairs, but neither A-T intercalating drugs nor RNA synthesis nor protein synthesis inhibitors, prevent processing. Horwitz & McGuire (1980) concluded that this inhibition of processing resulted from the direct effect of actinomycin D on DNA conformation. Processing does not occur, or only occurs at a reduced rate, in response to antioestrogens in rat uterine cells (Pavlik et al, 1979; Kassis & Gorski, 1981). It has been recently suggested that processing of nuclear receptor coincides with the appearance of salt resistant nuclear receptor, (Kasid et al, 1982; Strobl et al, 1982) - the fraction which was proposed by Clark and Peck (1976 ; 1979) to control the genetic response to oestrogen.

Auricchio's group have repeatedly proposed (Auricchio et al, 1981; Migliaccia & Auricchio, 1981) that the oestrogen receptor is controlled by phosphorylation and that processing is the dephosphorylation of the nuclear receptor. Although there is little evidence of phosphorylation of steroid receptors in vivo (Grody et al, 1980) control of other nuclear proteins, such as RNA polymerase (Dahmus, 1981), histone and non-histone proteins (Langan, 1978; Isenberg, 1979), by phosphorylation has been reported.

There are also two types of oestrogen binding in the nucleus, referred to as nuclear type I and nuclear type II (Eriksson et al, 1978; Markaverich

et al, 1980). The nuclear type I is the classical nuclear receptor which results from the translocation of the cytoplasmic receptor. The nuclear type II binding is also hormone and tissue specific, exhibits a lower affinity for oestrogen than the type I receptor but is not related to the cytoplasmic type II binding (Markaverich et al, 1980).

The function of nuclear type II is unknown but Kelner & Peck (1981) claim that it is absent in tissues such as the pituitary and hypothalamus, which contain oestrogen receptors but do not grow and divide in response to oestrogen.

The size of the classical nuclear receptor is generally accepted as 5S but a large percentage of 4S receptor has been found in purified nuclei (Linkie & Siiteri, 1978; Bailly et al, 1980). The nuclear oestrogen receptor from oviduct has been partially purified (Schneider & Gschwendt, 1980). The steroid nuclear receptor which has been best characterised is the progesterone receptor (Schrader et al, 1981) which consists of 2 subunits, A and B, both of which bind progesterone and which have been purified to homogeneity (Schrader et al, 1977; Coty et al, 1979). The A subunit shows DNA binding activity which is masked in the native form and which is exposed only upon a conformational change or upon its dissociation from the larger complex. (O'Malley et al, 1972; Coty et al, 1979). Protein B does not show DNA binding activity but appears to interact with chromatin at specific sites (Schrader et al, 1972). The native form has been shown to contain equal amounts of the A and B subunits (Birnbaumer et al, 1979).

The main methods of measuring nuclear oestrogen receptors are based on the exchange assays described by Anderson et al (1972). This involved the exchange of unlabelled hormone, bound to the receptor, with labelled oestrogen during an incubation at 37°c. The conditions of incubation have been altered, by various groups in an attempt to maximise the extent of hormone exchange and minimise the digestion of the receptor by protease action (Garola & McGuire, 1977a). Modifications of the assay include salt extraction of the receptor and the measurement of binding by receptor precipitation using protamine sulphate (Chamness et al, 1975), ammonium sulphate or hydroxylappatite (Garola & McGuire, 1977b).

1.9.2 NUCLEAR ACCEPTOR SITES

The concentration of receptor in the nucleus may reflect the hydrophilic nature (see Section 1.10) of the steroid receptor complex (Williams & Gorski, 1974; Sheridan et al, 1979) though a great deal of evidence has accumulated which suggests that about 90% of filled receptor is associated with chromatin (Spelsberg, 1974; Hemminiki, 1976). This led to the controversial suggestion of specific acceptor sites, for the hormone receptor complex, distributed throughout the chromatin (Chamness et al, 1974; Buller et al, 1975). The main disagreement is on whether receptor binding to purified nuclei is a saturable process (Higgens et al, 1973b; André & Rochefort, 1975) although Yamamoto & Alberts (1974 ; 1975) claim that specific sites could be masked by the vast excess of low affinity binding to DNA.

Reports have failed to agree on whether the acceptor sites are

distributed throughout the chromatin (Spelsberg et al, 1975) or concentrated in the 'inactive' (heavy) (Sala - Trepat et al 1977; Socher et al, 1976) or 'active' (light) (Stratling and O'Malley, 1976; Hemminiki & Vauhkonen, 1977) fraction of the chromatin. The response to hormone stimulation (Frenster et al, 1963; Webster et al, 1976) has traditionally been associated with the light chromatin. Fractionation of chromatin using nuclease dissection led to the suggestion that acceptor sites are well distributed throughout chromatin (Massol et al, 1978; Senior & Frankel, 1978). Davies et al (1980) found that the transcriptionally inactive portion of chromatin contained 2 - 3 times as many acceptor sites although these sites had a lower affinity for hormone receptor complex than those in the transcriptionally active fraction.

At some stage each component of the nucleus has been proposed as the acceptor due to its isolation in association with the steroid/receptor complex, e.g. ribonucleoproteins (Liao et al, 1973; Laing & Liao, 1974) the nuclear envelope (Jackson & Chalkley, 1974 a ; b; Smith & Von Holt, 1981), nuclear matrix (Barrack & Coffey, 1980) histone proteins (King & Gordon, 1967; Sluyser, 1969) non-histone protein (King & Gordon, 1972; Puca et al, 1974; Mainwairing et al, 1976; Thrall et al, 1978) and DNA (Higgens et al, 1973 a ; Yamamoto & Alberts, 1974 : 1975).

It is likely that DNA is an essential component of a three dimensional acceptor site but not the exclusive determinant of steroid receptor complex binding, since protease treatment releases receptor (King & Gordon, 1972) and the binding of receptor to DNA is of low affinity and unsaturable (Williams, 1974; Buller & O'Malley, 1976). Until

recently no difference in the affinity of the receptor complex for homologous or heterogenous DNA could be found (Yamamoto & Alberts, 1974). But recently, regions of DNA, cloned from hormone responsive genes, have been isolated from the chick oviduct which exhibit a higher affinity for the progesterone receptor than does bulk DNA (Compton et al, 1982; Mulvihill et al, 1982). Similar regions from the mouse mammary tumour virus exhibit a higher affinity for the rat liver glucocorticoid receptor (Payvar et al, 1981).

The second part of the acceptor activity is thought to be contained in the non-histone component of chromatin. A tightly bound protein fraction, extractable in 2M NaCl, has been found to have acceptor activity for the oestrogen receptor complex (Puca et al, 1974 ; 1975) and the androgen receptor (Mainwairing et al, 1976). So far the best characterised is the acceptor for the progesterone receptor (Spelsberg, 1974). The acceptor fraction is tightly bound to DNA and is sensitive to protease but not nuclease. The fraction is confined to chromatin but generally distributed within it, and the acceptor fraction is more active in target tissues than non-target tissue (O'Malley et al, 1972; Chytil, 1975; Pikler et al, 1976; Spelsberg & Toft, 1976). Extraction of histones and some non-histone proteins shows that the acceptor activity is present in all tissues but is masked in non target tissues (Spelsberg, 1974; Spelsberg et al, 1976). However, the techniques of tissue fractionation and reconstitution, used by Spelsberg's group, have been heavily criticised by a number of groups (Zasloff & Felsenfeld, 1976; Fulmer & Fasman, 1979; Stein, 1979).

Spelsberg (1976) and Weichman & Notides (1980) found that there were

three distinct classes of acceptor sites for the oestrogen receptor:-

i) the higher affinity sites (100 sites/cell) with a dissociation constant (Kd) of 10^{-12}M so that these sites would be completely filled at physiological plasma steroid concentration.

ii) intermediate affinity acceptor sites (900 sites/cell) with a Kd of a 5×10^{-10}M in chick oviduct which are probably involved in fine genetic control.

iii) lowest affinity acceptor sites (4000 sites/cell) with a Kd of 1×10^{-8}M, which are partially, but not all filled, at high concentrations of plasma oestradiol and their function is not fully defined.

In order to obtain the full spectrum of physiological response, all the middle class sites and a proportion of the lower affinity sites must be filled. Spelsberg has suggested that the majority of these sites must be filled. Furthermore Thrall et al, (1978) also reported three classes of acceptor sites for the progesterone receptor but which were present in a higher concentration per cell. However both studies report the total number of acceptor sites to be in excess of the 1000 - 2000 sites per cell that Clark & Peck (1976) calculated must be filled in order to get a full physiological response.

1.10 PROBLEMS IN THE TWO STEP MECHANISM

Recently doubts have been expressed on the validity of the two step mechanism. One flaw has been the discovery of unoccupied nuclear receptors in the cell-line MCF-7 and in three other cell lines from pleural effusions (Zava & McGuire, 1977; Horwitz & McGuire, 1978 a & b). The unoccupied nuclear receptor was thought to be responsible for the ability of these cell lines to grow in the absence of oestrogen.

Reports followed of a large number of breast cancers containing empty nuclear receptor (Garola & McGuire, 1977b; Panko & McLeod, 1978; Thorsen, 1979). Panko & McLeod (1978) proposed that the proportion of tumours containing unoccupied nuclear receptor corresponded to the proportion which contained receptor but which failed to respond to endocrine therapy. But, more recently, unoccupied nuclear receptor have been detected in immature rat (Carlson & Gorski, 1980), rat uterus and hypothalamus (Thrower et al, 1981) normal and malignant human endometrium (Levy et al, 1980; Geier et al, 1980) human myometrium (Giannopoulos et al, 1980), chick oviduct (Mester & Baulieu, 1972) and purified uterine nuclei from pigs (Jungblut et al, 1978). This suggested that unoccupied nuclear receptor was a product of the normal mechanism of oestradiol action in its target tissue. Recently Geier et al (1982) have correlated the appearance of unoccupied nuclear receptor with a decrease in cytoplasmic receptor after exposure to oestrogen. There have been many unsuccessful attempts to detect a weakly bound ligand, such as oestriol or oestrone, a metabolite of oestradiol or an adrenal analogue, which could be responsible for translocating the apparently empty receptor to the nucleus.

Estimates of the proportion of nuclear receptor that is uncharged have varied from 75% for MCF-7 (Horwitz & McGuire, 1978b) to 9 - 37% for human endometrium (Geier et al, 1980). One reason for the great variation may be that the method of tissue preparation profoundly affects the number and distribution of empty receptors detected (Clark & Peck, 1979; Martin & Sheridan, 1980).

Another possible reason for the inaccuracies in estimates of unoccupied

nuclear receptor levels may be the assumption, in most reports, that there is no exchange at 4°c, despite evidence against this in the original work by Anderson et al, (1972) and more recently by Blankenstein et al (1979) and Leake (1981b). Recently McGuire's group have claimed that unoccupied nuclear receptors in MCF-7 were an artefact resulting from cytoplasmic receptor adhering to the nucleus, and these receptors could be removed by purification of the nuclei through sucrose (Edwards et al, 1980a). In reply Geier et al (1981) found no change in the level of unoccupied nuclear receptor after purification of MCF-7 nuclei.

Much of the work in detecting unoccupied nuclear receptor has involved, as a first step, salt extraction of the receptor. There is still disagreement on whether the biologically functional nuclear receptor estimated at 20% of the total nuclear receptor, is contained in the 60% - 70% (Clark & Peck, 1979) which can be salt extracted. Many investigators have shown the presence of salt insoluble or resistant nuclear receptor in a number of systems, such as oestrogen receptor in rat uterus (Puca & Bresciani, 1968; Mester & Baulieu, 1975), chick liver (Lebeau et al, 1973) and human endometrium (Katzenellenbogen et al, 1980) and the androgen receptor from rat prostrate (Hiremath et al, 1981; Mulder et al, 1981). The 4S or cytoplasmic receptor has been detected in the nucleus (Linkie & Siiteri, 1978; Bailly et al, 1980). This disagrees with the proposal that activation and transformation to 5S are pre-requisites of receptor entry into the nucleus. This problem is complicated by an incomplete understanding of the mechanism of activation and transformation (Bailly et al, 1980; Gschwendt & Kittstein, 1980).

Translocation may only be the passive movement of the receptor to the nucleus driven by the greater free water concentration of the nucleus

(Horwitz & Moore, 1974). Sheridan et al (1979 ; 1981) maintain that the distribution of receptor as proposed in the two step mechanism is a result of the methods used to prepare the sub-cellular fractions. Sheridan proposes that receptors are in equilibrium between the cytoplasm and the nucleus and appear only in the soluble or cytoplasmic portion due to the large volume of buffer, relative to tissue volume, used in both the homogenisation step and the subsequent purification of nuclei. The conclusions of Sheridan et al (1979, 1981) and the resulting new model of steroid action (Martin & Sheridan, 1982) seem convincing despite their use of crude nuclear pellets (subject to cytoplasmic contamination) and of autoradiography (subject to visual and fixation artefacts). However Pietras & Szego (1977 ; 1979) believe that the cytoplasmic receptor in intact cells actively resides in the plasma membrane. This latter view would explain how steroid from the processed nuclear receptor complex can be excreted from the cell without recombining with empty soluble receptor.

1.11 BREAST CANCER THERAPY AND ITS RELATION TO OESTROGEN RECEPTOR STATUS

Breast cancer can be treated by radiotherapy, chemotherapy, immunotherapy and hormone therapy or some combination of the four (U.S. Dept. of Health, 1980). Once a malignant breast tumour has been detected and removed by surgery, with or without radiotherapy, one of the main problems is the selection of the treatment most suitable to prevent or control secondary disease.

The first properly documented use of hormone therapy was in 1896 by George Beatson when he induced regression of inoperable breast tumours

as a result of oopherectomy in some pre-menopausal women. In the intervening period it has been confirmed that only 25 - 30% of patients with advanced breast cancer experienced objective remission as a result of hormone therapy. Folca et al, (1961) began to provide some explanation, when they administered labelled hexoestrol to breast cancer patients about to undergo adrenalectomy and found a response only in patients whose tumours concentrated large amounts of label.

The first studies on cytoplasmic oestrogen receptors in breast tumours reported that 80% of oestrogen receptor positive (ER+) patients and 10% of those without receptor (ER-), responded to endocrine therapy (Jensen et al, 1975; Engelman et al, 1975; McGuire et al, 1975). A more realistic figure for the response rate of ER+ patients seems to be 40 - 60%. (Byar et al, 1979; Edwards et al, 1979; Hawkins et al, 1980). Even this figure is much more accurate than predictions based on clinical data (such as menopausal status, age, parity, disease free interval and sites of known secondaries) (Stoll, 1977).

The successful response of some receptor negative patients to hormone therapy may be explained either as (A) physiologically ER+ tissue in which the receptor was destroyed during 1) post-operative handling or 2) pre-operative therapy or (B) a response mediated by an indirect pathway eg. tamoxifen normally classified as an antioestrogen can also act by inhibition of prostaglandin synthetase (Tisidale, 1977) or (C) an assay error arising if the biopsy was atypical of the whole tumour and contained no, or a very low number of, tumour cells relative to fat and connective tissue.

The existence of ER+ patients that do not respond may be due to the detection of non-functioning oestrogen receptor. The receptor may fail to function because it cannot translocate to the nucleus or because it cannot bind to the appropriate acceptor sites. The number of ER+ patients that do not respond decreases when the nuclear receptor content is also assayed as an indication of which tissues contain 'functional receptor'. This additional assay detects where the receptor translocation mechanism is defective (Laing et al, 1977).

It has been reported that assaying for a product of oestrogen action also improves the response rate because it should indicate that the receptor system is able to induce a genetic response. Products that have been used in this way are casein mRNA (Rosen & Socher, 1977), α lactalbumin (Woods et al, 1979; Hall et al, 1979), peroxidase (De Sombre et al, 1978; Cantwell et al, 1980; Duffy & O'Connell, 1981), and the progesterone receptor (McGuire et al, 1976; Asselin & Labrie, 1978). The latter has become the most popular marker but synthesis of the progesterone receptor is not a complete guarantee of hormone dependence. Rat mammary tumours have been reported in which oestrogen and progesterone receptors are present but which are hormone independent. (McGuire, 1978; Allegra et al, 1979). Barnes et al (1979) have reported breast tumours which contain progesterone receptor but no oestrogen receptor. While oestriol has been shown to induce progesterone receptor but not DNA synthesis (Clark & Peck, 1979), or subsequent growth responses.

Westley & Rochefort (1979) have suggested that three intracellular proteins resulting from oestrogen stimulation, which are inhibited by tamoxifen, may prove to be better markers.

A tumour may contain a mixed population of ER+ and ER- cells. In this case response to endocrine therapy would be short term, until only the hormone independent cells are left (Sluyser & Evers, 1975). There is some evidence that tumours de-differentiate as they age since hormone dependent tumours which are serially transferred to immuno-suppressed rats eventually become autonomous. Human breast tumours which initially responded to endocrine therapy but eventually progressed to an apparently unresponsive state have been reported (Stoll, 1977; Leake et al, 1981a). It is not known whether, at the time of death, all metastases have become receptor negative (and so hormone indpendent) or if they retain receptor but no longer respond to endocrine therapy.

It is now generally accepted that hormone status of a tumour may be of prognostic value since, after stratification according to the extent of nodal involvement, patients with receptor positive disease have, on average, both a greater disease free interval before relapse and a longer total survival time than receptor negative patients (Hawkins et al, 1980; Leake, 1981a). This indicates that receptor positive tumours are generally slower growing. One reason for this may be that receptor negative tumours are less well differentiated and hence more aggressive. Early attempts at relating receptor status to histology showed no correlation (Feherty et al, 1971; Leclerq et al, 1973) though more recent, and possibly more careful, studies have found well differentiated tumours more likely to be receptor positive (McCarty et al, 1980; Parl & Wagner, 1980). Use of elastosis content and other histological parameters may be of value, but only when all tumours are classified by a single pathologist (Mills, 1980; Rolland et al, 1980).

1.12 OBJECTIVES

The object of this work is to study and compare the two standard methods used to measure nuclear oestrogen receptors. The conditions which influence the binding of œstrogens to the nuclear receptor will be examined in detail in an effort to explain some of the anomalies present in the literature about nuclear receptor stability and function. In particular, the exchange rate of the nuclear receptor at low temperature will be thoroughly assessed since it is on this method that the presence of the controversial unoccupied nuclear oestrogen receptor is dependent. In addition the practice of salt extracting nuclear receptor prior to its study will be evaluated, in view of recent criticisms that either the extracted fraction represents a non-functional complex, or that only a variable and inadequate proportion of the nuclear receptor is recovered by this technique.

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 FINE CHEMICALS were obtained as follows:-

Bovine serum albumin (fraction V)	Sigma, London
Deoxyribonucleic acid (DNA) (calf thymus type V sodium salt, highly polymerized)	Sigma, London
Monothioglycerol	Sigma, London
Norit A (activated charcoal)	Sigma, London
Phenyl methyl sulphonyl fluoride (PMSF)	Sigma, London
DTT (dithiothreitol)	Boehringer Corporation (London) Ltd.
N-2-hydroxypiperazine -N'-2-ethane sulphonic acid (HEPES)	Boehringer Corporation (London) Ltd.
Triton X-100 (purified)	Koch-Light Laboratories, Colnbrook
Trasylol (Aprotinin in isotonic solution containing 0.9% benzyl alcohol)	Bayer, Germany
Hydroxylapatite - spheroidal (HAP)	BDH, Dorset

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

2.1.2 STEROIDS

(6,7-(^{3}H)) Oestradiol-17β (40-80 Ci mmol^{-1}) and (2,4,6,7-(^{3}H)) Oestradiol-17β (100-120 Ci mmol^{-1}) were obtained from Amersham International, England and tested for purity on an LH20 column. Unlabelled Oestradiol-17β and Diethylstiboestrol (DES) were obtained from Sigma, London.

2.1.3 SCINTILLATION MATERIALS

Toluene (AR grade), 2,5 diphenyloxazole (PPO) and 1,4 di (2-(5-phenyloxazolyl))-benzene (POPOP) were obtained from Koch-Light Laboratories, Colnbrook, England. Triton X-100 was obtained from Rohm and Haas, Croydon, England and purified with silica gel prior to use.

2.1.4 HUMAN TISSUE

Breast tumour tissue and human endometrium were kindly supplied by Hospitals of the Greater Glasgow and Ayrshire Health Boards principally Stobhill General Hospital, Western Infirmary, Gartnavel General Hospital, Hairmyres Hospital, East Kilbride and Ballochmyle Hospital, Mauchline, Ayrshire.

2.1.5 MISCELLANEOUS

Sterile plastic pots used for human tissue collection were obtained from Sterilin Ltd., Teddington, England.

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

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

Glassware was washed and rinsed in glass distilled water as the assay of receptor was found to be adversely affected by the presence of divalent metal ions (Laing, 1980). All solutions were made up in glass distilled water.

All glassware contaminated with human tissue was soaked overnight in chloros, rinsed and then washed.

2.2 METHODS

2.2.1 TISSUE HANDLING

Tissue was obtained fresh from the operating theatre in an empty sterile container and transported on ice to the laboratory. When tissue was not used fresh it was stored, up to 4 weeks, at -20°c in sucrose-glycerol buffer (0.25M sucrose, 1.5mM $MgCl_2$, 10mM HEPES (pH 7.4) 50% (v/v) glycerol). Before use the tissue was rehydrated in 0.25M sucrose, 1.5mM $MgCl_2$, 10mM HEPES (pH 7.4) at 0°c for 15 minutes. The oestrogen receptor remains stable in both concentration and molecular form for at least three months under these conditions (Hyder & Leake, 1982).

Only breast tumour tissue which had been shown to contain oestrogen receptor in both the cytoplasm and nucleus was used. Endometrial samples were taken from women undergoing uterine curettage or hysterectomy for benign gynaecological conditions. When necessary, two to five endometrial biopsies were pooled for each experiment. A portion of tissue, adjacent to that used for experiments, was sent for a pathology report.

2.2.2 ASSAY OF OESTROGEN RECEPTOR

The oestrogen receptor content of the cytosol and nuclear fractions was assayed by the method of Laing et al, (1977). Briefly, a competition assay was used involving 7 concentrations of (^{3}H)-oestradiol ± 100 fold diethylstilboestrol, as a competitor. Approximately 150mg of tissue was homogenised by Kontes-Dual1 glass/glass grinder at 50mg/ml in sucrose buffer containing 0.25M dithiothreitol. The homogenate was then separated into soluble and nuclear fractions by centrifuging at 5000g for 5 minutes at 4°c. The crude nuclear pellet was washed three times with buffered saline. Both fractions were then incubated with 7 concentrations of (^{3}H)-oestradiol (between 10^{-10}M - 10^{-9}M) ± 100 fold diethylstiboestrol for either 18 hours at 4°c or 2 hours at 20°c. After incubation, unbound steroid was removed from the cytosol with activated charcoal (0.005%(w/v)) and from the nuclear pellet by trapping on a millipore filter and repeated washing with saline (total volume 15ml). Receptor affinity and concentration were calculated by Scatchard analysis using the established criteria. Briefly, a tissue was defined as receptor positive if three criteria were fulfilled, if 1) it yielded an unambiguous Scatchard plot, producing a straight line, 2) it yielded a Kd in the range 0.5 - 5.0 x 10^{-10} mol, 3) specificity was established by competition with excess DES (Cowan & Leake, 1982). Cells from pleural effusions and from tissue culture were assayed in a similar manner except that cells were disrupted by 2 short (5 sec.) bursts with a Dawie Soniprobe at the lowest power setting.

2.2.3 PROTEIN DETERMINATION

The protein content of each cytosol preparation was determined by the

method of Lowry et al, (1951).

2.2.4 DNA DETERMINATION

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

2.2.5a RATE OF DISSOCIATION OF (^{3}H) - OESTROGEN FROM THE NUCLEAR COMPLEX

Approximately 500 mg of tissue was homogenised by Kontes-Duall glass/glass grinder at 50mg/ml in sucrose buffer (0.25M sucrose, 1.5mM $MgCl_2$, 10mM HEPES (pH 7.4)) and 0.25M dithiothreitol. The homogenate was aliquoted and incubated for 30 min. at 37^{o}c in 2 x 10^{-9}M (^{3}H)-oestradiol in the presence or absence of 2 x 10^{-7}M unlabelled diethylstilboestrol (DES). If required, a wash in 0.1% triton X-100 was included at this stage to remove any contaminating blood.

The homogenate was centrifuged at 5000g for 5 minutes on a Beckman J2-21 centrifuge at 4^{o}c. The crude nuclear pellet was then washed three times with buffered saline to remove unbound steroid and then resuspended in the original volume of sucrose buffer +10% (v/v) glycerol. A glass/glass grinder was then used to ensure a homogeneous nuclear suspension. At various time points, throughout the experiments, nuclear integrity was checked by phase contrast microscopy on an Olympus IM phase contrast microscope.

The nuclei were incubated for 24 hours in 2 x 10^{-7}M unlabelled oestradiol. Duplicate (100 µl) aliquots were removed at various time points.

At 24 hours a 2ml aliquot was removed from each flask, to determine the amount of receptor remaining intact.

At each time point the duplicate 100 µl aliquots were added to 5ml 0.15M NaCl. The sample was then poured down the chimney of a millipore filter apparatus onto a pre-wetted glass fibre filter. The chimney was washed with 20ml saline, under suction. The filter was then removed to a plastic scintillation vial prior to drying overnight at 60^{o}c. 10ml toluene/PPO (5g/litre) scintillant was added and the samples counted at 30% efficiency in a Searle Mark III Liquid Scintillation analyser.

2.2.5b ESTIMATION OF RECEPTOR REMAINING AFTER EXCHANGE ASSAY

The concentration of nuclear oestradiol receptor which remained at the end of an exchange assay was measured in order to demonstrate that the decrease in specifically bound oestradiol was due to exchange of bound hormone and not degradation of the hormone-receptor complex by protease or nuclease action. At 24 hours a 2ml aliquot was removed from each flask, centrifuged at 5000g for 5 min. at 4^{o}c, washed three times and resuspended in sucrose buffer +10%(v/v) glycerol. The nuclei were then incubated in 2×10^{-9}M (^{3}H) oestradiol with or without 2×10^{-7}M unlabelled DES for 2 hours at 20^{o}c. Two 100 µl aliquots were removed and bound oestradiol determined as described above. DNA assays were performed on 1ml aliquots removed at 24 and 26 hours. The concentration of nuclear receptor at the start and completion of the experiment was calculated and compared.

2.2.6 PURIFICATION OF NUCLEI

After labelling and washing the 5000g pellet was resuspended in 1ml of sucrose buffer and layered over 12.5ml of 2.3M sucrose containing 1.5mM $MgCl_2$ and 10mM HEPES at pH 7.4. Tubes were then centrifuged in an SW-40 rotor at 105,000g for 1 hour at 4°c. The pellet was resuspended in the original volume of sucrose buffer containing 10%(v/v) glycerol. Nuclear integrity was checked by phase contrast microscopy before and after the 24 hour incubation.

2.2.7 SALT EXTRACTION OF NUCLEAR RECEPTOR

The labelled and washed 5000g nuclear pellet was resuspended in 6 volumes of sucrose buffer made 0.6M in KCl and 0.25mM dithiothreitol. The nuclear oestrogen was extracted for 60 minutes at 4°c, with vortexing every 15 minutes (Garola & McGuire, 1977b). The supernatant nuclear extract was obtained after centrifugation at 105,000g for 30 minutes at 4°c. Pellets were resuspended in sucrose buffer and salt resistant receptor measured by trapping nuclei on millipore filters (as described above). Crude extraction of radioactivity with ethanol yielded the same mean values of specifically-bound oestrogen, or salt resistant receptor but were subject to a much higher non-specific background.

2.2.8 HYDROXYLAPPATITE ASSAY OF NUCLEAR OESTROGEN RECEPTORS

Oestrogen receptors were precipitated from the nuclear extract by hydroxylappatite (HAP) as described by Garola & McGuire (1977b) and

Clark & Peck, (1979) except that HEPES buffer and dithiothreitol replaced TRIS buffer and monothioglycerol. The nuclear extract was diluted with phosphate buffer to produce a final protein concentration between 0.4 and 1.0 mg/ml and aliquoted into 200 µl units. A HAP slurry was prepared with a packed/liquid volume ratio of approximately 0.7 as described by Williams & Gorski, (1974). 250 µl of HAP slurry was added to the nuclear extract. The mixture was incubated on ice for 30 minutes and vortexed every 10 minutes to allow the receptor to absorp onto the HAP. The mixture was then centrifuged for 5 minutes at 800g and the supernatant discarded.

2.2.9 RATE OF DISSOCATION OF (^{3}H) OESTRADIOL FROM SALT EXTRACTED RECEPTOR

The HAP bound nuclear receptor was then resuspended and incubated in sucrose buffer +10%(v/v) glycerol plus 2 x 10^{-7}M oestradiol for varying times up to 24 hours. At each time point, three samples were washed twice with 0.1M phosphate buffer pH 7.4 and then extracted overnight in 2ml ethanol.

2.2.10 CHARCOAL TREATMENT OF RADIOACTIVE OESTRADIOL RELEASED FROM HYDROXYLAPPATITE

After incubation the HAP bound nuclear receptor was removed by pelleting HAP by centrifuging at 800g for 5 minutes. In order to determine whether the steroid was bound to receptor or free in solution, the supernatant was treated with charcoal.

500 µl 0.5% dextran coated charcoal solution (DCC), 0.5% w/v Norit A

charcoal, 0.005% w/v dextran T70 in 0.25M sucrose, 1.5mM EDTA, 10mM HEPES (pH 7.4) is added to 1ml of supernatant and the tubes mixed. The charcoal stripping was continued for 15 minutes at 0^{o}c with periodic mixing of the tubes. After the incubation, the charcoal was pelleted by centrifugation at 1000g for 5 minutes. 750 µl was removed to a scintillation vial and counted in triton toluene scintillant. Totals were calculated by counting 200 µl of untreated supernatant.

3. RESULTS

3.1 EXCHANGE RATE OF OESTROGEN FROM THE NUCLEAR RECEPTOR

Fig. 3.1 shows the theoretical basis of the measurement of exchange of oestradiol from the nuclear receptor. The first incubation at $37^{o}c$ fills all the nuclear receptor with labelled oestradiol. The first incubation uses homogenised tissue to maximise labelling of the receptor, use of tissue slices resulted in a failure to label all the receptor (especially in breast tumour tissue) even at $37^{o}c$. In the second incubation the labelled nuclei are incubated in a hundred fold excess of unlabelled oestradiol. Any (^{3}H) oestradiol which dissociates from the nuclear receptor is then replaced by unlabelled oestradiol causing a decrease in specifically bound radioactivity but maintaining the stability of the oestrogen receptor complex. The purpose of the third incubation is to demonstrate, by relabelling the nuclear receptor, that dissociation and not degradation of receptor complex has been responsible for the decrease in specifically bound counts. The calculation of specifically bound oestradiol is illustrated in Fig. 3.2. This involves subtracting the non-specific counts (labelled in the presence of a one hundred fold excess of DES) from the total counts (specific and non-specific binding, labelled by (^{3}H) oestradiol alone).

Fig. 3.3A demonstrates the effect of temperature on the exchange or 'off-rate' of (^{3}H) oestradiol from the nuclear receptor bound to nuclei from human endometrium. There is a significant rate of exchange at $4^{o}c$ ($K_1 = 1.39 \times 10^{-3}\ min^{-1}$) with approximately 50% of the specifically bound oestradiol exchanged during the 24 hour incubation. The rate

Figure 3.1 - Theoretical Basis of Off-Rate Experiments

This shows the theoretical basis of measurement of exchange of oestradiol from the nuclear receptor (Section 2.2.5.A). The first incubation at 37°c fills all the nuclear receptor with labelled oestradiol. In the second incubation the labelled nuclei are incubated in a hundred fold excess of unlabelled oestradiol. Therefore any (^{3}H) oestradiol which dissociates from the nuclear receptor is replaced by unlabelled oestradiol causing a decrease in specifically bound radioactivity. The object of the third incubation, at 20°c in (^{3}H) oestradiol, is to demonstrate that dissociation (⟶) and not degradation (--➤) of the nuclear receptor complex, has been measured.

Theoretical Basis of Off-rate Experiments

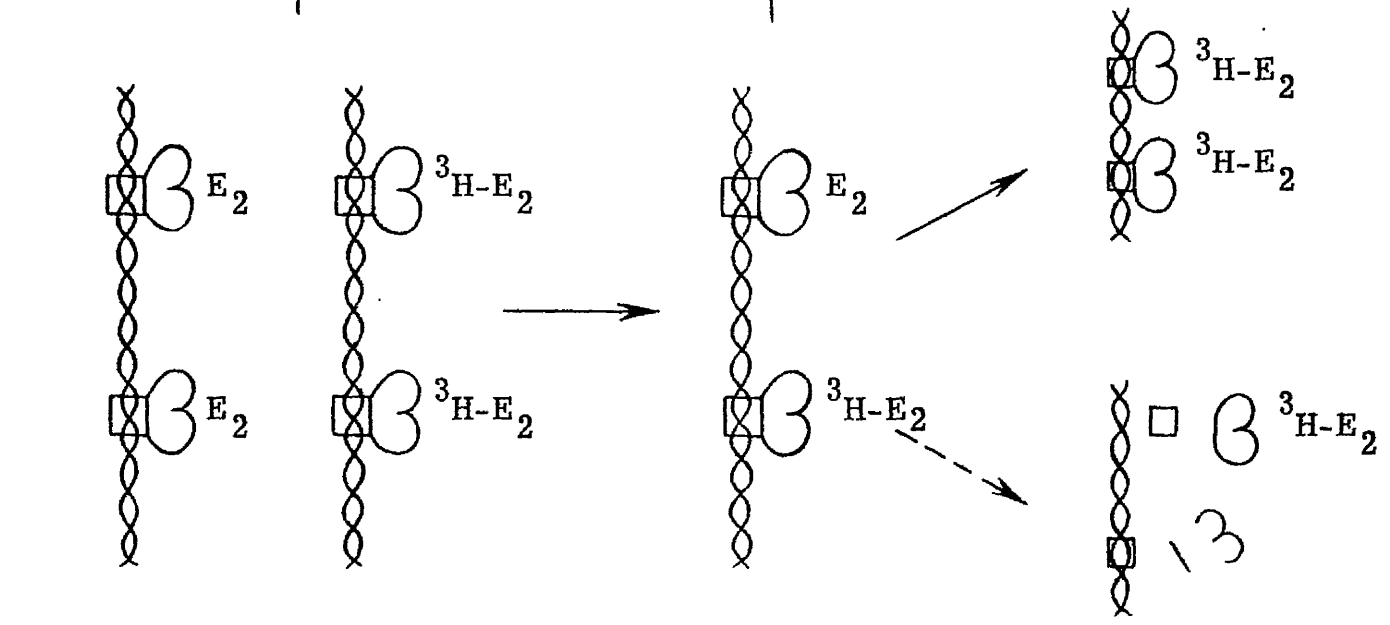


Figure 3.2 - Off-Rate of (^{3}H) Oestradiol from Pre-labelled Nuclear Receptor

Human nuclear oestrogen receptor was loaded with (^{3}H) oestradiol as described in Section 2.2.5.A. The off-rate is measured as a decrease in specifically bound oestradiol. This is calculated by subtracting the non-specific counts ((———) labelled in the presence of a one hundredfold excess of DES as described in Section 2.2.5.A) from total counts ((- · - · -) specific and non-specific binding labelled by oestradiol alone).

Off-rate of ^{3}H-E_2 from Pre-labelled Nuclear Receptor

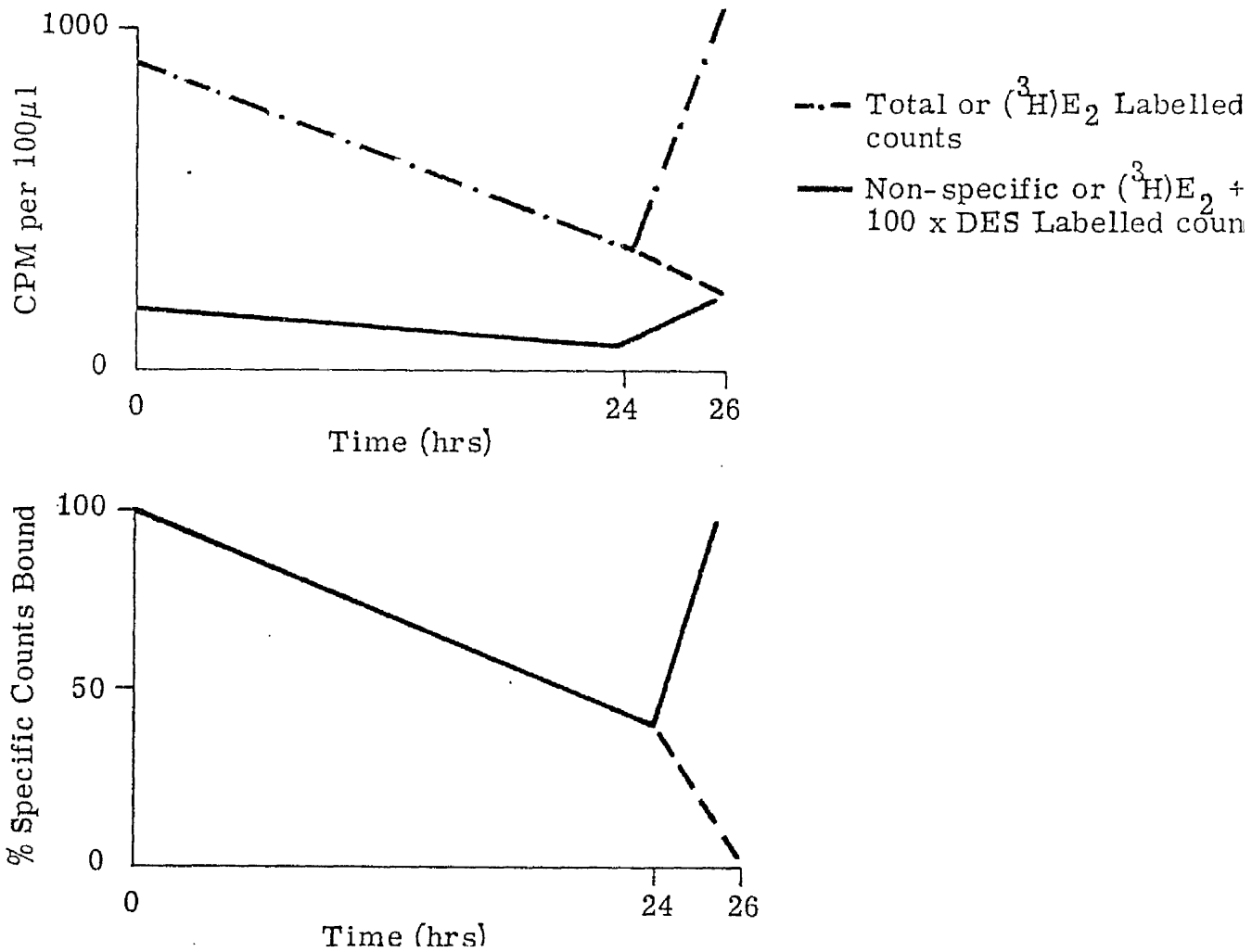
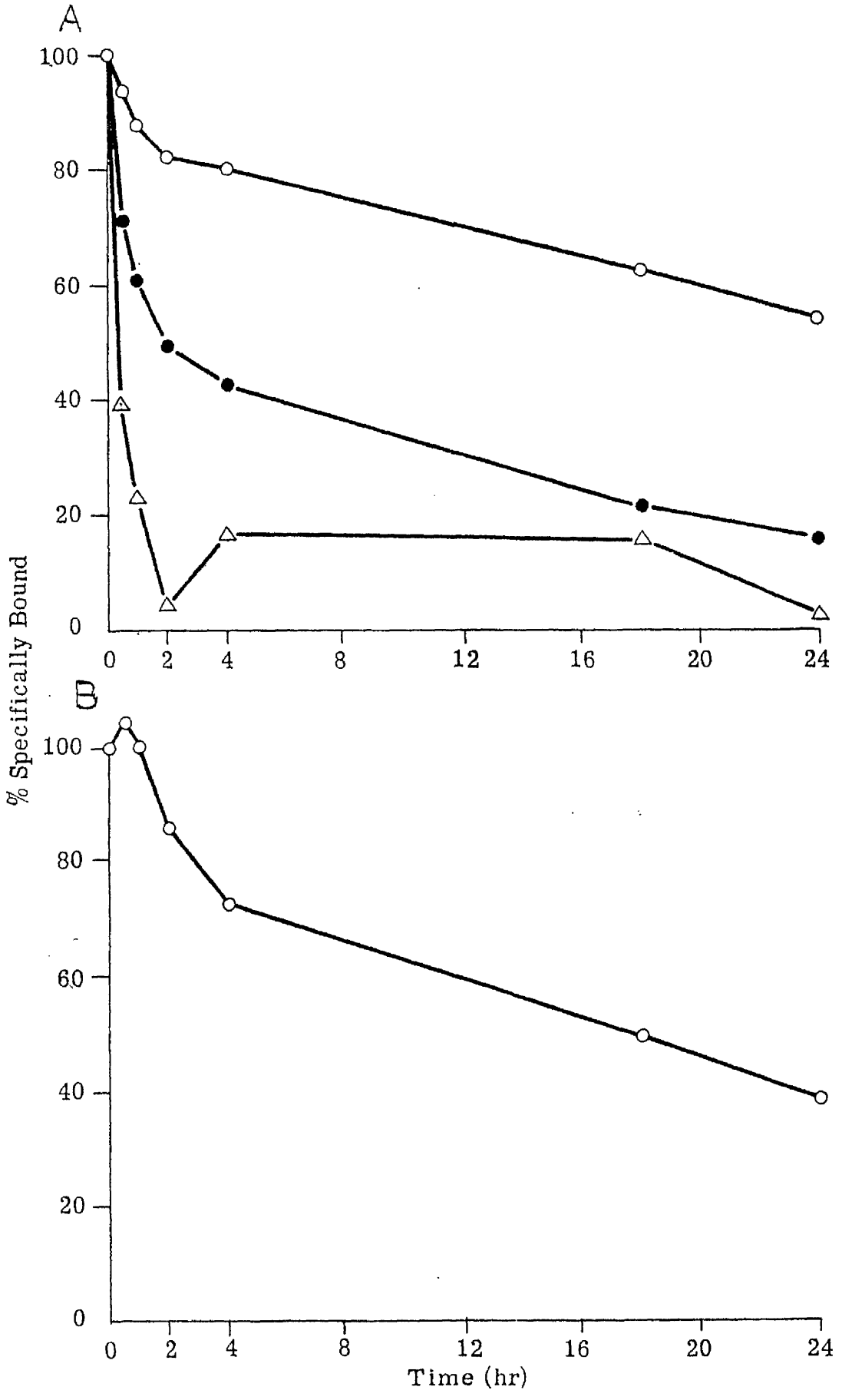


Figure 3.3 - The Effect of Temperature on the Dissociation of Oestradiol from the Nuclear Receptor

Figure 3.3.A Rate of dissociation of specifically-bound oestradiol from the human endometrial nuclear receptor. Nuclei were prepared, prelabelled with 2×10^{-9}M (^{3}H) oestradiol and then incubated in sucrose buffer containing 2×10^{-7}M unlabelled oestradiol as described in Section 2.2.5.A. The effect of temperature on the exchange rate was studied. Incubations were performed at O—O 4°c (30), ●—● 20°c (9), Δ—Δ 37°c (2). Results represent the percentage of specifically bound counts at the time indicated (Number of experiments). Experimental error on each point is ± 5% (within 95% confidence limits).

Figure 3.3.B Rate of dissociation of specifically-bound oestradiol from nuclear receptor from breast tumour tissue at 4°c. O—O (6) Nuclei were prepared and labelled as described in Section 2.2.5.A. Results represent the percentage of specifically bound counts at the time indicated (Number of experiments). Experimental error on each point is ± 5% (within 95% confidence limits).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of exchange was found to increase with increasing temperature, with K_1 values of 8.3×10^{-3} min^{-1} at $20^{o}c$ and 12.8×10^{-3} min^{-1} at $37^{o}c$. Complete exchange occurs within 2 hours at $37^{o}c$. The rate of exchange was found to be identical, whether stored or fresh tissue was used. This was true at all three temperatures.

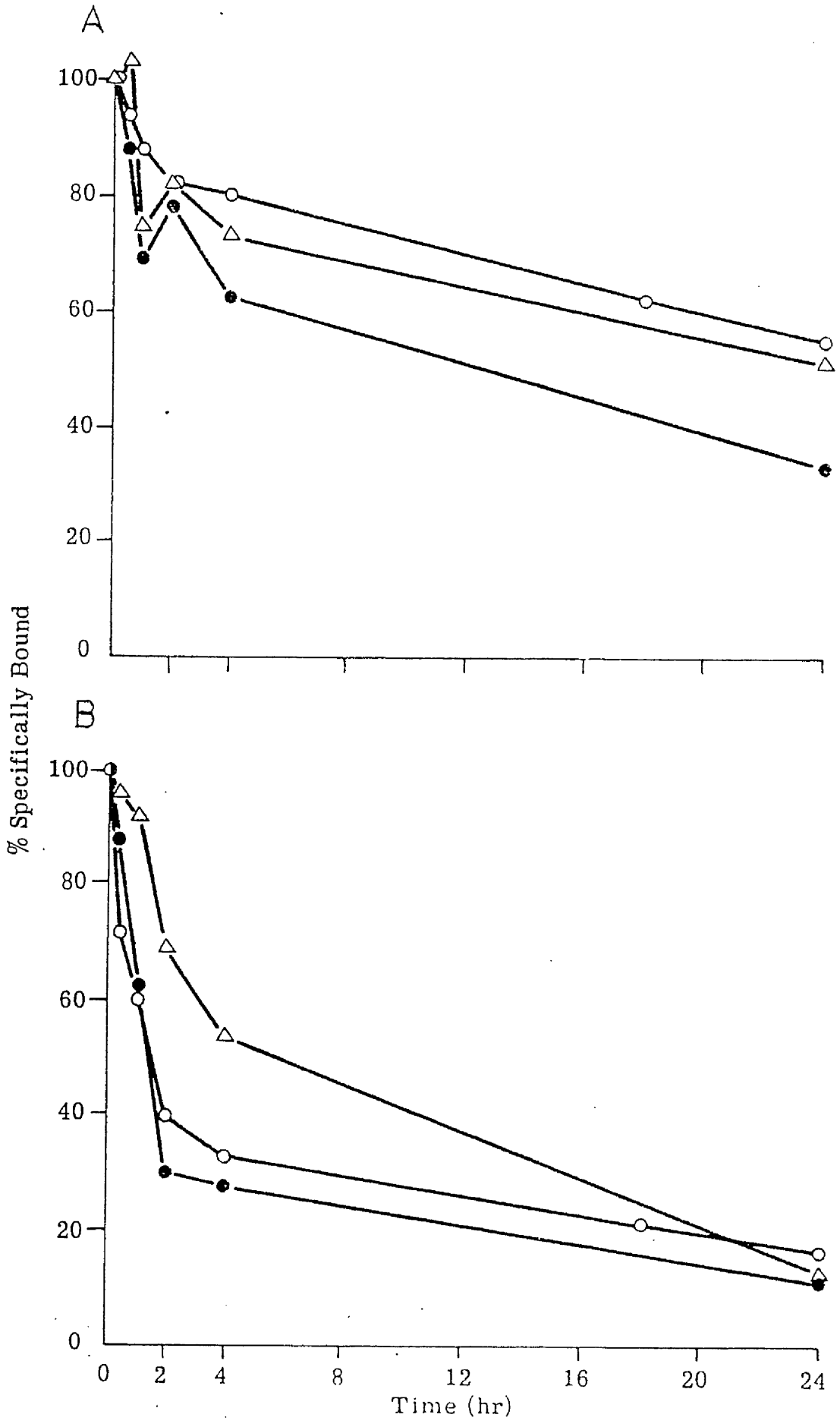
The rate of exchange from breast tumour nuclear receptor at $4^{o}c$ was found to be similar to that in human endometrium. However, despite a slightly lower initial rate of exchange ($K_1 = 1.18 \times 10^{-3}$ min^{-1}) there is a greater decrease in specifically bound oestradiol over the 24 hour incubation. This may result from degradation of the receptor by a protease present in malignant but not in normal tissue. Total recovery of receptor at 26 hours was approximately 87% for endometrium and approximately 76% for breast tumour.

In order to show that the decrease in specifically bound counts was not due to protease degradation the incubations were performed in the presence of the protease inhibitors trasylol or PMSF. At $4^{o}c$ neither of the protease inhibitors had an effect on the exchange rate, suggesting that the decrease in specifically bound oestradiol is not due to protease degradation of the receptor (Fig. 3.4A). However, at $20^{o}c$ PMSF, but not trasylol, markedly reduced the initial exchange rate (Fig. 3.4B). After 24 hours the level of specifically bound label was similar in both the control and PMSF treated nuclei. This result can be explained by the fact that PMSF is an irreversible serine protease inhibitor which is rapidly inactivated if it does not react with a protease. Therefore PMSF would inactivate protease molecules present at the beginning of the incubation. This leads to the

Figure 3.4 - Effect of Protease Inhibitors on the Dissociation of (^{3}H) Oestradiol from the Nuclear Receptor.

Figure 3.4.A Effect of protease inhibitors on the off-rate of (^{3}H) oestradiol from the nuclear receptor at 4°c. Nuclei from human endometrial tissue were labelled and incubated as described in Section 2.2.5.A. Results represent the percentage of specifically bound counts retained at the time indicated. Experimental error on each point is ± 5% (within 95% confidence limits). Incubation medium contained no additions (O——O) 2mM PMSF (Δ——Δ) (3) or 2000 units/ml Trasylol (●——●) (3) (number of experiments).

Figure 3.4.B Effect of protease inhibitors on the dissociation of (^{3}H) oestradiol from the nuclear receptor at 20°c. Symbols as described in Fig. 3.4.A above.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conclusion that part of the decrease in specifically bound counts at $20^{o}c$ is due to protease degradation of the nuclear receptor complex whereas only exchange is responsible for the decrease in specifically bound counts at $4^{o}c$.

Further experiments were performed to determine whether the hormone receptor remained intact throughout the course of the incubations. These experiments involved relabelling the nuclei by a third incubation for 2 hours at $20^{o}c$ in the presence of labelled oestradiol. Similar results could be achieved by incubating at a lower temperature for a longer period.

Provided no degradation of the hormone receptor has occurred then the level of specifically bound oestradiol at time 0 (the beginning of the second incubation) should equal the level at 26 hours (at the end of the third incubation). Table 3.1 shows that after the incubation at $4^{o}c$ for 24 hours almost all the nuclear receptor from human endometrium can be relabelled, indicating that only exchange of hormone has been measured and not protease degradation. However, at higher temperatures a large proportion of the nuclear receptor is not relabelled and so has been lost during the incubation. This shows that a considerable portion of the decrease in specifically bound counts at higher temperatures ($20^{o}c$ and $37^{o}c$) is due to degradation of the nuclear receptor.

At each time point during the incubations, samples were removed and studied by phase contrast microscopy. A large proportion of the nuclei were found to have lysed during the incubation, such that less than

Table 3.1 - Re-labelling of Nuclear Receptor After Exchange Assay

Figures represent specifically bound (^{3}H) oestradiol expressed as CPM/µg DNA, mean ± standard deviation (number of experiments), as indicated in Column 1. Nuclei from human endometrial tissue were labelled and incubated as described in Section 2.2.5.A. After 24 hours aliquots were re-incubated with 2×10^{-9}M (^{3}H) oestradiol for 2 hours as described in Section 2.2.5.B, and the amount of specifically bound steroid was determined and expressed relative to the DNA content, measured as described in Section 2.2.4.

Temp. °C. (No. of Exp.)	Time (hr)		
	0	24	26
4° (39)	18.8 ± 10.7	10.6 ± 7.0	16.2 ± 8.1
20° (14)	20.8 ± 13.3	2.48 ± 2.5	6.0 ± 4.6
37° (2)	14.2 ± 2.9	1.7 ± 1.0	1.0 ± 0.8

10% remained intact at 26 hours. This problem was overcome by centrifuging the nuclei through 2.4M sucrose. After purification, over 90% of the nuclei remained intact at 26 hours. Although the extent of exchange occurring over 24 hours was identical, the initial rates of exchange in purified nuclei showed some differences (Figs. 3.5 A & B). The initial rate of exchange from purified nuclei at 4^{o}c may be slightly greater because purification has reduced the amount of receptor leaking from damaged nuclei (since soluble receptor complex is less readily exchanged). The reduction in the initial exchange rate at 20^{o}c may be due to removal of contaminating cytoplasmic proteases on purification.

The exchange rate of oestradiol from the nuclear receptor can also be studied by an 'on-rate' measurement. Measurement of the 'on-rate' was carried out in a similar fashion to 'off-rate' measurements except that the receptor is first filled with unlabelled oestradiol and then incubated with an excess of tritiated oestradiol. Fig. 3.6 shows the binding of oestradiol to the nuclear receptor at 4^{o}c. This method of measuring the exchange rate also demonstrates that there is a significant exchange at 4^{o}c ($K_1 = 7 \times 10^{-3}$ min^{-1}). However, as shown in the off-rate measurements, prior purification of the nuclei caused a change in the initial rate of exchange ($K_1 = 3 \times 10^{-3}$ min^{-1}) but the extent of exchange occurring in 24 hours remained identical. This decrease in the exchange rate on purification of the nuclei cannot be attributed to removal of contaminating cytoplasmic receptor since this would result in an equivalent reduction in the extent of the exchange at 24 hours.

Figure 3.5 - Effect of Purification of Nuclei on the Exchange Rate of (^{3}H) Oestradiol from the Nuclear Receptor.

Figure 3.5.A Effect of purification of nuclei on the exchange rate of (^{3}H) oestradiol from the nuclear receptor at 4^{o}c. Nuclei from human endometrial tissue were labelled and incubated as described in Section 2.2.5.A. Nuclei were purified as described in Section 2.2.6. Results represent the percentage of specifically-bound counts retained at the time indicated. Experimental error of each point is ± 5% (within 95% confidence limits). Incubations were performed using either nuclei purified through 2.4M sucrose (●—●) (3) or untreated nuclei (○—○) (3) (number of experiments).

Figure 3.5.B Effect of Purification of Nuclei in the Exchange Rate of (^{3}H) Oestradiol from the Nuclear Receptor at 20^{o}c. Symbols as described in Fig. 3.5.A. above.

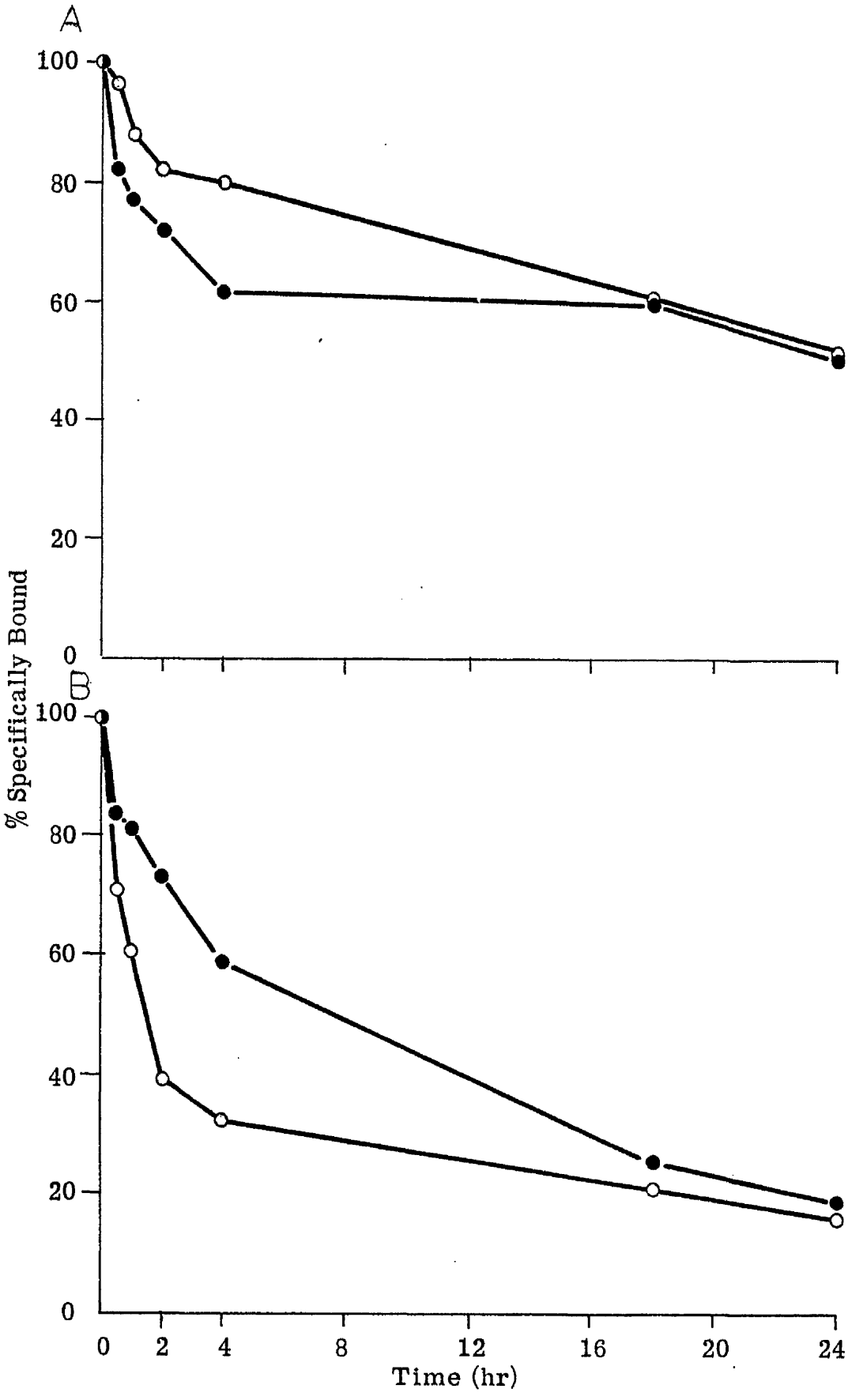
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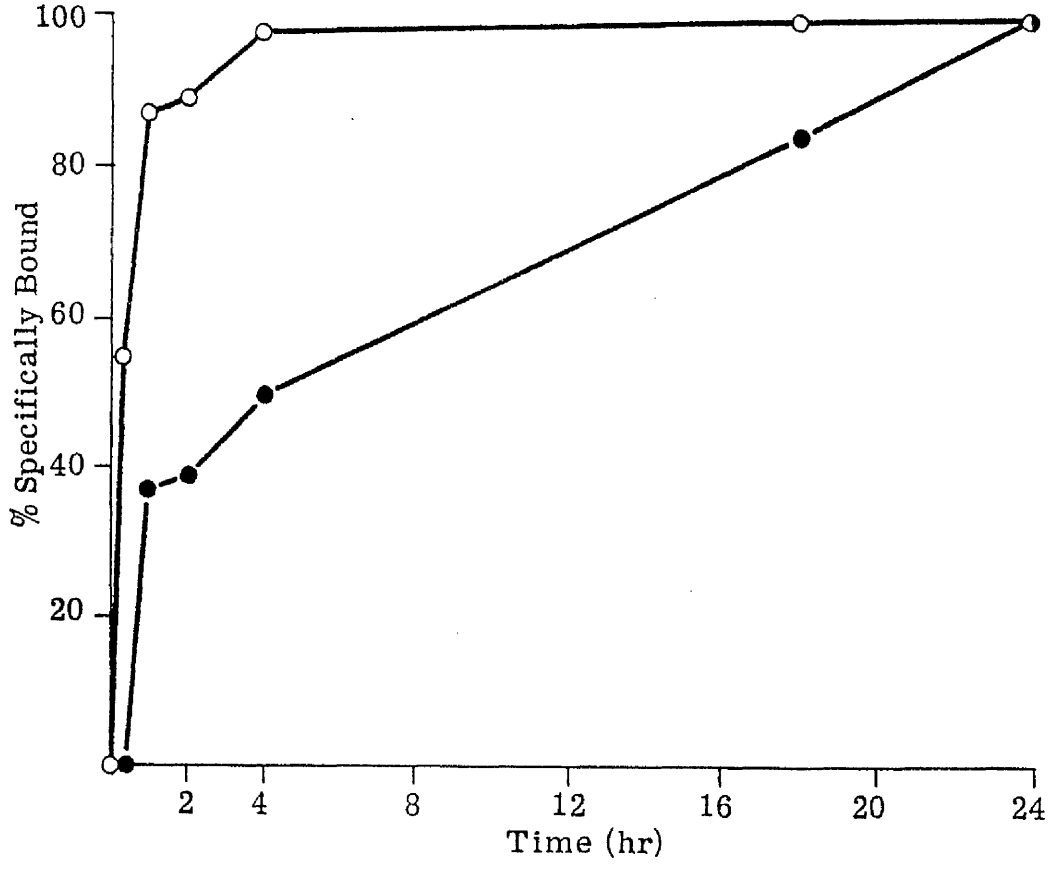
Figure 3.6 - Rate of Binding of (^{3}H) Oestradiol to Nuclear Receptor at 4^{o}c

Nuclei from human endometrial tissue were incubated with unlabelled oestradiol and then labelled with (^{3}H) oestradiol as described in Section 2.2.5.A. Results represent the percentage of specifically-bound counts retained at the time indicated. Experimental error on each point is $\pm$ 5% (within 95% confidence limits). Incubations were performed using either nuclei purified through 2.4M sucrose (●—●) (3) or untreated nuclei (○—○) (5) (Number of experiments).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3.2 SALT EXTRACTION OF NUCLEAR RECEPTOR

In order to determine the extent to which salt extracted nuclear receptor can be taken as typical of total nuclear receptor the percentage of specifically-bound oestrogen that was extracted in 0.6M KCl was determined. Salt resistant nuclear receptor was measured by trapping on millipore filters or by extracting the steroid overnight in ethanol. Crude extraction of radioactivity with ethanol yielded the same mean values of specifically-bound oestrogen in the nucleus as did the filter method, but was subject to a much higher non-specific background. Table 2 shows that 60% of the total nuclear receptor in human endometrium is resistant to salt extraction. Therefore, methods which rely on salt extraction as the first step toward assaying nuclear receptors begin with only a portion of total nuclear receptor.

The major method of detecting salt extracted nuclear receptor is to precipitate it from the salt with hydroxyla patite (HAP) and then exchange endogenously bound oestradiol for tritiated oestradiol. The rate of exchange of labelled oestradiol from the nuclear bound to HAP was measured and the results are shown in Fig. 3.7. At 4^{o}c, there is an extremely low level of exchange of bound hormone from the salt extracted receptor such that over 90% of the oestradiol remains bound after 24 hours. The rate of exchange from salt extracted receptor at 20^{o}c ($K_1 = 2.14 \times 10^{-3}$ min^{-1}) is also lower than the corresponding rate of exchange from receptor bound to intact nuclei ($K_1 = 8.3 \times 10^{-3}$ min^{-1}). This suggests that the salt extracted HAP-bound nuclear receptor complex is much more stable than hormone receptor complex bound to chromatin. A similar rate of exchange was detected in nuclear

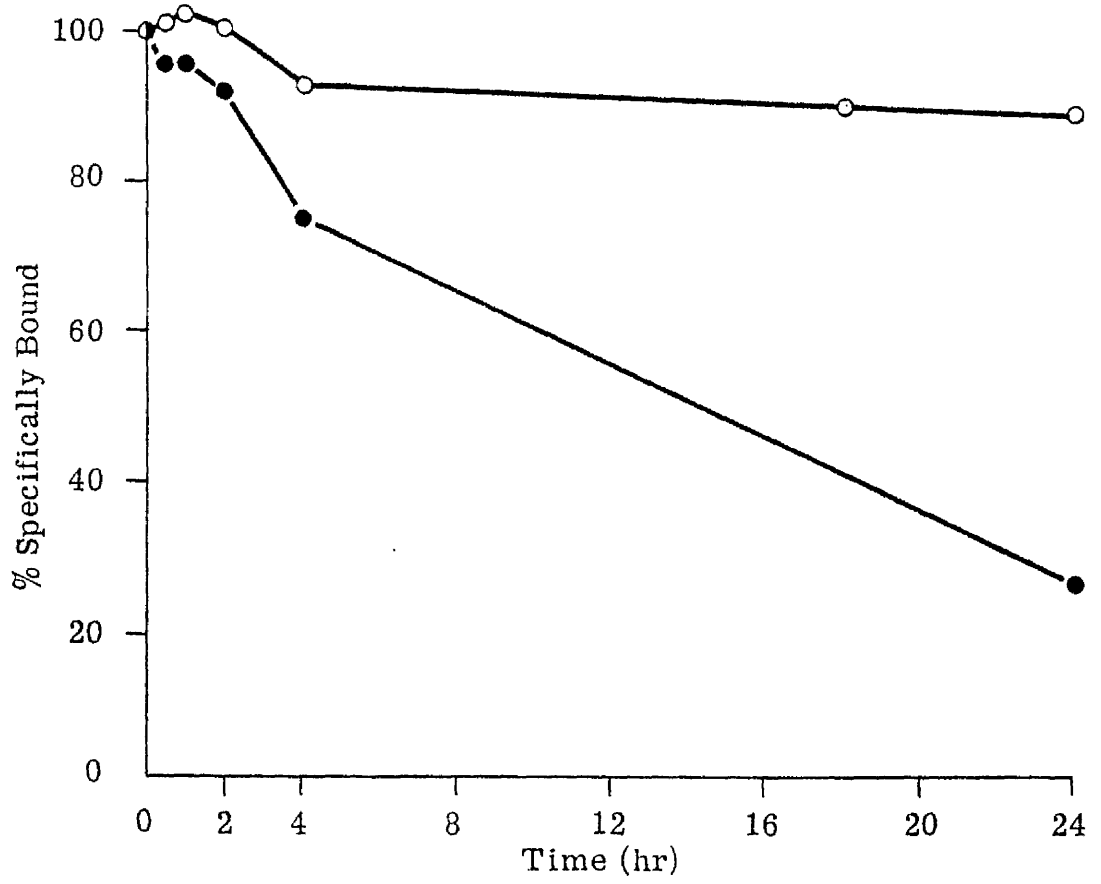
Table 3.2 - Salt Extraction of Nuclear Oestrogen Receptor

Nuclear receptor was extracted from human endometrial nuclei by incubation in 0.6M KCl for 1 hour at 4^{o}c as described in Section 2.2.7. Salt extracted nuclear receptor was measured by precipitation with HAP as described in Section 2.2.8. Salt resistant nuclear receptor was determined either by trapping nuclear receptor on millipore filters or by extracting steroid in ethanol overnight. Crude extraction of radioactivity with ethanol yielded the same mean values of specifically-bound oestradiol which remained bound to the nucleus, but was subject to a much higher non-specific background.

	Solubilised by 0.6M KCl	Salt Resistant	Standard Deviation
Specifically-bound (^{3}H) E_2 CPM/ug DNA	26.5	4.0.4	± 5.6
Percentage of specifically-bound (^{3}H) E_2	39.9	60.1	± 12.3

Figure 3.7 - The Rate of Exchange of (^{3}H) Oestradiol from Salt Extracted Nuclear Receptor at 4^{o}c and 20^{o}c

Endometrial nuclei were prelabelled at 37^{o}c with (^{3}H) oestradiol (Section 2.2.5.A) and the nuclear receptor was extracted with 0.6M KCl (Section 2.2.7). The nuclear receptor was then precipitated with HAP (Section 2.2.8) and incubated as described in Section 2.2.9. Results represent the percentage of specifically bound counts retained at the time indicated at 4^{o}c (O—O) (7) and 20^{o}c (●—●) (3) (number of experiments). Experimental error on each point is $\pm$ 5% (within 95% confidence limits).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receptor salt-extracted from breast tumour tissue.

However, several groups (Lan & Rutledge, 1979; Blankenstein et al, 1980) have reported a high rate of exchange of steroid from salt extracted receptor at low temperature. These groups appear to have measured the exchange rate from the receptor in a buffer containing high salt concentration. Therefore, the effect of ionic strength on the exchange of steroid from receptor complex was studied. Increasing the concentration of potassium chloride was found to increase the rate of dissociation of bound oestradiol from the salt extracted nuclear receptor (Fig. 3.8). A similar but less pronounced effect was obtained using sodium chloride. In order to check that dissociation of hormone from the receptor was responsible for the decrease in specifically bound radioactivity, rather than extraction of the entire hormone receptor complex from the HAP, the proportion of free and bound steroid was measured in the soluble fraction remaining after precipitation of the HAP. After measurement of total radioactivity present in the fraction, free steroid was removed by treatment with charcoal and so present as free steroid. This proves that increasing the salt concentration increases the rate of exchange and does not cause extraction of the receptor complex from the HAP.

The rate of dissociation of hormone from the proportion of nuclear receptor which was resistant to salt extraction was determined. After extraction of salt soluble receptor the nuclei were then incubated in unlabelled oestradiol and the specific binding determined by trapping nuclei on millipore filters. The results are shown in Fig. 3.9 and indicate that the rate of exchange is similar to that measured in

Figure 3.8 - Effect of Salt Concentration on the Exchange Rate of (^{3}H) Oestradiol from Salt Extracted Nuclear Receptor at 4^{o}c

Nuclear receptors were labelled and extracted as described in Sections 2.2.7 to 2.2.9. Results represent the percentage of specifically bound counts retained at the time indicated. Experimental error on each time point is ± 5% (within 95% confidence limits). Incubation medium contained 0.15M Na Cl (O—O) (7), 0.4M KCl (●—●) (2), and 0.6M KCl (Δ—Δ) (3) (number of experiments). At each time point, after removal of the HAP bound nuclear receptor, dextran coated charcoal was used to show that dissociation of oestradiol had been measured, and not extraction of the receptor complex from HAP, i.e. that the supernatant contained free oestradiol (Section 2.2.10).

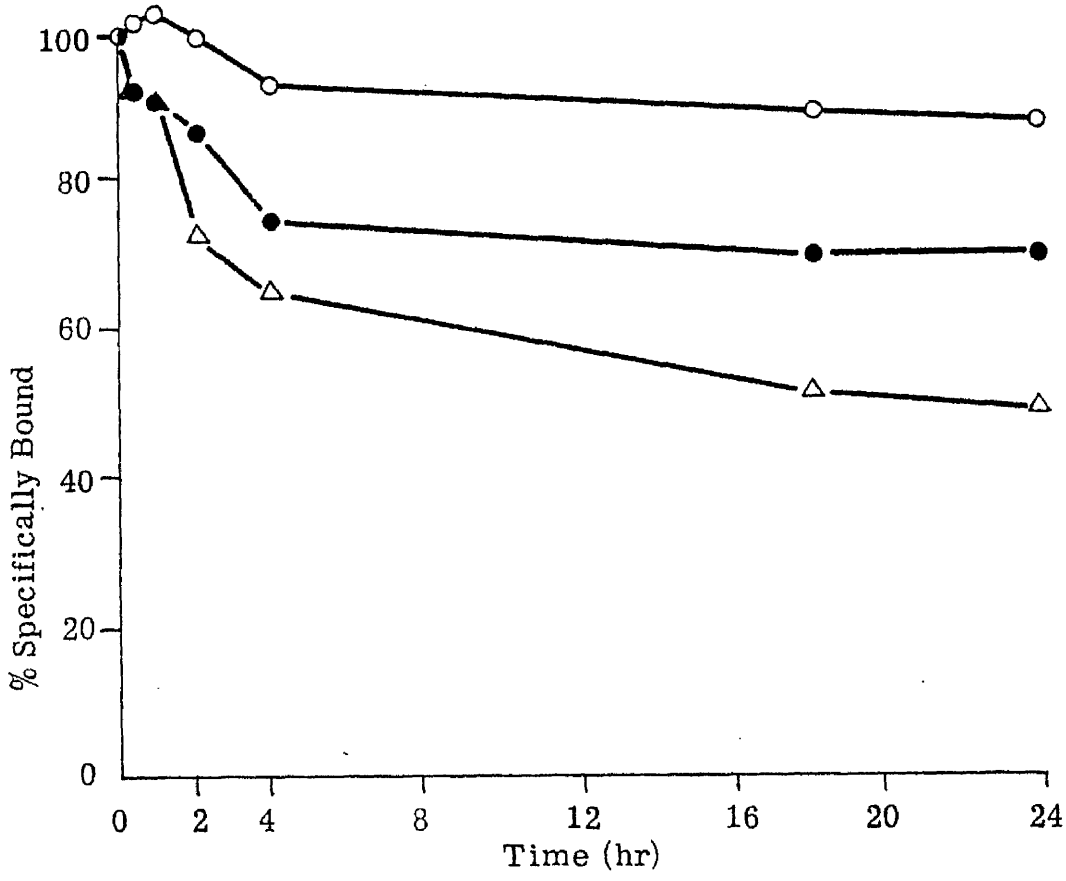
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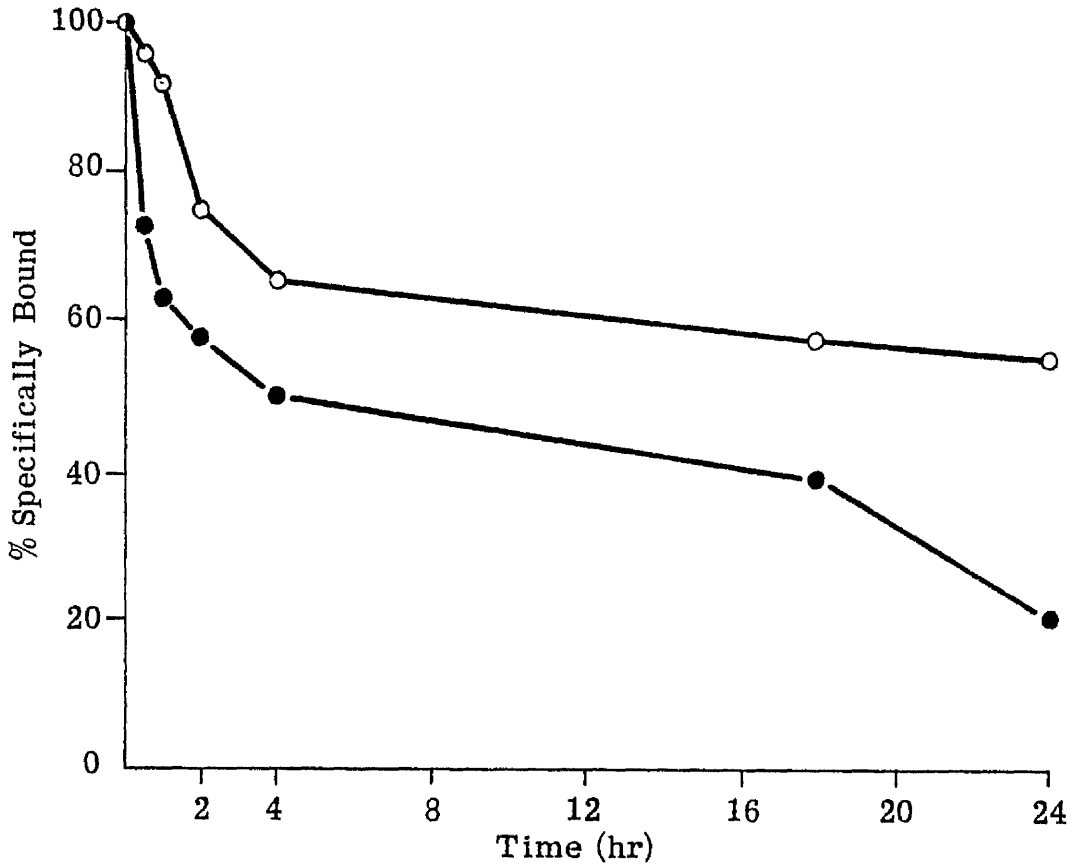
Figure 3.9 - The Rate of Dissociation of (^{3}H) Oestradiol from Nuclear Receptor Resistant to Salt Extraction

Endometrial nuclear receptor was labelled and salt extracted as described in Section 2.2.7 to 2.2.9. Nuclei were resuspended and an exchange assay performed as described in Section 2.2.5.A. The exchange rate of steroid by salt resistant nuclear receptor is shown at 4°c (O—O) (4) and 20°c (●—●) (2) (number of experiments). Results represent the percentage of specifically bound counts retained at the time indicated. Experimental error on each time point is ± 5% (within 95% confidence limits).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unextracted nuclei at either 4^{o}c or 20^{o}c. This is further evidence that exchange rates measured by this method are a property of the native nuclear receptor and that such receptor is not, therefore, contaminating cytoplasmic receptor.

4. DISCUSSION

4.1 EXCHANGE RATE FROM INTACT NUCLEI

The data obtained on the exchange rate of oestradiol from the salt extracted nuclear receptor (Fig. 3.7) confirm the observations of others, (Garola & McGuire, 1977a; Panko & Macleod, 1978; Geier et al, 1980; Levy et al, 1980) that there is no exchange of steroid at low temperature ($\leq 4^{o}c$) under these conditions. However, this result has been taken as evidence (Panko & Macleod, 1978; Thorsen & Stoa, 1979; Chamness & McGuire, 1980) that there is therefore no exchange of oestradiol at low temperature ($\leq 4^{o}c$) under any conditions. In fact, the original paper on exchange of the nuclear oestrogen complex in rat uterus (Anderson et al, 1972) showed that there was significant exchange at $4^{o}c$ within 2 hours. As can be seen in Fig. 3.3, the rate of exchange of oestradiol from both human breast tumour and human endometrial nuclear receptor complex in intact nuclei (as opposed to salt extracted receptor complex) occurs at a significant rate at low temperature. The exchange rate is such that after 24 hours incubation at $4^{o}c$, about half of the oestrogen binding sites (prefilled with unlabelled oestradiol) can be exchanged for labelled oestradiol. A similar exchange has been described for the nuclear glucocorticoid receptor from rat skin (Smith et al,1982).

As would be predicted, the rate of exchange of oestradiol from the receptor increased with increasing temperature (Fig. 3.3 & 3.7).

Although the initial off-rate of steroid from breast nuclear oestrogen

receptor was lower than that from human endometrial nuclear receptor, the level of exchange taking place in 24 hours was higher in breast tissue than in endometrium (Fig. 3.3.A and B). Some of the apparent off-rate could have been due to an increased level of protease action in malignant tissue. Garola & McGuire (1977a) and Hyder & Leake (1982) have reported a high and variable level of protease activity in the nuclear fraction of human breast tumour tissue.

In order to determine whether the decrease in specifically bound oestradiol was due to protease degradation, 1) the exchange rate was measured in the presence of protease inhibitors (Fig. 3.4.A and B) and 2) the nuclear receptor was relabelled by a 2 hour incubation at 20°c (Table 3.1). Both measurements suggest that the level of protease is high in human tissue. However, these proteases seem to be relatively inactive at 4°c since most of the receptor (~ 87%) can be relabelled. The activity of these protease(s) can be very different in different human breast tumours and, in some tumours, may even be significant at 4°c (Weichman & Notides, 1977; Hyder & Leake, 1982). Sherman et al (1981) have suggested that oestrogen receptor levels and molecular forms may be related to the protease levels, in particular to the level of plasminogen activator (known to be elevated in malignant disease). In the future it may be possible to use plasminogen activator levels as an indication of invasive potential and hence as a measure of the aggressiveness of tumour growth.

At higher temperatures there is a high level of degradation of hormone receptor complex, as shown by the failure to relabel all the nuclear

receptor (Table 3.1). The percentage of receptor which is degraded over 24 hours at these temperatures appears very high, but nuclear exchange assays, using elevated temperatures, should use a much shorter incubation time. However, it is probable that at least a portion of the nuclear receptor complex is degraded even during short incubations at high temperatures. Katzenellenbogen et al (1973) have reported that the cytoplasmic form of the rat oestrogen receptor is very labile at temperatures above 20°c while Braunsberg & Pflaeger (1981) have reported that a portion of the nuclear oestrogen receptor is labile above 22°c. It is likely that the degradation of the hormone receptor complex is due solely to protease digestion though Schoenberg & Clark (1980) have reported loss of oestrogen-binding sites due to nuclease action.

The binding of (^{3}H) - oestradiol to nuclear oestrogen receptor (pre-filled with unlabelled oestradiol) at low temperature ('on-rate') also indicates that there is a significant exchange rate at 4°c (Fig. 3.6). However, the 'on-rate', or association rate, appears much more rapid than the corresponding 'off-rate'. This is particularly marked for the binding of oestradiol to receptor from the crude nuclear pellet. Hansen et al (1976) reported that the association rate constant of the progesterone receptor varied more with temperature than did the dissociation constant. Weichman & Notides (1977) reported that the association constant, unlike dissociation constant, of oestrogen receptor from calf uterus was unchanged by salt concentration or by sodium thiocyanate. These two reports indicate that changes in one of the binding constants is not necessarily reflected in its opposite rate constant, though the significance of this is unclear. However, the

much slower 'on-rate' noted with purified nuclei might indicate that the early rapid binding could be due to damaged nuclei or to a small portion of contaminating cytoplasmic or membrane bound receptor (Szego & Pietras, 1977; 1979). The difference is most likely ascribed to damaged nuclei since the levels of membrane or cytoplasmic contamination of the crude nuclear pellet appear to be fairly small. The final receptor concentration of receptor per μg of DNA was the same for both the 'crude' and 'purified' nuclear preparations.

The initial off-rate from 'purified' and 'crude' nuclear pellets also shows small differences (Fig. 3.5) but these changes seem inconsistent. However the overall extent of exchange of 4^{o}c and 20^{o}c is the same in both crude and purified nuclei suggesting that these differences may not be significant.

4.2 SALT EXTRACTION

In agreement with the observations of Katzenellenbogen *et al* (1980) and King (1982) 0.6M KCl was found to solubilise only about 50% of the human nuclear oestrogen receptor. This reinforces the original criticisms of salt extraction of nuclear receptor (Clark & Peck, 1976), that the salt resistant fraction is the fraction which is attached to the high affinity specific acceptor, or gene regulator sites. Although widely accepted, this has been criticised by a number of groups (Muller *et al*, 1977; Traish *et al*, 1977; Borgna & Ladrech, 1982). Recent reports by Lippman's group (Stobl *et al*, 1982; Kasid *et al*, 1982) have linked 'processed' nuclear receptors with salt resistant nuclear receptors by demonstrating that the processed receptor exhibits a

slower exchange rate and an increased resistance to salt extraction. In these experiments the human tissue would already have been exposed to oestrogens and therefore the nuclear receptor would be present in the processed form. However from Table 3.2 it can be calculated that roughly 5000 salt resistant sites are present in each nucleus. Such a calculation is necessarily inaccurate but the number of sites involved appears to be in excess of the number of sites of genetic regulation even allowing for co-operative effects of nuclear receptors and multiple interaction sites (Leake, 1981 a; Palmiter et al, 1981).

The method which is used to distinguish between nuclear receptors controlling gene expression (salt resistant nuclear receptors) and the bulk of nuclear receptors (salt soluble receptors) which have no effect on gene regulation, was arrived at empirically, after studies on the oestrogen receptor in rat uterus, (Clark & Peck, 1976). It may be that the greater proportion of salt resistant nuclear receptor in human tissue compared to rat uterus reflects a species difference and indicates that oestrogen receptor in human tissue is bound to chromatin with a higher affinity.

One of the principal reasons for the variation in the published estimates is the different rate of exchange in chromatin bound and salt extracted nuclear oestrogen receptor (Fig. 3.3 and Fig. 3.7). However the various differences in the off-rate of oestradiol from salt extracted nuclear receptor reported in the literature may, at least in part, be due to interlaboratory differences in salt concentration (Weichman & Notides, 1977; Lukola et al, 1981) although the dissociation constant of oestrogen from its receptor has also been

reported as sensitive to triton (Pavlik & Rutledge, 1980; De Boer & Notides, 1981) and high concentrations of oestradiol (Borgna & Ladrech, 1982). In particular De Boer & Notides (1981) have concluded that the dissociation rate reflects the conformational state of the oestrogen receptor. The rate of exchange of oestradiol from HAP bound receptor is lower at 20°c (Fig. 3.7) than that from chromatin-bound receptor suggesting that the extracted nuclear oestrogen receptor complex is generally more stable than when still bound to chromatin. This contrasts with a report by Sala-Trepat & Reti (1974) that nuclear receptor complex from calf uterus was less stable at 22°c after salt extraction. However both Sala-Trepat & Reti (1974) and De Boer & Notides (1981) reported that binding salt-solubilised nuclear receptor to HAP increased the stability of the hormone-receptor complex.

Although most of the differences in the reported extent of exchange at low temperature can be attributed to the different methods used, two papers (Blankenstein et al, 1979; Pavlik & Rutledge, 1980) which reported a high rate of exchange at 4°c from HAP bound nuclear receptor remain unexplained. These two papers had measured the exchange rate in 0.6M KCl, in contrast to the usual HAP method at low ionic strength, suggesting that ionic strength of the medium could affect the rate of exchange.

As reported by Lukola et al, (1981) increasing the ionic strength was found to increase the rate of exchange of steroid from HAP-bound nuclear receptor at 4°c. Fig. 3.7 and 3.8 indicate that increasing the ionic strength can cause almost as large a change in the exchange

rate as increasing the temperature. The corollary to this result is that salt extraction of nuclear receptor, usually for 1 hour at 4^{o}c in 0.6M KCl, would cause an apparent increase in the amount of unoccupied nuclear receptor which is detected. Such stripping of endogenously filled nuclear receptor, confirmed by the demonstration that the lost radiolabelled oestradiol can be removed as unbound steroid (Fig. 3.8 legend), suggests that the HAP method of measuring nuclear receptor may give rise to an underestimate of filled nuclear receptor content and will necessarily lead to an overestimation of the proportion of unoccupied nuclear sites.

The use of different extraction methods (different salt concentrations and incubation times) may explain why there has been a wide variation 9 - 75% (Horwitz & McGuire, 1978b; Geier et al, 1980) in published estimates of the proportion of nuclear oestrogen receptors which are unoccupied. Unoccupied nuclear receptors were originally proposed as the reason for oestrogen receptor positive tumours failing to respond to endocrine therapy (Panko & McLeod, 1978; Thorsen & Stoa, 1979). The more recent reports (Carlson & Gorski, 1980; Lévy et al, 1980; Thrower et al, 1981) which demonstrate unoccupied nuclear receptors in most oestrogen target tissues suggest that they are a general phenomenon resulting from oestrogen action. However it seems unlikely that the actual function of unoccupied nuclear receptors will be elucidated until the amount of this class of nuclear receptors can be accurately measured. Therefore, in future, more attention should be given to the effects of salt and temperature on the levels of unoccupied nuclear receptors detected.

Fig. 3.9 indicates that the salt resistant nuclear receptor has the same exchange rate, at both 4^{o}c and 20^{o}c, as the total receptor content of untreated nuclei in the crude nuclear pellets. This shows that the rate of exchange cannot be used to differentiate between salt resistant and salt extractable nuclear receptor.

There is now increasing evidence that measurement of nuclear oestrogen receptor content in breast tumours, whether measured in intact nuclei or in a salt extract can improve the value of receptor as a clinical index of both potential response of advanced breast cancer to endocrine therapy and of prognosis of primary disease (Leake, 1981b). It is therefore important that the estimates of both filled and empty nuclear receptor should be as reliable as possible.

Therefore, in conclusion, a number of factors can affect the stability of the human oestrogen receptor. These factors, and the differences between the human and rat forms of the oestrogen receptor, should be considered when receptor levels are measured.

PART II

Growth of breast tumour cells *in vitro*.

5. INTRODUCTION

5.1 PRIMARY CULTURE OF MAMMARY TUMOUR CELLS

It has been recognised for many years (Lasfargue & Ozzello, 1958) that primary culture of epithelial cells from human mammary tissue would be valuable, for example there exists no reliable guide for the selection of the optimal therapeutic regime, or combination of regimes, for the treatment of individual patients. A method for culturing mammary tumour biopsies could provide both a system for individually testing the efficacy of potential treatments and be of great value in the screening of new drugs. A specific objective in developing mammary tumour cell culture would be to provide a method to examine oestrogen induced stimulation of epithelial growth and the mechanism of its inhibition by tamoxifen. This should both identify patients suitable for tamoxifen therapy and extend our understanding of the mechanism of action of anti-oestrogens in human tissue. Physiological defects which are not apparent in a receptor assay may become evident under culture conditions (Burstein et al, 1971; Wellings & Jentoff, 1972; Aspergen et al, 1975). There are certain physiological defects which would be particularly suitable for study using tissue culture. These include 1) the presence of oestrogen receptors in apparently hormone-independent tumours, 2) clonal variation within a breast tumour, i.e. tumours which contain a mixed population of hormone dependent and independent cells and 3) the possibility of transition of an individual cell or group of cells from hormone dependence to independence.

Primary culture of breast tumour biopsies could provide an alternative

in vitro model of breast cancer. At present there are three systems available for the study of hormonal involvement in mammary tumours - 1) The DMBA induced rat mammary tumours, which can be hormone dependent or independent (even within the same rat) (Huggins et al, 1959), though these tumours are frequently more sensitive to peptide hormones such as insulin (Cohen & Hilf, 1974) and prolactin (Asselin & Labrie, 1978), 2) Small pieces of breast tumour biopsies can be maintained for short periods in organ culture but the validity of these experiments has been questioned (see Section 5.4.2) because of cell damage both during preparation and due to anoxia in culture. The cells within each explant in organ culture remain viable for only a few days, 3) Cell lines derived from pleural effusions, the most studied of which is MCF-7 (Soule et al, 1973). This cell line has been shown to contain receptors for androgens, oestrogens, glucocorticoids and progesterone, and growth can be stimulated by oestrogen (Horwitz et al, 1975) (for problems with MCF-7 see Section 5.4).

A method for the primary culture of solid tumours would provide a useful model for studying both the mechanism of hormone action in tumours and the effects of anti-tumour drugs. In the past, it has proved very difficult to culture epithelial cells from solid tumours (Whitescarver, 1974) though it has been possible to culture normal breast epithelium (Ebner, 1961; Stampfer et al, 1980). The main reasons given for the lack of success are the low yield of epithelial cells and their slow growth rate. The yield of epithelial cells is low due to the large proportion of dead cells in a solid tumour as a result of previous therapy, necrosis or calcification (Buehring & Williams, 1976). Breast fibroblasts replicate at a much higher rate than epithelial cells (Owens

et al, 1976). They grow over epithelial colonies (Stoker et al, 1978) and coat them in collagen (Lasfargue, 1973) limiting access to nutrients and preventing dispersal of epithelial cells during sub-culturing, which effectively prevents epithelial growth.

Another reason, proposed to account for the low yield, is damage to the very fragile epithelial cells during tissue disaggregation (Wiepjes & Prop, 1970). The method used to establish the first cell lines from solid tumours (Lasfargue & Ozzello, 1958) involved a dissection technique in which epithelial cells were spilled from the tissue by careful slicing. More recently enzymatic disaggregation using collagenase has become the preferred method. (Hamburger et al, 1982). Unlike trypsin, (Lasfargue & Moore, 1971) collagenase digests stroma, causes little epithelial cell damage and is not inactivated by serum, calcium or magnesium (Ceriani et al, 1978). Epithelial cells can endure long exposure to collagenase and still recover (Freshney, 1972).

Only a small percentage of the attempts to establish permanent cell lines from solid human breast tumour biopsies have succeeded. These include BT-20 (Lasfargue & Ozzello, 1958) A1-Ab (Reed & Grey, 1962) G-11 and HBT-39 (Plata et al, 1973) SW-613 (Liebovitz, 1973) and B-O (Nissen et al, 1980). The very low level of success in establishing cell lines suggests that these cell lines are due to the mutation of a single cell, or that the original tumours were abnormal in some way. Such cell lines have not been widely utilized, compared with MCF-7, and this must imply doubts about their validity as a model of breast cancer.

More recently there have been reports of successful maintenance of primary

cultures of breast tumour cells for short periods (Buehring & Williams, 1976; Kirkland et al, 1979). This has allowed a comparison between growth of tumour cells and normal breast epithelium, and the effects of hormones and growth factors to be studied (Klevjar - Anderson & Buehring, 1980; Yang et al, 1980a; Smith et al, 1981a). It is much more reasonable to expect short-term primary cultured cells to reflect those of the parent tumour than it is for established cell lines to do so.

5.2 SELECTION OF EPITHELIAL CELLS

The most successful method of selecting for epithelial cells has been to cover the culture vessel to prevent stromal attachment. This was first achieved by coating the surface of the flask with rat tail collagen (Ehrmann & Grey, 1956). This method is still in use (Yang et al, 1980b) but because rat tail collagen gels have a tendency to tear (allowing fibroblast growth) the use of either floating collagen gels (Katiyer et al, 1978) or agar (Asano & Mandel, 1981) has become more popular.

A variation of this method is to grow a monolayer of appropriate cells then arrest division by irradiation or mitomycin C treatment.

This 'feeder layer' aids growth by preventing stromal attachment (Rheinwald & Green, 1975 a; b) and possibly by conditioning the medium (Armstrong & Rosenau, 1978). The fibroblast line of Swiss 3T3 cells has been successful and breast foam cells (non-dividing epithelial cells from human milk) (Taylor-Papadimitrou et al, 1977b) have also been used as a feeder layer.

Other methods have relied on the more stringent growth requirements of fibroblasts. The omission of foetal calf serum (Puck et al, 1958) or the addition of the D forms of certain essential amino acids, e.g. valine (Gilbert & Migeon, 1975) prevents or retards the growth of fibroblasts while permitting epithelial cells to grow.

Fibroblast attachment can be selectively inhibited by controlled exposure to proteases (Owens, 1974; Matsumara et al, 1975). Separation of epithelial cells from stromal cells can be achieved by using Ficoll (Sykes et al, 1970) or Percoll (Pertoft et al, 1977) density gradients. Cis-hydroxy proline has been added to cultures to prevent collagen formation and so inhibit smothering of epithelial cells (Kao & Prockop, 1977; Liotta et al, 1978). Edwards et al (1980c) have isolated a monoclonal antibody to fibroblasts and regular treatment with the antibody plus complement consistently killed fibroblasts, eventually leaving sheets of epithelial cells.

5.3 STIMULATION OF EPITHELIAL CELL GROWTH

5.3.1. GROWTH FACTORS

The mitogen epidermal growth factor (EGF) was first discovered in the mouse submaxillary gland (Cohen, 1962; Cohen & Elliot, 1963). It was first reported to specifically stimulate epithelial cells (Turkington, 1969) but the glycoprotein receptor for EGF has also been found in fibroblasts (Hollenberg & Cuatercases, 1973; Cohen et al, 1975) where EGF stimulates cell proliferation and reduces the serum requirement (Armelin, 1973; Carpenter & Cohen, 1979). EGF increases colony formation and growth of breast epithelial cells on feeder layers (Rheinweld &

Green, 1977; Taylor-Papadimitrou et al, 1977a) and normal substrate (Stampfer et al, 1980; Yang et al, 1980a).

Yang et al (1980c) also reported the agents that increased cellular cAMP, in particular cholera toxin, favoured mammary epithelial proliferation though the response of tumour cells was more inconsistent than normal cells. Smith et al (1981a) have routinely used cholera toxin to maximise the growth of both malignant and nonmalignant breast epithelia. The inconsistent response of tumour cells may be due to the variable in situ elevation of cAMP levels in malignant breast tissue (Elliot et al, 1981).

Conditioned media (spent medium from other cell lines) from human epithelial or myoepithelial cell lines have been found to increase the growth of both normal and malignant breast cells in culture (Stampfer et al, 1980; Smith et al, 1981a). This suggests that some factor or factors are secreted by growing cells which are required for the growth of breast epithelial cells but which are not present in suitable amounts in normal serum of calf or human origin. The cell lines used to supply the conditioned media are usually human epithelial cells of foetal or embryonic origin (Armstrong & Rosenau, 1978; Stampfer et al, 1980) suggesting that the factor(s) may only be able to cross-react between certain compatible cell types.

Though serum is apparently not essential for epithelial cell culture (Puck et al, 1958) it does stimulate growth. Human serum has a greater effect than bovine serum (Foley & Aftanomus, 1965). Page et al, (1982) have recently reported a factor in human serum which is required for a

response to oestrogen in MCF-7 cells.

Exposure to carcinogenic phorbol esters has aided in the establishment of primary cultures by stimulating the growth rate (Steele et al, 1978). Crude extracts of human urine, kidney or brain have been found to stimulate growth of normal breast epithelial cells (Yang et al, 1980a). The active ingredient(s) in these extracts has not been identified but might simply be human EGF known to be present in human urine (Carpenter & Cohen, 1979).

5.3.2 HORMONES

The growth and development of breast cells depends on the stimulation and interaction of a number of hormones (Topper, 1970; Young et al, 1978). Much of the information regarding the action of hormones has been provided by studies of explants of immature mouse mammary gland in vitro. Cortisol (Mills & Topper, 1970) and insulin are required for the differentiation of mammary epithelial secretory cells. Prolactin stimulates milk secretion including the synthesis of milk proteins. For example, the synthesis of casein and of α lactalbumin can be induced in mammary gland tissue previously incubated with cortisol and insulin in vitro and then exposed to prolactin (Turkington et al, 1967; Turkington, 1970).

During pregnancy prolactin acts synergistically with insulin to promote development of the mammary gland by increasing the synthesis of protein kinases. Insulin has been shown to stimulate the growth and development of the mammary gland (Topper, 1970). Oestrogen causes the development

of the alveolar system in the mammary gland. Progesterone inhibits the formation of the milk proteins casein and ∝lactalbumin while allowing growth and differentiation of mammary epithelial cells. With the expulsion of the placenta at parturition and the decreased progesterone levels, the synthesis of casein and ∝lactalbumin increase rapidly (Conn & Cowie, 1980). There is considerable species differences in the hormonal control of mammary gland development (Forsyth & Hayden, 1977) with the human breast being the most oestrogen sensitive of all primates (Short & Drifie, 1977).

5.4 HUMAN MAMMARY CARCINOMA IN CULTURE

5.4.1. CELL LINES FROM SOLID TUMOURS

No routine method has been established for the formation of cell lines from solid breast tumour biopsies. Only a small number (see Section 5.1) of the attempts to culture solid tumours have succeeded in the formation of a cell-line (Lasfargue & Ozzello, 1958; Reed & Grey, 1962; Liebovitz, 1973; Plata et al, 1973) while Cailleau et al, (1978), Owen et al (1976), Trempe (1976), and Whitescarver (1974) have all reported no success from a large number of attempts. These cell lines are not regarded as good in vitro models of breast cancer and have not become as popular a research tool as MCF-7 cells for several reasons: 1) Despite numerous attempts, only a small number of cell lines have been established and this has led to doubts on whether the cell lines are representative of the tumour from which they were derived or of breast tumours in general 2) poor growth rate of these cell lines (relative to MCF-7) and 3) many of the cell lines contain no steroid receptors while others show little response (in terms of increased cell growth) to steroids.

5.4.2 ORGAN CULTURE OF HUMAN BREAST TUMOURS

Most attempts at organ culture of human breast cancer appear to have been unsuccessful. Most authors were unable to maintain viable tumour tissue for even a few days (Wellings & Jentoff, 1972; Lagois, 1974) although one group have reported routinely culturing biopsies for up to 14 days. Some authors have attempted a tissue survival test (Chayen et al, 1970; Hobbs et al, 1973) and reported that hormone supplementation could improve the survival of some tumours but not others. These methods have been strongly criticised (Heuson & Tagnon, 1973) and testing the action of hormones on dying tissue seems meaningless. Experiments attempting to identify hormone-dependent DMBA induced rat mammary tumours by measuring hormone sensitivity in organ culture concluded that results varied with length of incubation time (Aspegren et al, 1975) and that a hormone receptor assay on a fresh tumour section was more accurate (Kinoshita, 1980).

5.4.3. CELL LINES FROM PLEURAL EFFUSIONS

There has been much more success in establishing cell lines from pleural effusions (Illiger et al, 1975) probably because of the very low stromal content (Soule et al, 1973; Cailleau et al, 1978). The most studied cell line of this type is MCF-7 which was derived from the pleural effusion of a patient with an oestrogen receptor positive breast tumour. It has been rigorously characterized with respect to morphology (Russo et al, 1976 ; 1977) chromosomal studies (Soule et al, 1973) and secretion products (Rose & McGrath, 1975; Lippman et al, 1976). MCF-7 cells respond to hormonal stimulation (Lippman & Bolan, 1975; Lippman

et al, 1976) and contain receptors for oestrogen (Brooks et al, 1973) androgen, glucocorticoids, progestins (Horwitz et al, 1975), insulin (Osborne et al, 1978), prolactin (Shiu, 1979) and vitamin D_3 (Eisman et al, 1980). The injection of MCF-7 cells into nude or athymic mice results consistently in the rapid formation of metastases, frequently with structural morphology similar to the original tumour.

The cell line has been widely used to study oestrogen receptors (Edwards et al, 1980a; Geier et al, 1981), mechanism of action of hormone/receptor complex in relation to chromatin (Horwitz & McGuire, 1978 a & b; Sheridan et al, 1979) oestrogen stimulation of DNA polymerase (Edwards et al, 1980b) interaction of androgens with oestrogen metabolism (Zava & McGuire, 1978 a ; b), effects of tamoxifen (Allegra & Lippman, 1978) and has been proposed as an in vitro model for studying oestrogen responsive breast cancer (Lippman et al, 1976).

Cells from a pleural effusion may derive from a highly selected section of the breast tumour and, therefore, may not be representative of the tumour as a whole. This, perhaps, reduces their potential. Problems have arisen because MCF-7 lines held in different laboratories no longer appear identical and there have been various 'sub-clones' reported (Nawata et al, 1981 a ; b). There have been reports of a decrease in (Briand & Rose, 1981) or loss of (Nawata et al, 1981b; Page et al, 1982) response to oestrogen, loss of certain hormone receptors, dependence of oestrogen stimulation on a serum factor(s) (Page et al, 1982) and translocation of the oestrogen receptor to the nucleus followed by stimulation of cell growth after treatment with 17α oestradiol, (Edwards & McGuire, 1980). Page et al (1982) found a change in the number of

chromosomes in their MCF-7 line from the original 88 reported by Soule et al (1973) (compared with 46 for a normal cell).

5.4.4. PRIMARY CULTURE OF BREAST BIOPSIES

Recently a limited amount of success has been achieved in maintaining breast tumour cells in culture for short periods of time (Kirkland et al, 1979; Klevjer - Anderson & Buehring, 1980; Smith et al, 1981a). Most of these reports have concentrated on attempting to maximise 1) the rate of growth 2) the survival time and 3) the ability to survive and replate after passaging (subculturing). All three of these functions are normally poor in comparison with other types of primary cultures. These methods rely on utilizing a feeder layer or adapted substrate and/or medium to prevent stromal growth and require rich media (including high levels of serum) and combinations of hormones, growth factors and other mitogens.

Although most reports agree that insulin causes increased growth (Gaffney & Pigott, 1978; Stampfer et al, 1980; Yang et al, 1980a) they disagree on the extent of stimulation caused by insulin. Growth factors, such as EGF, and mitogens, such as cholera toxin, while increasing the growth rate of normal breast epithelium, were found to have a more variable effect on breast tumour cells (Kirkland et al, 1979; Yang et al, 1980a). Opinions vary over the effect of steroid hormones. Oestrogens have been reported as having no effect (Kirkland et al, 1979), a varied effect (Barker & Richmond, 1971), or occasional stimulation (i.e. 2 tumours out of 12) while the glucocorticoids have been more consistently reported as causing an increase in growth rate (Gaffney &

Pigott, 1978; Kirkland et al, 1979; Stampfer et al, 1980).

Although most studies have been concentrated on improving the growth conditions, a few groups have begun to use the technique of primary culture of breast cells, to study surface antigens of normal and abnormal breast tissue (Peterson et al, 1978; Sasaki et al, 1981) adriamycin sensitivity (Smith et al, 1981c) and the relationship of concanavalin A reactivity with early disease recurrence (Furmanski et al, 1981).

Cell culture has been criticised (Pasteel et al, 1976) because only one cell type is studied, in isolation, and there is no collagen matrix with which the cells may interact. But primary culture has several advantages when compared with either organ culture or use of established cell lines. The cells in primary culture do not remain in culture long so that mutation and adaptation to culture conditions can be minimised. They respire and divide 'normally' and a large number of biopsies can be studied so that the heterogeneity of human tumours can be investigated. In the future, primary culture of breast cells may be useful in raising and testing monoclonal antibodies against breast tumour antigens.

5.5 IDENTIFICATION OF CELL TYPE

Once a cell line is established it is important that it is fully characterised. This involves demonstrating the presence of certain specific markers which are indicative of the cell type and origin.

5.5.1. HUMAN MAMMARY MARKERS

Specific products of the normal mammary gland can be used as markers. Products which have been used as markers include αlactalbumin (Rose & McGrath, 1975; Woods *et al*, 1979) which is often retained in tumours, casein (Hurilmann *et al*, 1976; Franchimont *et al*, 1977) which is occasionally retained and milk fat globule proteins (Ceriani *et al*, 1977; Sasaki *et al*, 1981).

Other possible breast markers are :- gross cystic disease protein (Haagensen *et al*, 1978) believed to be an epithelial cell secretory product; lactoferrin (Hurilmann *et al*, 1976) and non-histone nuclear antigen (Shiu *et al*, 1977).

Markers which indicate mature breast cells to be both human and malignant include :- Carcinoembryonic antigen (CEA) (Cantwell *et al*, 1980; Walker, 1980) Human chorionic gonadotropin (hCG) (Horne *et al*, 1976) Pregnancy associated α_2 Glycoprotein (Stimson *et al*, 1977) and Gamma foetal proteins (Edynak, 1972). All of these have been reported as present in human breast cancers *in vivo*.

Sasaki *et al* (1981) have studied human mammary epithelial antigens which are expressed in culture. They found four major protein components which are expressed in high levels in primary culture of normal breast and in lower levels in breast cell lines from pleural effusions and breast tumour cells in primary cultures (Peterson *et al*, 1978).

5.5.2 MARKERS OF MALIGNANCY

There has been no correlation found between structural differentiation and malignancy except that tumour cells display a polyploidy which normal breast cells rarely do (Macpherson, 1970). Periodic chromosomal examinations (Nelson - Rees et al, 1974) are also important when using established cell lines because in the past several lines have been found to consist totally or largely of HeLa cells (Buehring & Hackett, 1974).

Injection of cells into thymectomized (or nude) mice (Fogh & Giovanella, 1978) can show that the cells are malignant, but the absence of a tumour does not indicate that the cells are not malignant (Marshall et al, 1977). Tumours can be raised in nude mice from transplanted solid tumours (Giovanella et al, 1978) or from cells cultured from solid tumours (Giovanella et al, 1974) or pleural effusions (Shafie, 1980). The rate of success from all solid tumours is about 50%, with breast tumours being the most unsuccessful (Shimosata et al, 1976; Giovanella et al, 1978). The success rate for established cell lines is much higher, depending largely on the number of cells innoculated (Giovanella et al, 1974; Shafie, 1980). The tumours raised in nude mice usually closely resemble the morphology of the original tumour (Shimosata et al, 1976) and growth of MCF-7 cells in nude mice is truly oestrogen dependent, whereas in culture they will grow in the absence of oestrogen (Shafie, 1980; Shafie et al, 1981).

The capacity to grow in soft agar has been used to demonstrate malignancy (Das et al, 1974). Tanigawa et al (1982) have recently reported

growing breast tumour cells in soft agar although caution must be exercised since, in one report, colonies formed in soft agar from breast tumours have been subsequently identified as T lymphocytes (Asano & Mandel, 1981). The levels of cathepsin B, which digests collagen and proteoglycan, are elevated in malignant cells (Poule et al, 1978). However this would not be the ideal test for cells in culture since 1) it relies on detecting a relative increase in the level of enzyme, 2) changes may have occurred because the cells are in culture and 3) the cell population may be mixed.

Recently plasminogen activator has become favoured as a specific marker of malignancy. The major form has been characterised as a serine protease of 39,000 molecular weight with arginine specificity which preferentially cleaves one fraction of plasminogen (Unkles et al, 1973; 1974). High levels of plasminogen activator have been reported as one of the first detectable changes resulting after carcinogen administration and tumour formation in rats (Hince & Roscoe, 1978). The release of plasminogen activator is inhibited by interferon (Schorder et al, 1978). Release of plasminogen activator can be demonstrated by a technique measuring the digestion of a fibrin overlay (Jones et al, 1975) or by measuring release of fluorescent tagged (Whur et al, 1980), or radioactively labelled, fibrin by plasminogen activator in spent medium.

Plasminogen activator has been found in breast and cervical tumours (Pearlstein et al, 1976; Tucker et al, 1978) but not in normal cells of the same origin. It is also present in a range of human tumour cell lines (Nagy et al, 1977). However doubts have been raised by studies of rat liver where no correlation has been found between its production

and malignancy (San et al, 1977; Howett et al, 1978). More recently Yang et al (1980c) have shown that plasminogen activator is released from both normal and malignant mammary cells in culture. However Wilson et al (1980) have shown that different molecular weight forms of plasminogen activator are produced by normal and malignant cells in culture. The need to determine molecular weight would increase the complexity of the assay but this would be justified if it was confirmed as a reliable marker of malignancy. The assay of plasminogen activator would also prove very useful if the release of this and other proteases could be related to the invasive potential of the breast tumour.

5.5.3 MARKERS OF EPITHELIAL CELLS

Epithelial cells can easily be distinguished in culture by growth pattern and morphological characteristics (Bassin et al, 1972) such as the 'paving-like' arrangement of cells or the formation of domes, (Young et al, 1978; Smith et al, 1981b). The electron microscope is the most routinely used method to define the epithelial nature of cultures by identifying desmosomal junctions, tonofilaments, microvilli and large irregular nuclei (Owens et al, 1976; Cailleau et al, 1978). The presence of tonofilaments can also be demonstrated using monoclonal antibodies raised recently by Lane (1982).

The mammary epithelial antigens (Section 5.5.1) used by Peterson et al, (1978) are present in normal breast epithelial cells in situ in high levels but in lower amounts in cultured normal or malignant cells. They are also found in trace amounts in breast fibroblasts. The ability of epithelial cells to coat collagen gels and protect them from

collagenase has been used to identify them (Kanoza et al, 1978) but this requires a large number of, or rapidly growing, epithelial cells and is, therefore, unlikely to be of use as a marker in primary culture of breast cells.

5.6 OESTROGEN RECEPTOR ASSAY IN TISSUE CULTURE

As yet there is no simple and accurate method for detecting hormone receptor in a small number of cells. Present receptor assays, such as those using dextran coated charcoal, require a minimum of 100 mg of cells per assay and so are of value only for cells with rapid growth rates, similar to MCF-7's, where a large number of cells can be quickly generated.

A number of groups have published autoradiographic methods for use in tissue culture (Arnold, 1981; Sheridan et al, 1981; Stumpf et al, 1981) though it may prove difficult to distinguish between specific and non-specific binding of labelled steroid.

Recently there have been a large number of reports of histochemical methods for detecting oestrogen binding (Pertschuk et al, 1978; Walker et al, 1980; Nenci et al, 1981). As yet none of the methods have come close to fulfilling all the requirements for a histochemical receptor assay to be considered valid (Chamness et al, 1980). The main drawback is in the concentration of oestrogen analogue needed. The lowest effective concentration is 10^{-7}M which is 100 - 1000 fold in excess of the physiological level. This suggests that the type II binding sites (Markaverich et al, 1980) are being detected rather than

the functional cellular oestrogen receptor. If it can be shown that type II occur only if type I is present then the method may be of value for dinical use in rapidly predicting tumours which will respond to oestrogen. However the histochemical methods must become more reliable and quantitative before they can be used as a research tool.

A natural oestrogen coumestrol may be of use in detecting the oestrogen receptor in culture since it absorbs at 340nm and fluoresces at 410 nm. It can only be used in cell culture because in tissue, elastase and collagen fluoresce strongly at 410 nm (Pertschuk et al, 1980).

The development of monoclonal antibodies to the oestrogen receptor (Greene et al, 1977; Greene et al, 1979; Coffer et al, 1980) may provide an alternative method. But problems still remain with detecting receptor after the cells or tissues have been fixed. Furthermore, the antigenic sites do not seem to be limited exclusively to the oestrogen receptor.

The use of antibodies against the oestrogen molecule itself, has been proposed as a method for detecting tissue which contains oestrogen receptor (Nenci et al, 1976 a ; b). Unfortunately it would seem extremely unlikely that antibodies would be able to bind oestrogen while it was bound to the oestrogen receptor. Furthermore, oestrogen, particularly at the levels used, will almost certainly be retained within membranes.

It may prove possible to adapt the technique for measurement of oestrogen receptor by isoelectric focusing to detect receptor in small volume

extracts from tissue culture. This technique has been found to correlate well with the dextran coated charcoal assay (Lloyd *et al*, 1982).

5.7 OBJECTIVES

The purpose of this work is to develop a method for the routine growth in culture of cells from human breast tumour biopsies. This will involve determining the conditions which maximise tumour cell growth and survival. Cells grown from biopsies will be characterised as fully as possible to demonstrate their origin, cell type and malignancy. An attempt will be made to evaluate the possible use of primary culture to detect 1) hormone responsive tumours 2) to act as an *in vitro* model of breast cancer and 3) to test anti-tumour drugs.

6. CELL CULTURE MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 MEDIA

Ham's F 10 (10x) (Ham, 1963) was obtained from Flow Laboratories, Irvine, Scotland. A full list of ingredients is provided in the Flow laboratories catalogue page 51.

6.1.2 ANTIBIOTICS were obtained from the following :-

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

6.1.3 FINE CHEMICALS were obtained as follows:-

Foetal Calf Serum	Gibco Bio-Cult, Paisley, Scotland & Flow Laboratories, Irvine, Scotland
Glutamine (200mM)	Gibco Bio-Cult, Paisley, Scotland
N-2-hydroxypiperazine-N'-2-ethane sulphonic acid (HEPES) 1M	Gibco Bio-Cult, Paisley, Scotland
Non-essential amino acids	Gibco Bio-Cult, Paisley, Scotland
Sodium Bicarbonate (7.5%)	Flow Laboratories, Irvine, Scotland

Hanks balanced salt solution (BSS)	Flow Laboratories, Irvine, Scotland
Giemsa stain	BDH, Poole, Dorset
Solvent methanol (Microscopical grade reagent)	BDH, Poole, Dorset
Depex	Hopkin and Williams, Essex
Glutaraldehyde 25%	TAAB Laboratories, Reading, Berkshire
Osmium tetroxide	TAAB Laboratories, Reading, Berkshire
Propylene oxide	EM Scope Laboratories, Ashford, Kent
Araldite mixture	EM Scope Laboratories, Ashford, Kent
Vitrogen 100 collagen	Flow Laboratories, Irvine, Scotland

All other reagents used were BDH "Analar" grade.

6.1.4 HORMONES AND GROWTH FACTORS were obtained as follows:-

Oestradiol -17β	Sigma, London
Insulin	Sigma, London
Cortisol	Sigma, London
Prolactin (ovine)	Sigma, London
Epidermal Growth factor	Collaborative Research Inc., Mass., U.S.A.
Cholera toxin	Sigma, London

6.1.5 ENZYMES were obtained as follows:-

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

6.1.6 PHOTOGRAPHY

D 19 Developer	Kodak Ltd., England
Kodak Electron microscopy film 4489	Kodak Ltd., England
AR10 fine grain autoradiographic stripping film	Kodak Ltd., England
Ilford Panatomic X	Il ford Ltd., England
Amfix	May & Baker, Dagenham, Kent

6.1.7 CELL LINES

3T3 : Swiss mouse fibroblast line (Todaro & Green, 1963)

6.1.8 RADIOCHEMICALS

(methyl-^{3}H) thymidine (25 Ci mmol^{-1})

(6-7-^{3}H) 17β oestradiol (54 Ci mmol^{-1})

(1,2,6,7 -^{3}H) cortisol (30 Ci mmol^{-1})

were supplied by Amersham International.

6.1.9 MISCELLANEOUS

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

Nalgene filter units were obtained from Nalge Co., Division of Sybron Corporation, Rochester, New York, U.S.A.

Millex Filtration Units were obtained from Millipore, London.

Disposable plastic ware was obtained from Sterilin, Teddington, England.

Plate glass strips for glass knives were obtained from LKB Productia, Sweden.

Moulds for constructing araldite blocks from TAAB Laboratories, Reading, Berkshire.

Electron microscopy grids (3.0 mm copper, HF34, 200 mesh) were obtained from Graticules Ltd., Tonbridge, England.

Cells were routinely examined using a Leitz diavert microscope.

Electron microscopy was carried out on a Phillips 301 electron microscope.

6.1.10 LIVESTOCK

Nude or athymic mice were kindly supplied by the animal house of the Dermatology Department, Glasgow University. These animals were bred from MF 10 LA albino mice and supplied from the Mordan Institute for Animal Diseases, Edinburgh. Athymic mice were maintained in an isolator under sterile conditions throughout the period of experiments.

6.1.11 HUMAN TISSUE

Breast tumour tissue was kindly supplied by Health Board Hospitals, principally Stobhill General Hospital, Western Infirmary, Gartnavel General Hospital, Hairmyres Hospital, East Kilbride and Ballochmyle Hospital, Mauchline, Ayrshire.

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

6.1.12 ANTIBODIES

The monoclonal antibody (LE65) was kindly supplied by Dr. E.B. Lane (Lane, 1982).

Fluorescein conjugated rabbit anti mouse IgG was obtained from the Scottish Antibody Production Unit, Law Hospital, Carluke, Scotland.

6.2 TISSUE CULTURE SOLUTIONS

6.2.1 COMPOSITION OF MEDIA

(i) Basic media (F10)

Ham's F10 (10x)	45 ml
deionised, distilled water	350 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	

Media is then "tested" for 3 - 4 days at $37^{o}c$ to ensure sterility.

(ii) Standard working media (F10/10) for maintenance and growth of established cell lines.

The following were added to the Basic Medium:-

Foetal calf serum	50 mls (10%)
Glutamine	2mM
Penicillin	50 units/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

(iii) Primary working media (F10/20)
for maintenance and growth of primary cultures.

The following were added to the Basic medium:-

Foetal calf serum	100 ml (20%)
Glutamine	2 mM
Penicillin	50 units/ml
Insulin	5µg/ml

iv) Digestion media (F10/20/PSK)

The following were added to the primary working medium:-

Kanamycin	10 µg/ml
Streptomycin	100 µg/ml

v) Collection medium (F10/PSFK)

The following were added to the Basic medium:-

Penicillin	250 units/ml
Streptomycin	250 µg/ml
Kanamycin	100 µg/ml
or	
Gentamicin	50 µg/ml
Amphotericin β fungizone	2.5 µg/ml

vi) Phosphate buffered EDTA

Na Cl	8g
K Cl	0.2g
$Na\ H_2\ PO_4$	1.15g
$K_2H_2\ PO_4$	0.2g
EDTA	0.2g

added to 1 litre of distilled water and pH raised to 7.2

(vii) Formal Saline

100 ml of 40% (w/v) formaldehyde plus 900ml 0.08M NaCl, 0.2M Na_2SO_4.

6.2.2 ANTIBODIES

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

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

Gentamicin and Amphotericin β were obtained in sterile solution.

6.2.3 ENZYMES

(i) Collagenase - made up in Hanks BSS at a concentration of 2000 units/ml.

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

Sodium chloride	6.0g
Trisodium citrate	2.96g
Tricine	1.79g
Phenol red	0.005g
distilled deionised water	700 ml

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

(iii) Protease neutral Dispase II - 2% solution made up in Hanks BSS.

6.2.4 HORMONES

Insulin was made up in Hanks BSS to 200 µg/ml and the pH raised to 8 to facilitate solubilisation.

Steroid hormones were dissolved and stored in ethanol at -20°c. When required the ethanol was blown off and the hormone redissolved in F10/20 at the following concentrations, diluted with F10/20 as required and stored at 4°c.

stock concentrations

oestradiol 17β	10^{-7}M
cortisol	10^{-5}M

To ensure that oestradiol and cortisol were completely soluble at these concentrations, stock solutions of tritiated hormones were made up and diluted in primary working medium. The level of radioactivity in each dilution was counted and found to be identical to the level obtained when hormones were aliquoted directly in small volumes of ethyl alcohol. Therefore steroid hormones are completely soluble in F10/20 at these concentrations.

Cholera toxin and Epidermal Growth Factor (EGF) were stored, lyophilized, at 4°c. When required they were dissolved in F10/20 as stock solution (10 times required concentration) and stored at 4°c. The bulk of reconstituted EGF was stored at -20°c.

Prolactin was dissolved in F10/20 containing phenol 3mg/ml and glucose 50 mg/ml at a concentration of 100 µg/ml. Equal volumes of prolactin vehicle were added to control flasks.

Sterilization of solutions in Sections 6.2.2 - 6.2.4 was carried out using either Nalgene or Millex filtration units, depending on the volume involved.

6.3 TISSUE CULTURE METHODS

6.3.1 PREPARATION OF PRIMARY CELL CULTURES FROM SOLID TISSUES

6.3.1.1 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. A portion of the tumour was retained for routine pathology, a second for oestrogen receptor determination and the remainder transferred asceptically to collection medium and stored at $4^{o}c$. Although viable cells could be recovered after storage overnight, a much greater yield was obtained when tumour cells were cultured within 4 hours of the removal of the biopsies.

6.3.1.2 TISSUE DISSECTION

The tissue was transferred to a 9 cm. diameter petri dish containing 25ml 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 carefully chopped into pieces of about 2mm cubed, 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 (i.e. fresh scalpel blades used after approximately 30 cuts) and the chopping

completed as quickly as possible. The pieces were then transferred by inverted pipette to a sterile universal container. The pieces were allowed to settle by gravity. Any remaining fatty tissue, which floated on top of the supernatant, was removed at this stage. The supernatant was removed to a second sterile universal. Fresh dissection BSS was added to the pieces, and again removed to a universal 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 (F10/20/PSK). The suspension was transferred using an inverted pipette, to a $25cm^2$ culture flask, collagenase added to a final concentration of 200 units/ml, and hormones or growth factors were added as required. The flasks were then incubated at $37^{o}c$ for 3 - 7 days until disaggregation of tissue was apparent by the formation of small clumps of cells and free cells on shaking the flask. The timing of this incubation varied with different types of tumour probably reflecting the level of collagen in the tumour.

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 200g for 5 minutes in an MSE bench centrifuge. The pellet was then resuspended in F10/20 medium and transferred to 1) fresh $25cm^2$ culture flasks containing a feeder layer or collagen gel (see Section 6.3.3) or 2) a glass coverslip, coated with a monolayer of FHI, in a 24-well plate. The flasks were maintained in a hot room at $37^{o}c$ and the coverslips in multiwell plates, were maintained in an incubator with 5% CO_2 to allow growth of the cells. Medium and hormones were renewed regularly as indicated to be necessary

by a change in colour of the phenol red indicator. The cells were routinely inspected, before transfer or feeding, using an inverted microscope. The methodology is summarised in figure 6.1.

6.3.1.3 "LASFARGUE SPILLAGE METHOD"

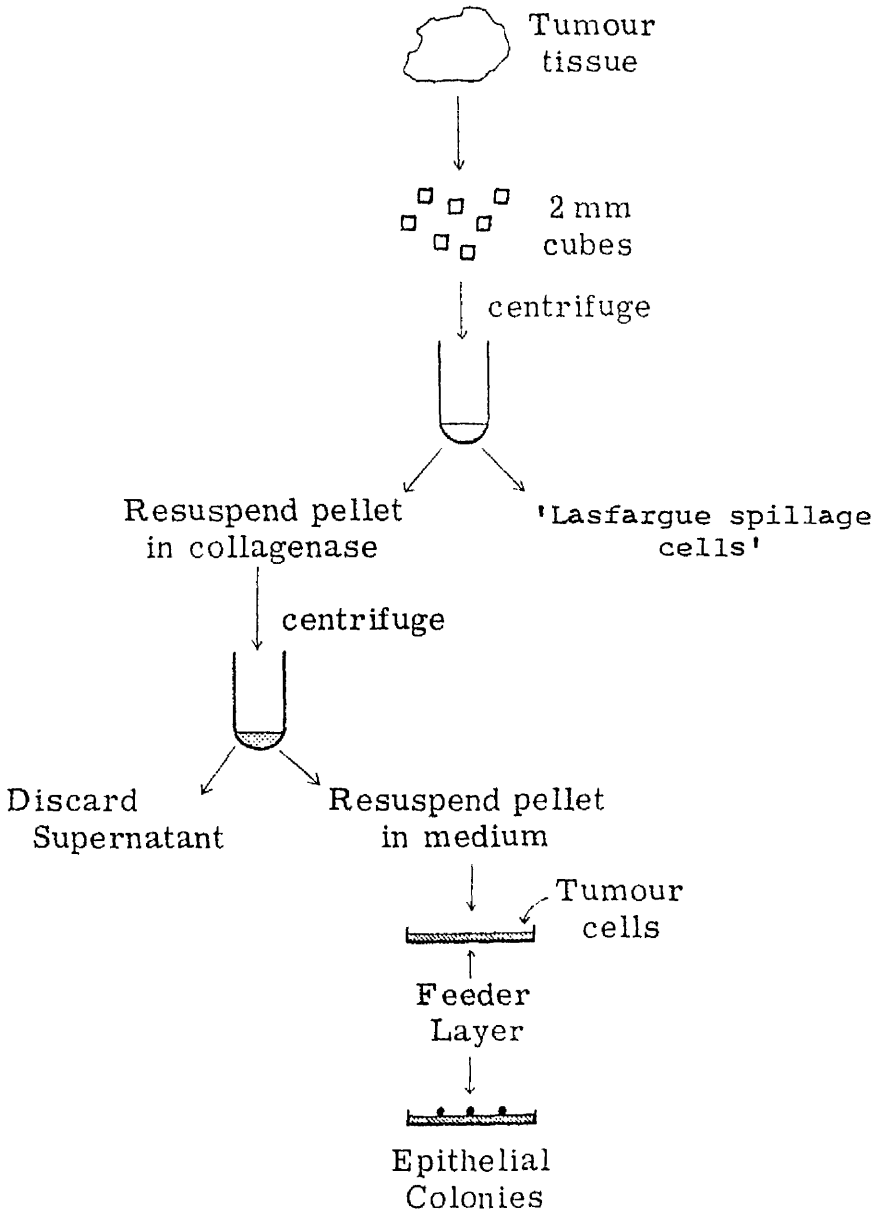
Cells released by 'spillage' (Lasfargue & Ozzello, 1958) on slicing the tumour, were recovered from the original dissection BSS and subsequent washes by centrifuging at 200g for 5 minutes. The pellet containing 'spilled' cells and a large amount of cellular debris, was washed once with dissection BSS and resuspended in F10/20. The cells were examined using an inverted microscope and then treated as cells released by collagenase digestion.

6.3.1.4 PLEURAL EFFUSIONS AND ASCITES FLUID

Fluid was collected from the patient in a buffered antibiotic solution (penicillin/streptomycin) and the solution cooled on ice prior to transport to the laboratory. Cells were recovered by centrifugation at 200g, washed 3 times in dissection BSS and resuspended in F10/20/PSK. They were then plated onto a $25cm^2$ flask and grown at $37^{o}c$ as described by Soule et al (1973) and Cailleau et al, (1974). Cells attached to the substrate slowly over 2 weeks, with some clumps of cells growing in suspension during this period. Prior to feeding the cells, those in solution were centrifuged at 200g for 5 minutes and resuspended in fresh F10/20.

Figure 6.1 - Procedure for the Dissociation of Breast Tissue into Cells

SLIDE OF TISSUE CULTURE METHOD



6.3.2 SUBCULTURE OF CELLS

6.3.2.1. TRYPSINISATION

Trypsin treatment was used to remove cells from attachment to the flask prior to counting or passaging into fresh flasks. 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 and removed after 30 seconds exposure. 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.

6.3.2.2 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 primary working medium (0.2% final concentration. The flask of cells was then incubated at 37°c until the desired cell type was observed detaching from the surface. The medium was then removed and the cells sedimented by centrifugation at 200g for 5 minutes followed by washing twice in medium to avoid any subsequent contamination by residual dispase. The selected cells could then be resuspended in primary working medium and used as required.

6.3.3 PREPARATION OF CELLS FOR USE AS FEEDER LAYER MATERIAL

6.3.3.1 HUMAN FOETAL INTESTINE

An undifferentiated epithelial cell line (FHI-4) was produced from foetal intestine by the "evertion method". A long piece of foetal 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 in either a concentrated collagenase solution (2,000 units/ml) at 37°c for 30 minutes or a more dilute solution of collagenase (1,000 units/ml) at 37°c for 2 hours. The dilute solution of collagenase usually gave the best results. At the end of the digestion period the suspension was centrifuged at 200g for 5 minutes and the resuspended material set up in 25 cm^2 culture flasks which were incubated at 37°c. This method was used to set up lines of FHI which were frozen and stored in liquid nitrogen at -70°c. Cells were brought up from nitrogen as required.

A fibroblast cell line (FHI-F) was produced from foetal intestine by the "flushing method". A long piece of intestine was cut into smaller pieces (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 a 0.25% (v/v) trypsin solution. The other end was then also sealed and the piece of intestine left at 37°c for 30 minutes of trypsin digestion. After incubation, the end of the piece was opened and the trypsin or collagenase digest collected. This was then centrifuged at 200g for 5 minutes and the resulting pellet of cells

resuspended in F10/20/PSK. The suspension was innoculated into 25 cm^2 culture flasks and incubated at 37°c. FHI-F cells were frozen and stored in liquid nitrogen until required.

6.3.3.2 INHIBITION OF CELL DIVISION OF FIBROBLAST CELLS

In order to use 3T3 or FHI-F cells as a feeder layer, division was prevented when cells reached a confluent monolayer. This was achieved by the action of mitomycin C, a safer and cheaper technique than X-ray irradiation (MacPherson & Bryden, 1971). The medium was removed from a monolayer of fibroblast cells and fresh F10/10 medium added. Mitomycin C was added to a final concentration of 10 µg/ml and incubated for 2 hours at 37°c. The cells were then washed with medium and incubated for 2 hours at 37°c. The feeder layer of FHI-F or 3T3 was then suitable for use. The monolayer of fibroblasts was stable and did not divide for up to 2 weeks at which time the cells began to slough off into the medium.

6.3.3.3 VITROGEN 100 COLLAGEN COATED DISH PREPARATION

A thin layer of vitrogen 100 collagen was placed inside the dish to be coated. The vitrogen was evaporated to dryness with a stream of sterile air and then rinsed with sterile phosphate buffered saline. The coated surface was then washed twice with F10/20 medium to hydrate the collagen.

6.3.4 CELL COUNTING

The flask of cells to be counted was trypsinised, as described in

Section 6.3.2.1. The cells were suspended in an arbitrary volume of medium and 20ml counting fluid added to 0.4ml cell suspension. This was then counted on a Coulter Counter at settings of threshold and current apperture suitable for the cell type being studied.

6.3.5 CELL FIXING

6.3.5.1 CELL FIXING USING METHANOL

Medium was removed from cells to be fixed and Hanks balanced salt solution (BSS) was then added to cover the cells and remove any remaining medium. This was poured off, and a (1:1 v/v) mixture of BSS and methanol was added to the culture. After this was discarded, methanol alone was added to cover the cells, and exposure maintained for 10 minutes. Then the cells were washed once with fresh methanol and dried. This method of fixation was suitable for photography since it reduced the thickness of cell layers.

6.3.5.2 CELL FIXING USING FORMAL SALINE

Medium was removed from cells to be fixed, and they were washed twice with Hanks BSS. The cells were washed once with formal saline, and then fixed in fresh formal saline for 30 minutes at 20^{o}c and air dried at room temperature.

6.3.6 CELL STAINING

Giemsa stain was added to cover cells and allowed to stand for 2 minutes. This was then diluted with 10 volumes of water, allowed to stand for

a further 10 minutes and then discarded. The cells were then washed with distilled water until no more stain could be detected and the flasks left to dry.

6.3.7 ASSAY FOR THE EFFECT OF HORMONES OR GROWTH FACTORS ON COLONY GROWTH

6.3.7.1 DIRECT MEASUREMENT

Tumour cells, isolated after collagenase treatment, were transferred onto a monolayer of FHI cells. Medium and hormones were replaced every 2 days and after 4 - 6 weeks the cells were fixed and stained. The colonies were then counted and the total colony area in a flask determined using a dissection microscope and this was expressed relative to a control flask.

6.3.7.2 AUTORADIOGRAPHIC MEASUREMENT

Tumour cells isolated after collagenase treatment were transferred onto a monolayer of FHI cells grown on glass coverslips. Medium and hormones were replaced every 2 days and, after 2 - 4 weeks of culture, medium was replaced for a final time and cells were grown in the presence of 10μCi/ml (^{3}H) thymidine for three hours. The medium was removed and the cells fixed with formal saline (as described in 6.3.5.2). The coverslips were removed, placed in racks and the acid soluble nucleotides removed by washing twice with 5% (v/v) TCA at 0^{o}c for 5 minutes, and twice with water at 0^{o}c for 4 minutes. The coverslips were finally rinsed in ethanol and dried. Coverslips were mounted (cells uppermost) with Depex mounting fluid onto microscope slides and processed for autoradiography.

Preparation of Microscope Slides for Autoradiography

Microscope slides were dipped in gelatin/chrome alum and dried at room temperature. They were then covered with AR10 fine grain autoradiographic stripping film, dried under a stream of cold air and placed in a light tight box. Autoradiographs were stored at room temperature in the dark. The period of exposure varied depending on the extent of radioactive incorporation.

After the exposure period, slides were developed with D19 developer at 20^{o}c for 5 minutes, washed in water for 2 minutes and fixed for 5 minutes, with a 1 : 5 dilution (v/v) of Amfix. After washing for 4 minutes with water, slides were stained using freshly diluted Giemsa (1 : 20 in water) for 1 minute. The stained washed autoradiographs were air dried and second coverslips mounted on top with Depex. The number of cells growing in a colony and the extent of (^{3}H) thymidine incorporation could be measured by grain counting. The changes in these parameters, as a result of hormone treatment, were then calculated.

6.3.8 PHOTOGRAPHY

After fixing and staining cells were photographed on a Leitz diavert microscope using a Leitz camera attachment and using Panatomic X film.

6.3.9 ELECTRON MICROSCOPY OF CELLS

Cells in culture were prepared for electron microscopy by the method described by Kuhn (1981). Briefly this involved fixing the cells

for 30 minutes at 4°c with 2% (v/v) glutaraldehyde in Sorensons' phosphate buffer (pH 7.4). Cells were washed twice with phosphate buffer at 4°c. The cells were stained with 2% osmium tetroxide for 30 minutes at 4°c, and washed twice with phosphate buffer. The cells were dehydrated for 30 minutes in 75% ethyl alcohol, and 10 minutes each in 3 washes of absolute alcohol. The monolayer was then scored with a scalpel and covered with propylene oxide. When suitable areas of cells floated from the substrate they were picked up and transferred to araldite with fine forceps. The araldite blocks were cured for 24 hours at 60°c. Thick sections were cut down the araldite block and mounted, stained and viewed by light microscopy. When the desired cell type was reached, thin sections were cut. Sections were cut with glass knives using an LKB ultratome (Model 4801A with control unit 4802A). Ribbons of thin sections were floated off the glass knives and picked up on copper grids.

Grids were then stained with uranyl acetate and lead citrate as described by Mercer & Birbeck, (1966). The grids were then air dried at room temperature and sections viewed in a Phillips 301 electron microscope.

6.3.10 STEROID HORMONES IN FOETAL CALF SERUM

The concentration of steroid hormones in foetal calf serum was determined by radioimmunoassay which was kindly performed by Dr. G. Beastall (Glasgow Royal Infirmary).

6.3.11 CARCINOEMBRYONIC ANTIGEN ASSAY

The concentration of carcinoembryonic antigen (CEA) was measured by enzyme linked immunoassay using a kit purchased from Abbot Diagnostics. This assay was kindly performed by Mrs. M. Meiklejohn.

6.3.12 FLUORESCENT LABELLING OF TONOFILAMENTS

Coverslips were fixed for 20 seconds in acetone to remove all membranes, and then mounted, cells upward, on a slide. The coverslips were then covered with a $\frac{1}{10}$ dilution of stock solution of the monoclonal antibody LE65 (the monoclonal antibody was produced from mice immunized with a crude preparation of tonofilaments from Ptk_1 cells, Lane (1982)). This was incubated at $37^{o}c$ for 30 minutes in a humid atmosphere. The coverslip was then washed twice with phosphate buffered saline for 15 minutes at room temperature. The coverslips were then covered with a film of fluorescein - conjugated rabbit anti-mouse IgG. A second coverslip was mounted on top of the coverslip on the slide. The coverslips were then examined with a Leitz photomicroscope using U.V. illumination, and filters to reduce background fluorescence.

7. RESULTS

7.1 BREAST STROMAL CELLS

All attempts to grow breast epithelial cells after standard collagenase treatment, in absence of a feeder layer, resulted in overgrowth by fibroblasts. The fibroblasts grew rapidly, coating the flask in less than one week and no epithelial growth was ever noted.

If the tumour pieces were maintained in collagenase for 2 weeks and then the collagenase concentration was slowly reduced, some epithelial growth resulted, but the epithelial cells were quickly overgrown once the fibroblasts had adapted and begun to attach to the substrate.

Feeder layers, in particular Foetal Human Intestine (FHI-4) were able to retard but not completely prevent, fibroblast growth (Fig. 7.1, 7.14). In some cases epithelial colonies, after 2-3 months growth with no visible fibroblastic growth, would become overgrown with fibroblasts in the space of one week. The trigger for this rapid increase in fibroblast growth was not evident, though in several cases passaging a primary culture onto a fresh feeder layer apparently stimulated stromal growth. This overgrowth does not occur in all tumours. However, fibroblast cells could be easily grown from any grade or class of breast tumour. Five fibroblast cell lines were established (B-ECOF, B-MCPF, B-LIMF, B-GORF and B-JECF) and could be cultured indefinitely in F10/10 medium. The cells grew and divided rapidly and were not contact inhibited. This resulted in cells piling up in layers, several cells thick. The spindle

Figure 7.1 - Fibroblast Colony

A fibroblast colony growing on FHI-4 feeder layer from a tumour B-123. Cells had been in culture for 3 months before fibroblast growth began. Cells were fixed with ethanol and stained with Giemsa as described in Section 6.3.5 to 6.3.6 (Magnification X 120).

Figure 7.2 - Breast Fibroblast Cells

Cells from the breast fibroblast cell line B-MCPF.
This demonstrates the alignment of the spindle shaped
fibroblast cells which results in the characteristic whirls
Cells were fixed with ethanol and stained with Giemsa
as described in Section 6.3.5 to 6.3.6 (Magnification X 360

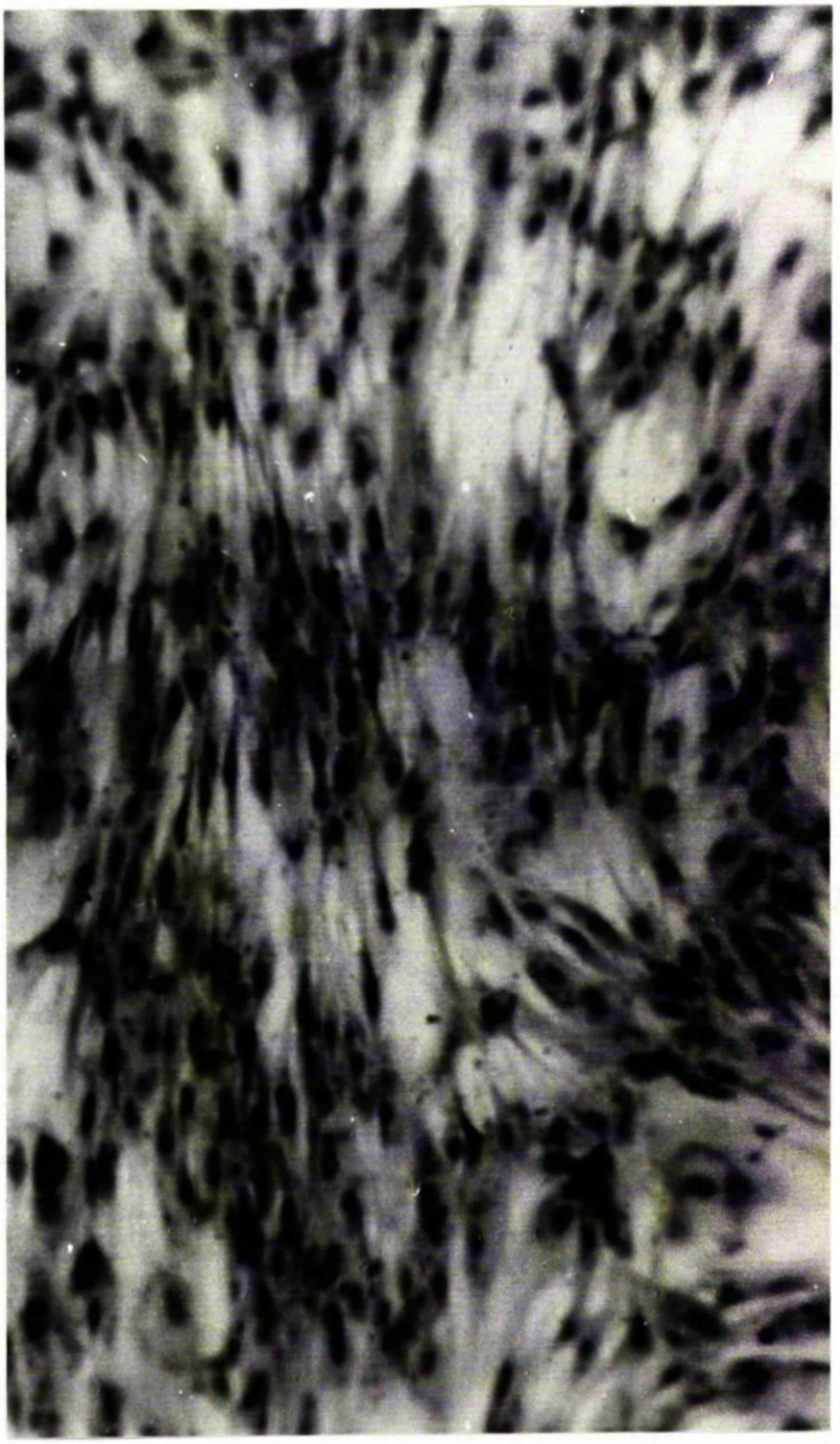
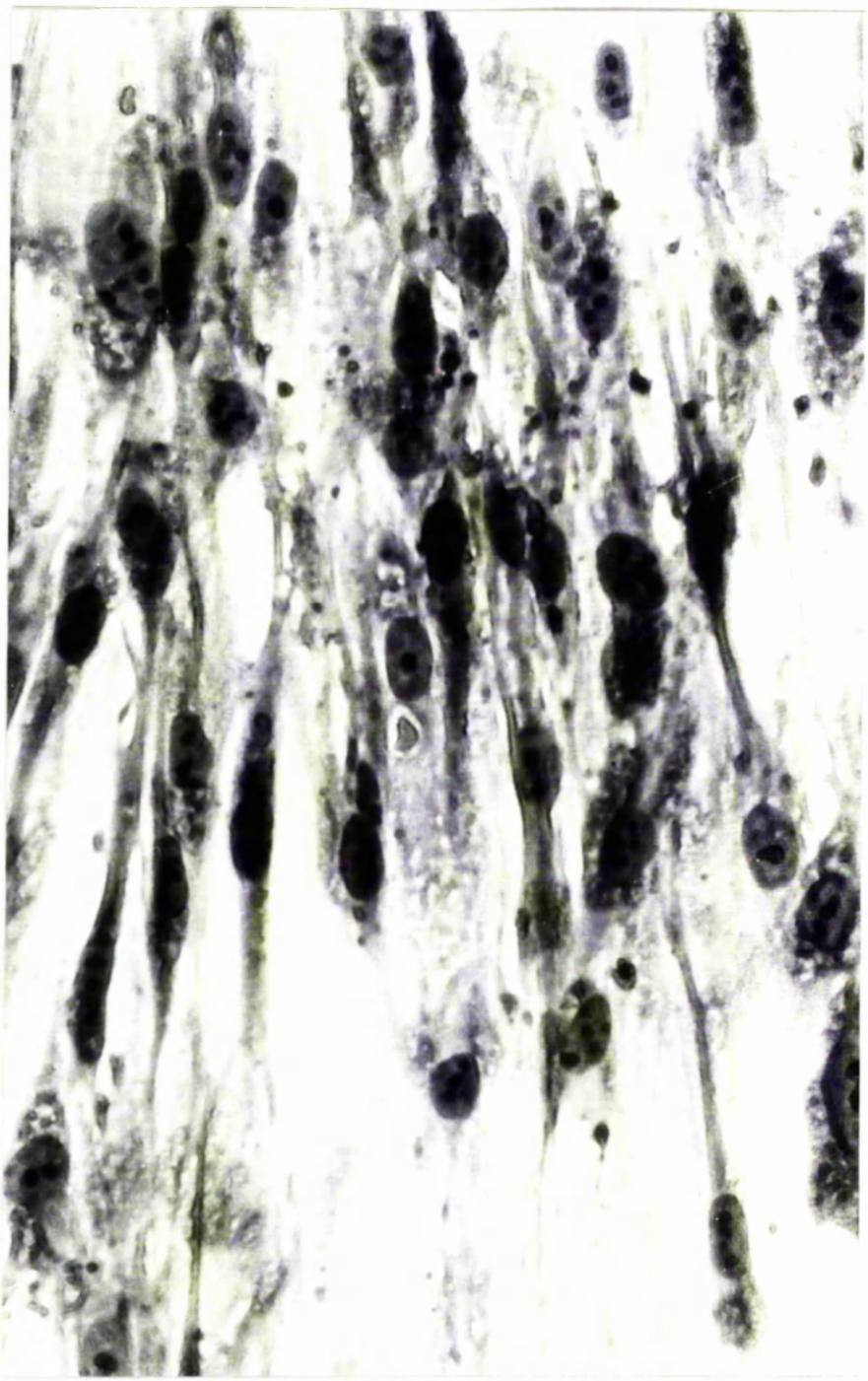


Figure 7.3 - Breast Fibroblast Cells

Cells from the breast fibroblast cell line B-MCPF. This illustrates the alignment of the spindle shaped cells in a layer several cells thick. Cells were fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 960).



shaped fibroblast cells grow aligned in the same direction and form characteristic whirls (Fig. 7.2 and 7.3).

A standard oestrogen receptor assay was performed on the five fibroblast cell lines, grown in the presence and absence of oestrogen (10^{-9}M) and cortisol (10^{-7}M). All five were found to be receptor negative in both the cytosol and nuclear fraction.

7.2 FOETAL HUMAN INTESTINE (FHI-4)

The most successful way of preventing overgrowth of epithelial cells by fibroblasts has been to coat the surface of the flask. This prevents the attachment of the fibroblasts to the substrate and so inhibits their growth. The plating efficiency and growth of breast epithelial cells on three different feeder layers and on collagen coated flasks were determined (Table 7.1). While growth of breast epithelial colonies was achieved on all the surfaces, the most successful method was a feeder layer of human epithelial cells (FHI-4) derived from foetal intestine. However growth, albeit at a lower level, was routinely achieved using either of the mitomycin C treated fibroblast cell lines. Therefore these fibroblast feeder layers could be used as an alternative if problems arose in using FHI-4, because of similarities between this cell line and breast epithelial cells. Although growth of colonies was achieved on collagen coated flasks sporadically, only a very low number of small colonies grew. Collagen coating the substrate was also the least effective method of preventing fibroblasts attaching and growing.

Figure 7.4 - Foetal Human Intestine Cells (FHI-4)

A monolayer of Foetal Human Intestine (FHI-4) after growth in culture for 4 months without passaging. The cells were fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 960)

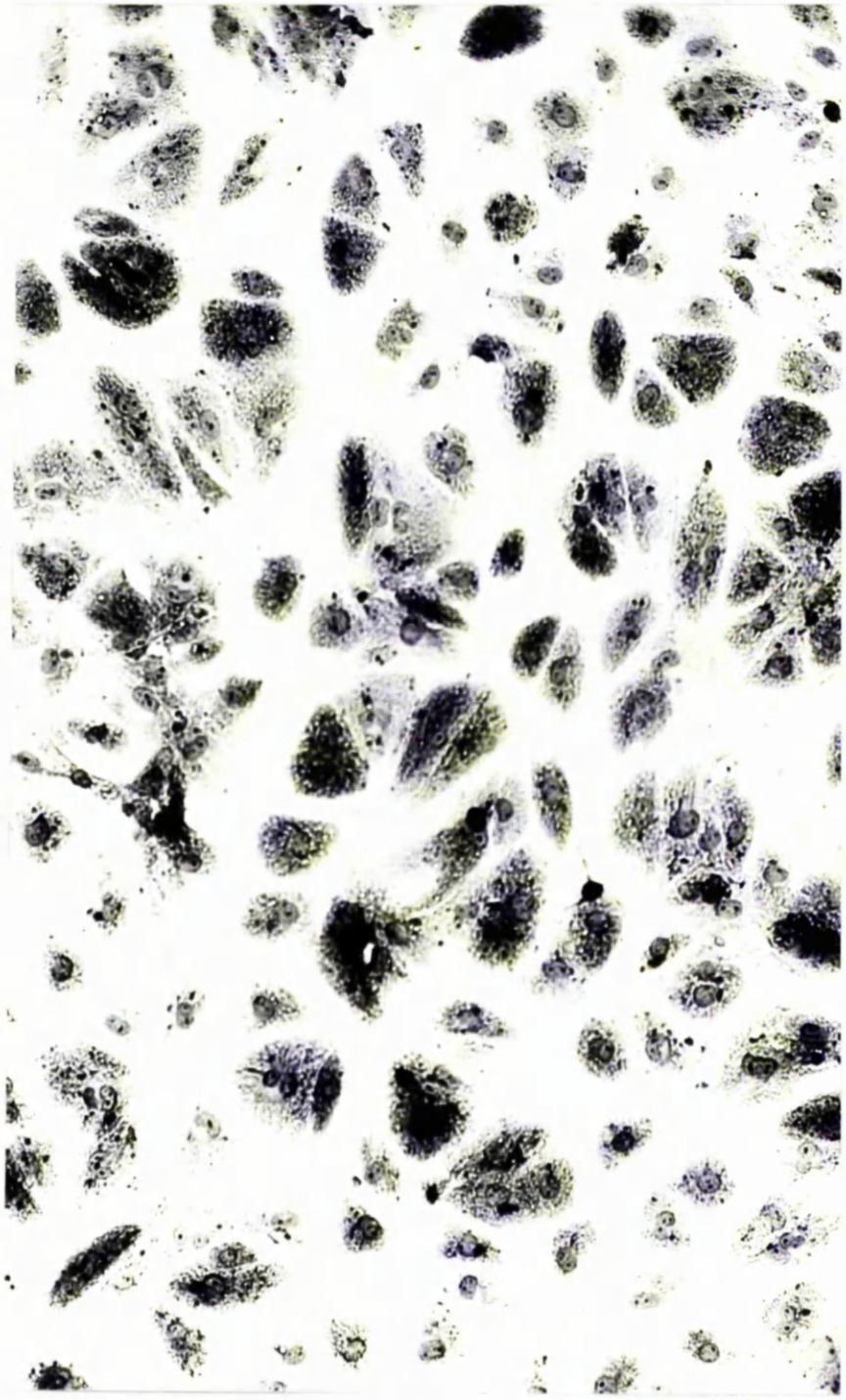


Table 7.1 The Effect of the Substrate on Growth of Cultured Breast Tumour Cell

After digestion of tumour pieces with collagenase was completed, single and clumps of epithelial cells were recovered by centrifuging at 200g for 5 minutes. The pellet of cells was washed once with, and resuspended in, fresh medium and the cells grown on the support indicated for 3 weeks at 37^{o}c. The cells were then fixed and stained with Giemsa. The number and size of the epithelial colonies was counted using a dissecting microscope (at 40 x magnification). Results are expressed as mean $\pm$ the standard deviation.

Substrate	Number of Experiments	Number of Colonies	Area (mm^2)
Vitrogen 100 collagen	6	5.2 ± 2.9	4.0 ± 2.4
3T3 (Mitomycin C treated)	7	19.4 ± 11.5	15.0 ± 9.9
FHI-F (Mitomycin C treated)	12	17.1 ± 9.4	27.6 ± 20.5
FHI-4 (Epithelial cell-line)	22	75.9 ± 26.9	184.8 ± 98.9

The cell line, FHI-4, is an undifferentiated epithelial cell line established from human foetal intestine by collagenase treatment. The cells are contact inhibited such that they grow to a confluent monolayer and then stop dividing. The cells remain as a monolayer, with no piling up, for several months (Fig. 7.4). This strong contact inhibition means that treatment with mitomycin C is unnecessary.

Attempts to plate FHI-4 cells onto a monolayer of the same cells resulted in no change in the monolayer. This means that breast epithelial cells on a feeder layer of FHI-4 can be passaged onto a fresh feeder layer with no interference from the original feeder layer.

However FHI-4 have a relatively slow growth rate compared with either of the fibroblast cell lines. The cell density must be maintained fairly high on passaging (cells can only be split up to 1:4) otherwise the cells stop dividing. The FHI-4 have a finite life in culture such that after 20-25 passages ($\sim$1 yr) the cells stop growing. But these disadvantages are outweighed by the superior growth of the breast epithelial cells on this type of feeder layer.

The FHI cells were assayed for oestrogen receptors and found to be receptor negative in both cytosol and nuclear fractions.

The epithelial origin of the cells was demonstrated using electron microscopy. Fig. 7.5 shows that FHI-4 cells grow as a monolayer. To the right of the figure there is a small amount of surface activity with

Figure 7.5 - Electron Micrograph of FHI-4 Monolayer

A monolayer of foetal human intestine cells (FHI-4) was prepared and sectioned for electron microscopy as described in Section 6.3.9. Thin sections were cut at right angles to the plastic substrate (Magnification X 16,870).

Figure 7.6 - Electron Micrograph of FHI-4 Monolayer

A monolayer of foetal human intestine cells (FHI-4) was prepared and sectioned for electron microscopy as described in Section 6.3.9. Thin sections were cut at right angles to the plastic substrate (Magnification X 81,000).

a line of tonofilaments just below the cell membrane. The cell is relatively inactive in terms of secretory products as shown by the high nuclear to cytoplasmic ratio, the small amount of golgi apparatus (to left of nucleus) and a low number of inactive mitochondria distributed throughout the cell. The interdigitation between cells and a tight junction are illustrated in Fig. 7.6. A small number of glycogen granules can be seen in the cytoplasm of both cells.

7.3 CULTURE OF NORMAL BREAST CELLS

Cells were cultured from 3 mammoplastic reductions after slicing and digestion with collagenase. Growth on a normal plastic substrate resulted in only fibroblast growth in all three cases. There was no evidence of any epithelial growth on a feeder layer of FHI-4 cells.

7.4 BREAST TUMOUR CELLS FROM PLEURAL EFFUSIONS AND ASCITES FLUID

Attempts were made to culture cells from one pleural effusion (B-PES) and one ascites fluid (AF-ES). Cells from both tumours grew in solution and stuck down slowly over a period of 2 weeks. The cells then grew in culture for 4 months in F10/20 medium with a steadily decreasing growth rate. The cells grew both on a feeder layer of FHI-4 cells (Fig. 7.7 and 7.8) and on a normal plastic substrate (Fig. 7.9). The different morphology appears to be a result of the differences in substrate since cells could be transferred from plastic onto a feeder layer with the resultant change in morphology. The cells of the pleural effusion (Fig. 7.8) appear to be pushing

Figure 7.7 - Cultured Cells from a Pleural Effusion

Two epithelial colonies from the pleural effusion B-PES, grown on a feeder layer of FHI-4 cells, are shown. Cells were prepared as described in Section 6.3.1.4. Cells were grown in culture for 10 days then fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 120).

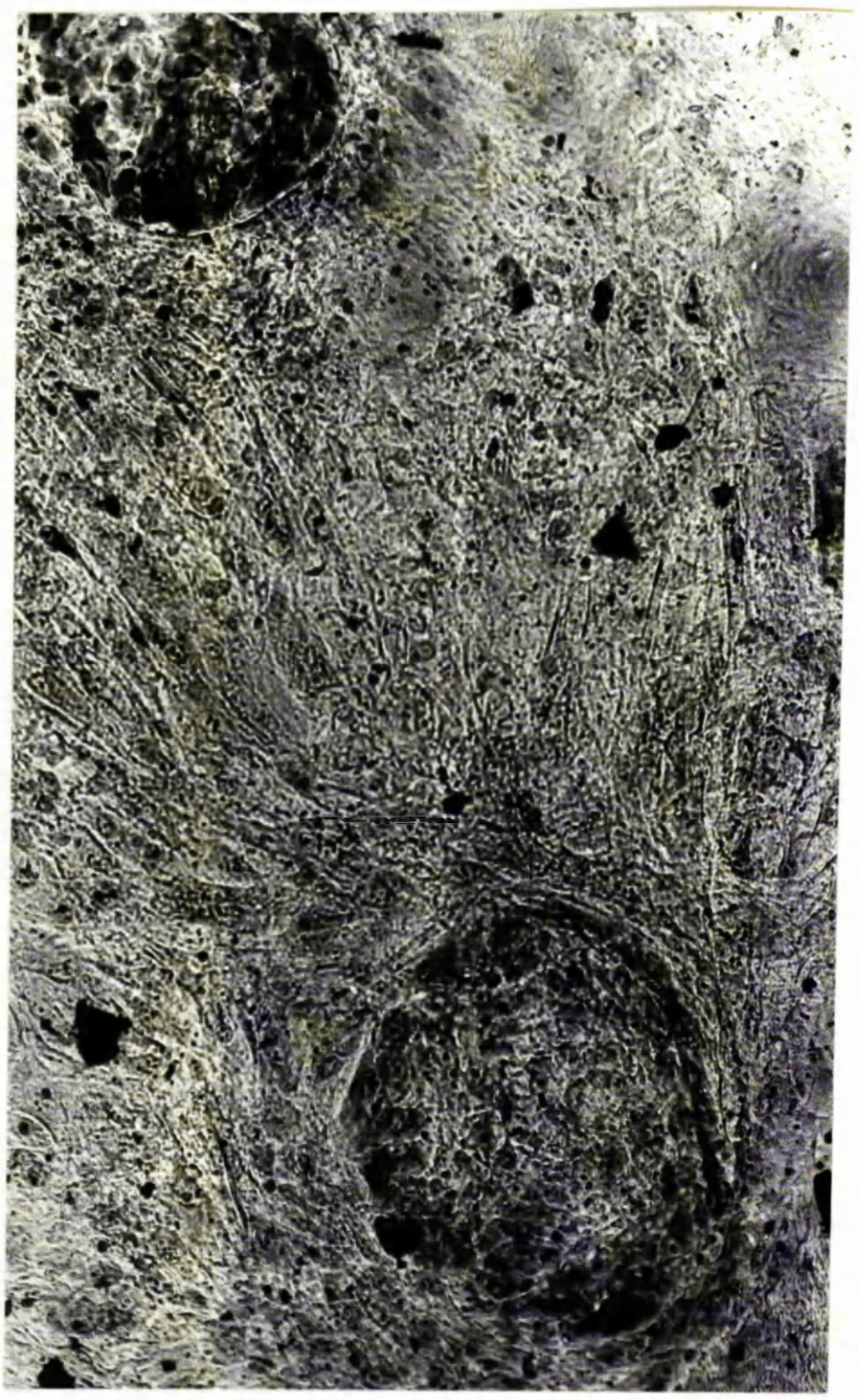


Figure 7.8 - Cultured Cells from a Pleural Effusion

An epithelial colony from the pleural effusion B-PES, grown on a feeder layer of FHI-4 cells. Cells were prepared as described in Section 6.3.1.4. The cells were grown in culture for 10 days, fixed with ethanol and stained with Giemsa. Note the increase in density of feeder layer at the edge of the epithelial colony (Magnification X 300).

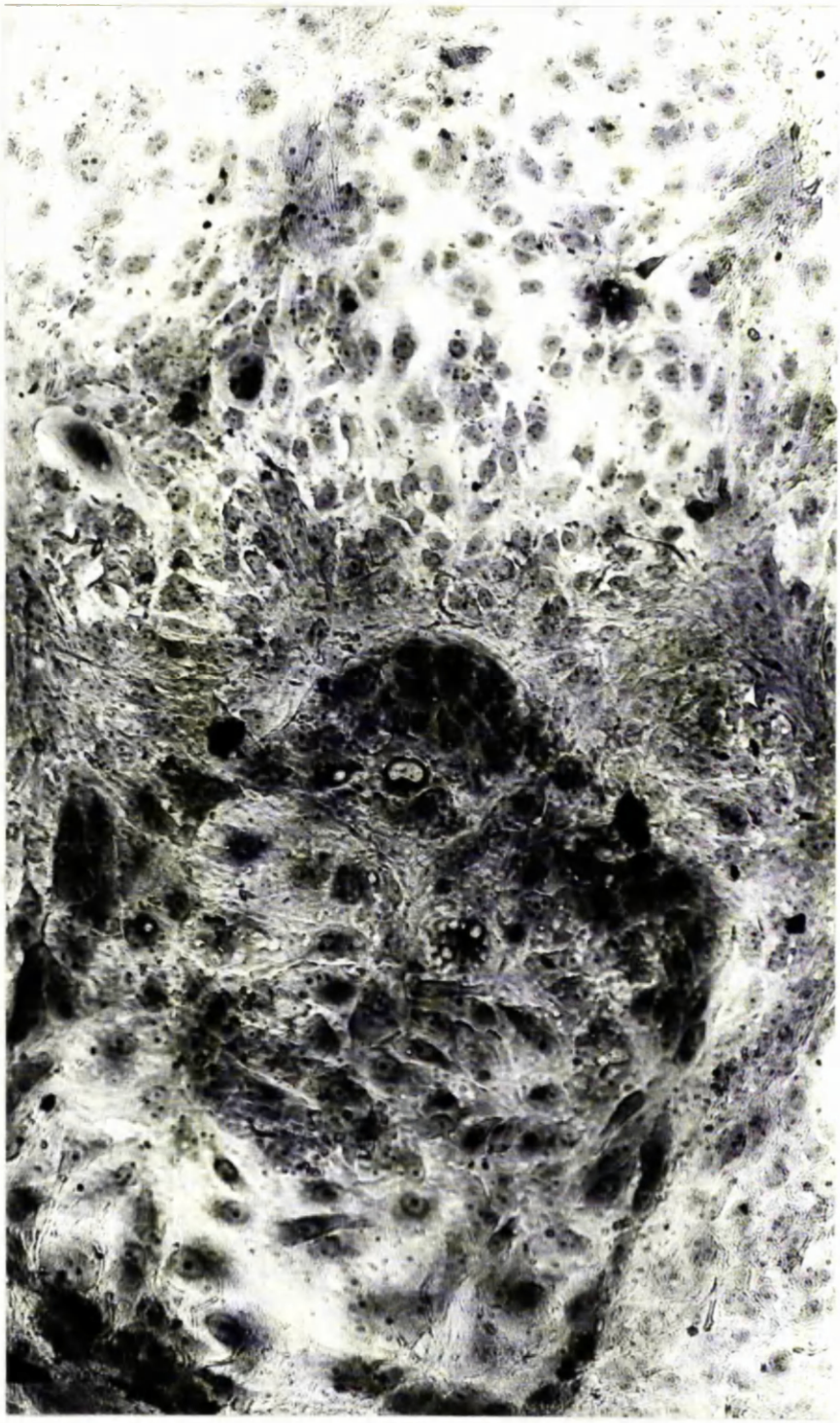


Figure 7.9 - Cultured Cells from a Pleural Effusion

Cells from the pleural effusion B-PES on a plastic substrate. Cells were prepared as described in Section 6.3.1.4. After growth in culture for 2 weeks cells were fixed with ethanol and stained with Giemsa. A number of the cells shown appear to be multinucleated. This property was not shown by cells grown on a feeder layer (Magnification X 300).

back the feeder layer, as shown by the increase in density of the cells round the colony.

During four months in culture there was no evidence of stromal growth. The original cells from both the pleural effusion and the ascites fluid were oestrogen receptor negative. In culture neither B-PES nor AF-ES showed an increase in growth after treatment with oestradiol (10^{-9}M) and cortisol (10^{-7}M).

7.5 CULTURE OF BREAST TUMOUR CELLS

Attempts to culture cells from breast tumour biopsies were most successful when, after digestion of tumour pieces in collagenase, the suspension of breast cells was transferred onto a feeder layer of FHI-4 cells. They settled down onto the feeder layer and grew slowly over a period of weeks as individual colonies (Fig. 7.10). These colonies grew from small clumps of tissue (2-10 cells probably epithelial since they were resistant to collagenase) and had a distinctive ring at the edge of the colony.

In total, 65 from 76 biopsies gave rise to ring colonies (85%) while the remainder gave rise to only fibroblasts. The ring colonies could be derived from 1) both primary and secondary tumours, 2) tumours of all histological grades and 3) both oestrogen receptor positive and negative tumours.

Figures 7.11 - 7.13 show a typical ring colony derived from an oestrogen receptor positive tumour, B-MGR. In the centre of the colony there

Figure 7.10 - Flasks of Cultured Breast Cells

25 cm^2 flasks of FHI-4 with ring colonies of breast tumour cells (staining more heavily). Cells were prepared as described in Section 6.3.1.2. Cells were grown for 4 weeks, fixed with ethanol and stained with Giemsa. The flask on the right was treated with oestradiol (10^{-9}M) and cortisol (10^{-7}M) and this caused an increase in the number of ring colonies.

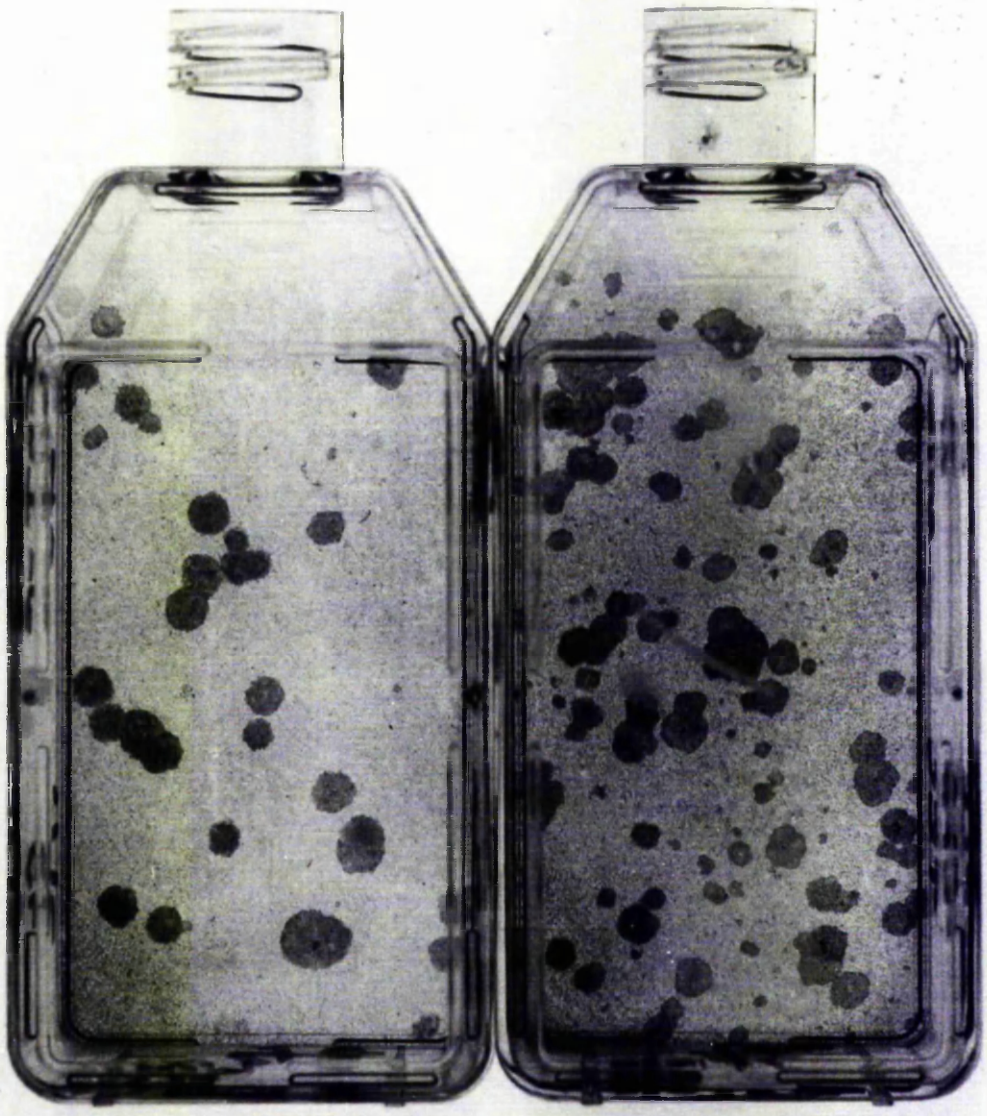


Figure 7.11 - Cultured Breast Tumour Cells

A ring colony (B-MGR) growing on a feeder layer of FHI-4. Cells were prepared as described in Section 6.3.1.2. from an oestrogen receptor positive tumour. The cells were grown in culture for 4 weeks then fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 120).

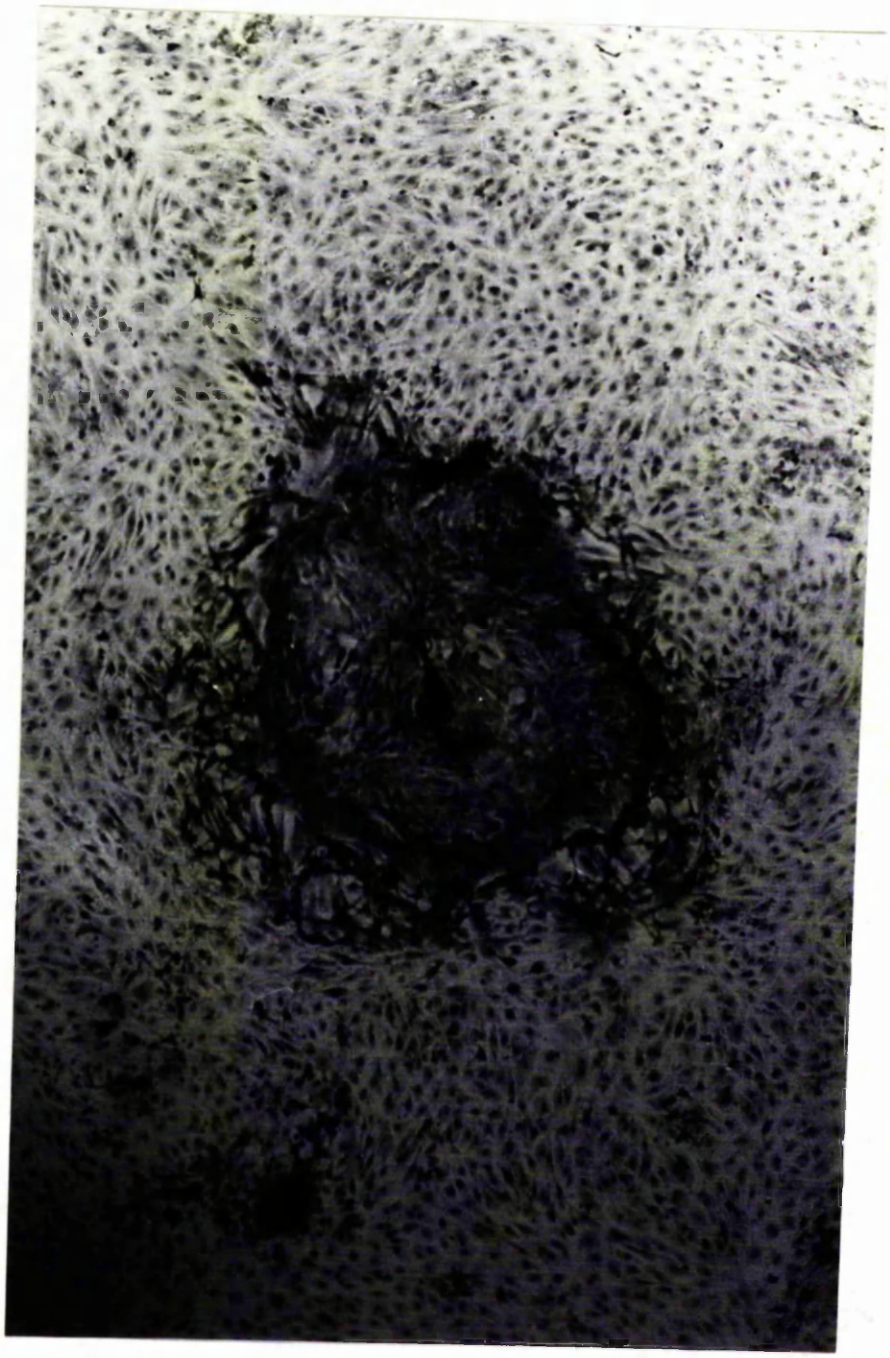


Figure 7.12 - Cultured Breast Tumour Cells

A ring colony (B-MGR) growing on a feeder layer of FHI-4. Cells were prepared as described in Section 6.3.1.2. from an oestrogen receptor positive tumour. The cells were grown in culture for 4 weeks then fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3. (Magnification X 300). The heavily stained area in the centre of the colony is probably the tissue from which the colony grew.

is a small heavily stained area which is probably the piece of tissue from which the colony grew. In this case the ring structure appears outwith the colony, on the feeder layer cells, as if the breast cells are causing the death of the feeder layer cells. At higher magnification, this colony is unusual because there are cells with two different types of morphology within the same colony (Fig. 7.13). The cells at the top edge of the colony are spindle shaped and almost fibroblast like in morphology. It is possible that this colony may contain a mixed population of epithelial and fibroblast cells though the slow growth rate makes a fibroblast origin of these cells unlikely.

The second colony (Fig. 7.14 - 7.16) was derived from another oestrogen receptor positive tumour (B-MHE). In Fig. 7.14 there is a small fibroblast colony above the top edge of the colony and its growth has been inhibited by the feeder layer. In this colony the ring structure appears within the colony and may be due to alignment of cells at the colony edge (Fig. 7.15). At certain points the colony can be seen growing over the feeder layer with apparently no ill effects to the feeder layer. At the highest magnification (960 X) in Fig. 7.16 the morphology of the ring colony cells (staining darker) can be compared with FHI-4 cells, to the top of the figure. The large spherical nuclei and prominant nucleolus shown by both cell types, are characteristic of epithelial cells.

Fig. 7.17 demonstrates further that some ring colonies can grow on top of the feeder layer with no ill effects to the FHI cells. This shows a tear in a ring colony derived from an oestrogen receptor negative tumour (B-EMA) with the feeder layer cells visible below.

Figure 7.13 - Cultured Breast Tumour Cells

The edge of a ring colony (B-MGR) growing on a feeder layer of FHI-4. This illustrates the differences in morphology, occasionally seen, between cells within a colony. Cells were prepared as described in Section 6.3.1.2. The cells were grown in culture for 4 weeks and then fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 300).

Figure 7.14 - Cultured Breast Tumour Cells

A ring colony (B-MHE) growing on a feeder layer of FHI-4. The heavily stained area at the top edge of the colony is a small fibroblast colony. Cells were prepared as described in Section 6.3.1.2. from an oestrogen receptor positive tumour. The cells were grown in culture for 4 weeks then fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 120).

Figure 7.15 - Cultured Breast Tumour Cells

The edge of a ring colony (B-MHE) growing on a feeder layer of FHI-4. This illustrates the 'ring' structure at the edge of the colony. Cells were prepared as described in Section 6.3.1.2. The cells were grown in culture for 4 weeks then fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 300).

Figure 7.16 - Cultured Breast Tumour Cells

This shows the edge of a ring colony (B-MHE) on a feeder layer of FHI-4 (lighter staining cells). The cells were prepared as described in Section 6.3.1.2. from an oestrogen receptor positive tumour. Cells were grown in culture for 4 weeks then fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 960).

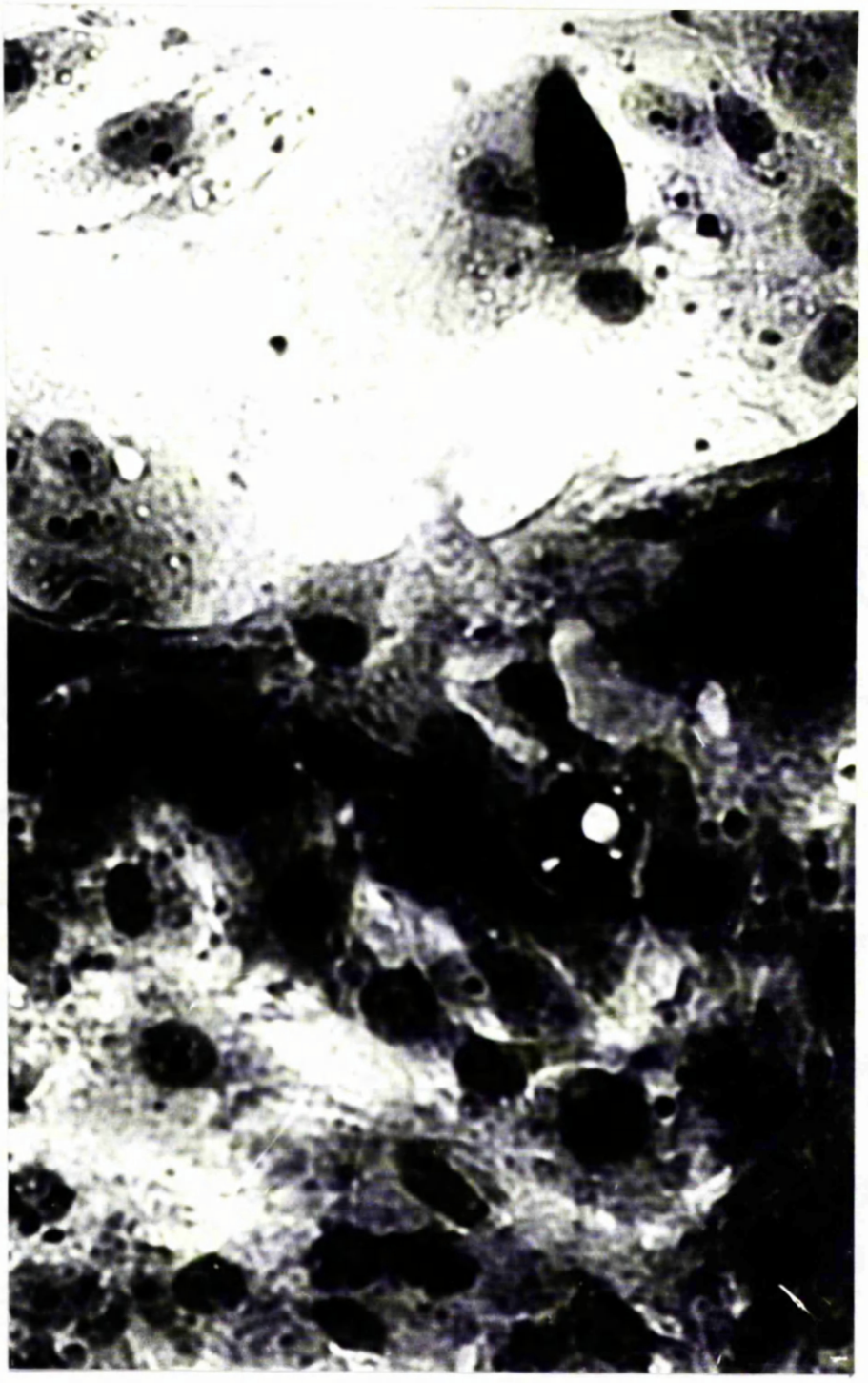


Figure 7.17 - Cultured Breast Tumour Cells

A gap in cells of a ring colony (B-EMA) showing the feeder layer cells (FHI-4) below. Cells were prepared as described in Section 6.3.1.2. from an oestrogen receptor negative tumour. Cells were grown in culture for 4 weeks, then fixed with ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 960).

7.6 SUBCULTURING BREAST TUMOUR CELLS

Trypsin digestion, although a harsher method, was eventually selected as the routine method of subculturing. Use of dispase was discontinued because the ring colonies and feeder layer usually detached simultaneously as a sheet of cells. This sheet of cells then had to be disrupted mechanically which was probably very damaging to the cells and resulted in small clumps of cells. The removal of dispase by centrifuging and washing is time consuming. In both cases only a small percentage of the cells grew after passaging. In approximately half of the samples, subculturing was found to cause a rapid increase in fibroblast growth. For this reason the effect of hormones and growth factors was determined on primary cultures and not passaged cells.

7.7 BREAST TUMOUR CELLS BY "LASFARGUE" METHOD

Breast tumour cells released on the slicing of the biopsy were recovered by centrifuging and washing. While a large number of cells could be recovered (in some cases $> 10^6$ cells/ml) a large proportion ($> 85\%$) appeared damaged when viewed by phase contrast microscopy and tested for exclusion of Trypan Blue. However, if these cells were placed in culture, approximately 30% (5 biopsies from 16) gave rise to epithelial colonies. In all cases there was a very low contamination with stroma though in most cases this grew to a significant level within 2 weeks. Figs. 7.18 and 7.19 show a typical colony prepared by the Lasfargue spillage technique from an oestrogen receptor positive tumour, B-PST-L. In Fig. 7.18 several individual fibroblast cells can be seen round the colony.

Figure 7.18 - Cultured Breast Tumour Cells

Breast tumour cells from an oestrogen receptor positive tumour (B-PST-L) on normal substrate. Cells were prepared by the Lasfargue 'spillage' method as described in Section 6.3.1.3. Cells were grown in culture for 10 days then fixed in ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 300).

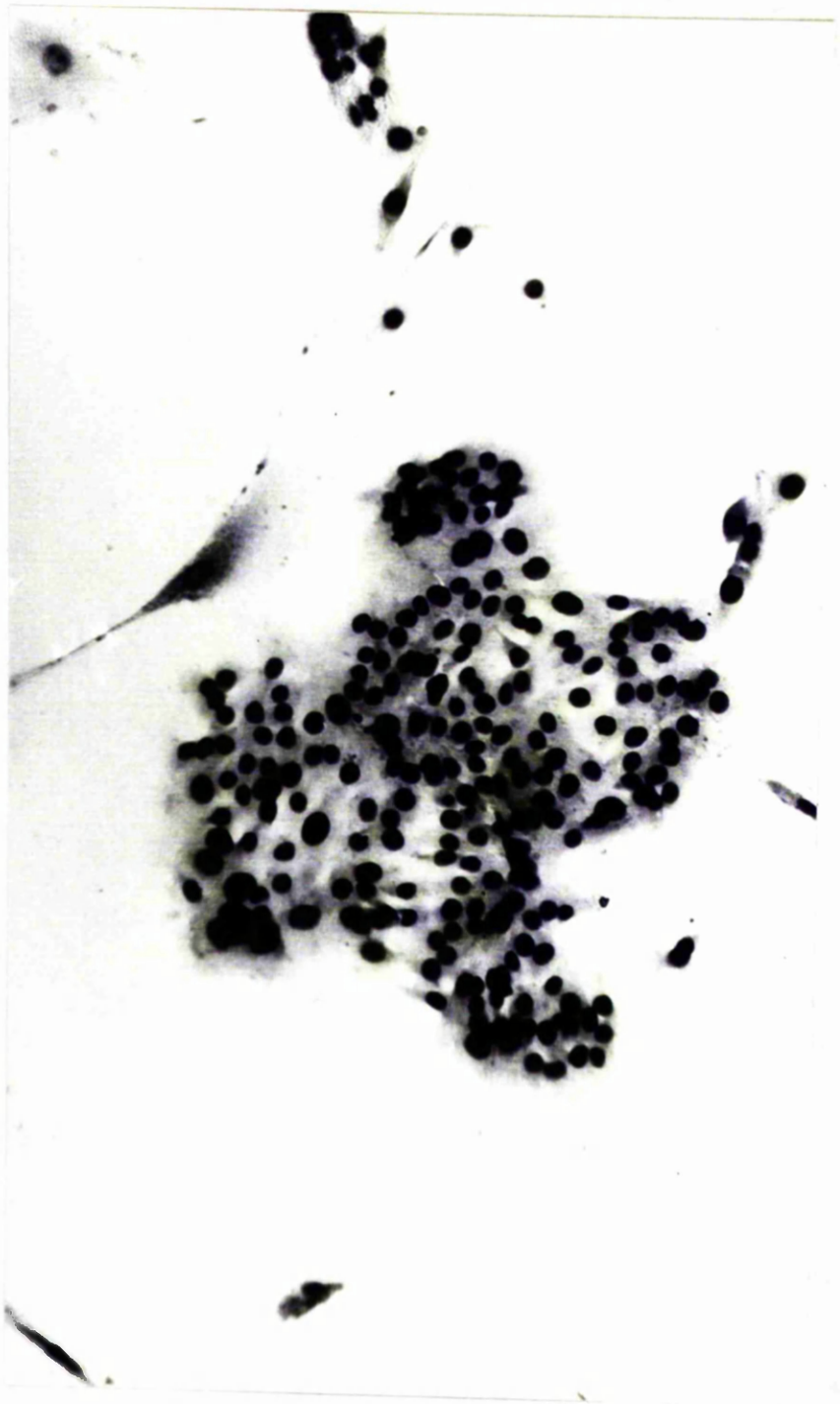
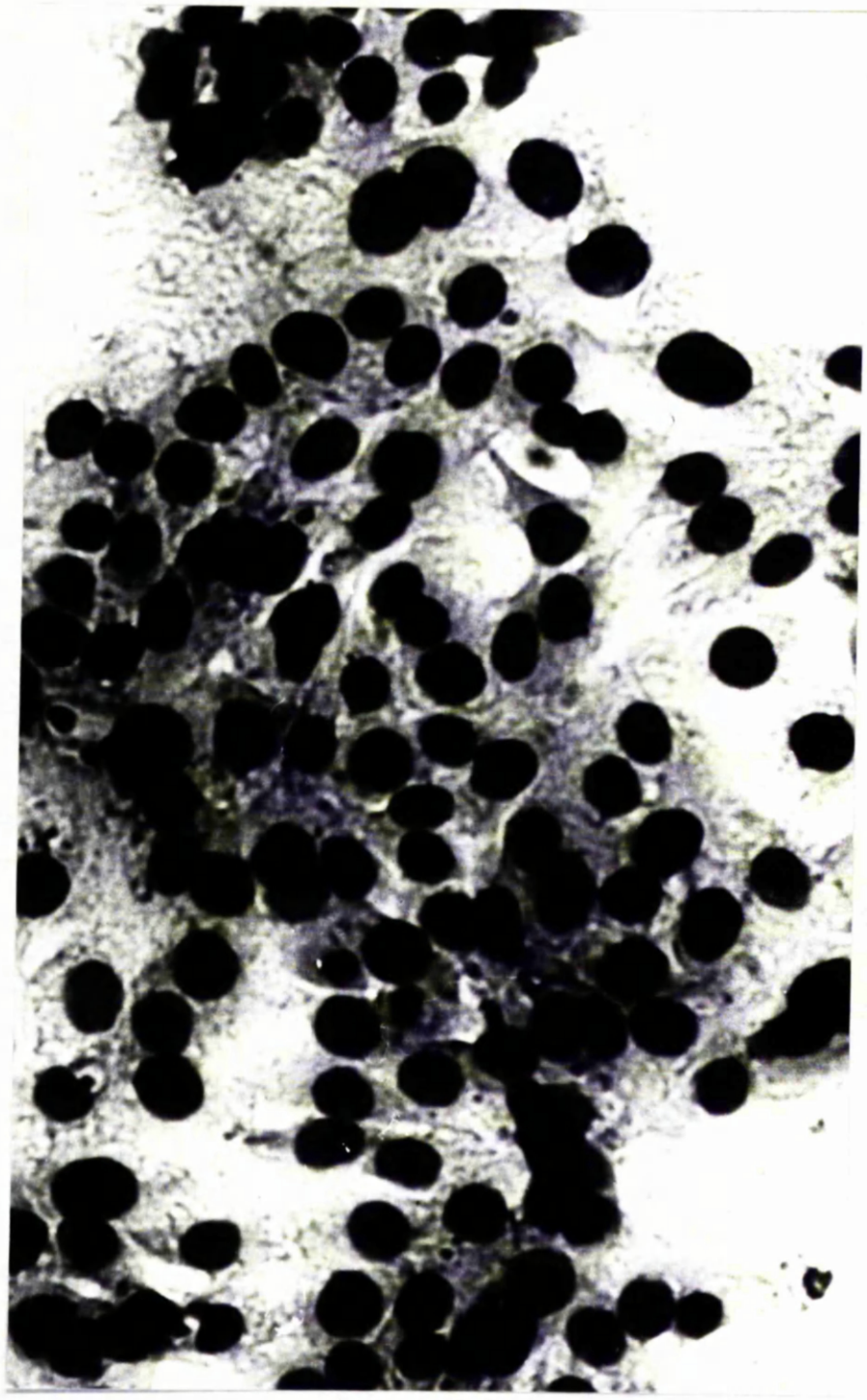


Figure 7.19 - Cultured Breast Tumour Cells

Breast tumour cells cultured from an oestrogen receptor positive tumour (B-PST-L) on normal substrate. Cells were prepared by the Lasfargue 'spillage' method as described in Section 6.3.1.3. Cells were grown in culture for 10 days then fixed in ethanol and stained with Giemsa as described in Section 6.3.5. to 6.3.6. (Magnification X 960).



When the 'spillage cells' were transferred onto a feeder layer of FHI-4 then colonies were established in approximately 70% of attempts (8 from 11 biopsies). These colonies were identical in morphology to those formed after collagenase treatment. In the small number of experiments performed the response of the 'spillage' cells to hormonal stimulation was identical to that observed in colonies derived by collagenase treatment.

7.8 CHARACTERISATION OF CELLS IN RING COLONIES

In order to demonstrate that the ring colonies are human breast cancer cells they must be shown to be both epithelial and malignant.

7.8.A Evidence of the Epithelial Nature

1. The morphology of the cells under light microscopy is consistent with them being epithelial. The cells form the characteristic paving-like arrangement seen in culture and have large spherical nuclei and prominent nucleoli (Fig. 7.16). However in some cases the morphology differs from that of a classical epithelial cell such as in Fig. 7.13 where the cells are more spindle shaped and fibroblast like.

2. The electron micrographs, shown in Figs. 7.20 - 7.22, further demonstrate the epithelial nature of these cells. Fig. 7.20 shows that this ring colony is several cells thick. These cells are involved in active synthesis as demonstrated by 1) low nuclear to cytoplasmic volume, 2) large amounts of rough endoplasmic reticulum (RER) which is swollen, probably with secretory

products, 3) large amounts of glycogen granules, 4) a number of active mitochondria some of which are very large, and 5) the large number of ribosomes. The epithelial nature of the cells is shown by 1) the surface activity (evident between cells), 2) tonofilaments, 3) tight junctions (thickening of cell membrane at areas of cell contact) which may be desmosomes, and 4) the prominent nucleolus in the lower cell.

The contrast between a healthy and damaged cell within a ring colony is shown in Fig. 7.21. The lower cell contains extensive golgi apparatus and large amounts of mitochondria and RER. However, the upper cells show extensive vacolation and 4-5 degenerating mitochondria. Anoxia is known to cause similar symptoms in the cell ultrastructure.

Fig. 7.22 shows a cell with a large amount of tonofilaments and tight junctions (possibly desmosomes) with tonofilaments going into them. The upper cell again shows a large number of organelles.

The fact that all figures show that the ring colony is several layers thick implies that the sections have been cut close to a piece of tissue released by the collagenase digestion, since the ring colonies appear to consist largely of a monolayer when studied by light microscopy.

3. The ring colonies were screened for the presence of tonofilaments using a monoclonal antibody (LE65) raised against a small molecular weight component of tonofilaments (Lane, 1982). The cells were fixed with acetone to remove all membranes and labelled with

Figure 7.20 - Electron Micrograph of Cultured Breast Tumour Cells

Cells from an oestrogen receptor positive tumour (B-TMC) were cultured on a monolayer of FHI-4 cells. The cells were prepared and sectioned for electron microscopy as described in Section 6.3.9. Thin sections were cut at right angles to the plastic substrate as described in Section 6.3.5. to 6.3.6. (Magnification X 22,050).

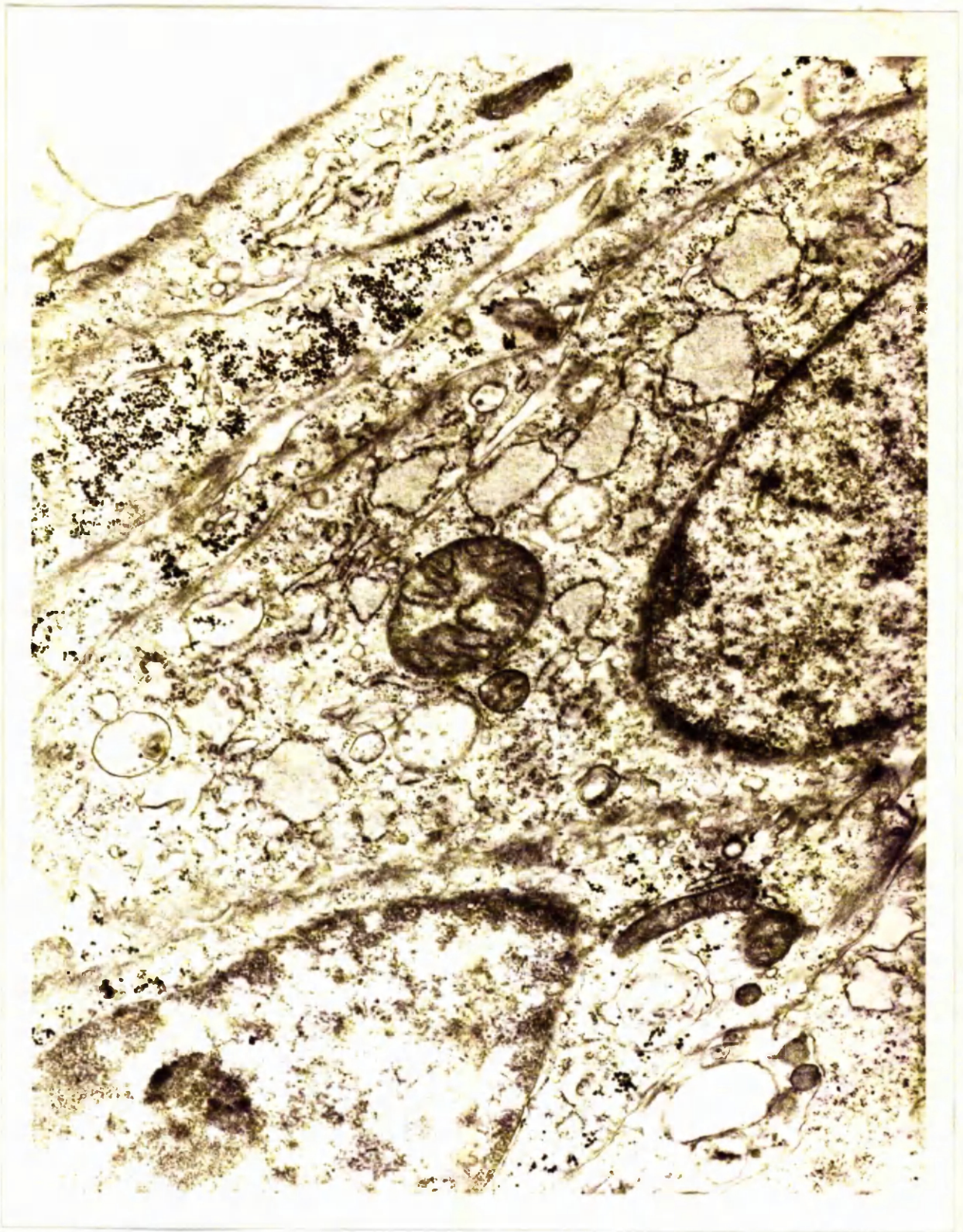


Figure 7.21 - Electron Micrograph of Cultured Breast Tumour Cells

Cells from an oestrogen receptor positive tumour (B-TMC) were cultured on a monolayer of FHI-4 cells. The cells were prepared and sectioned as described in Section 6.3.9. Thin sections were cut at right angles to the plastic substrate (Magnification X 22,050).

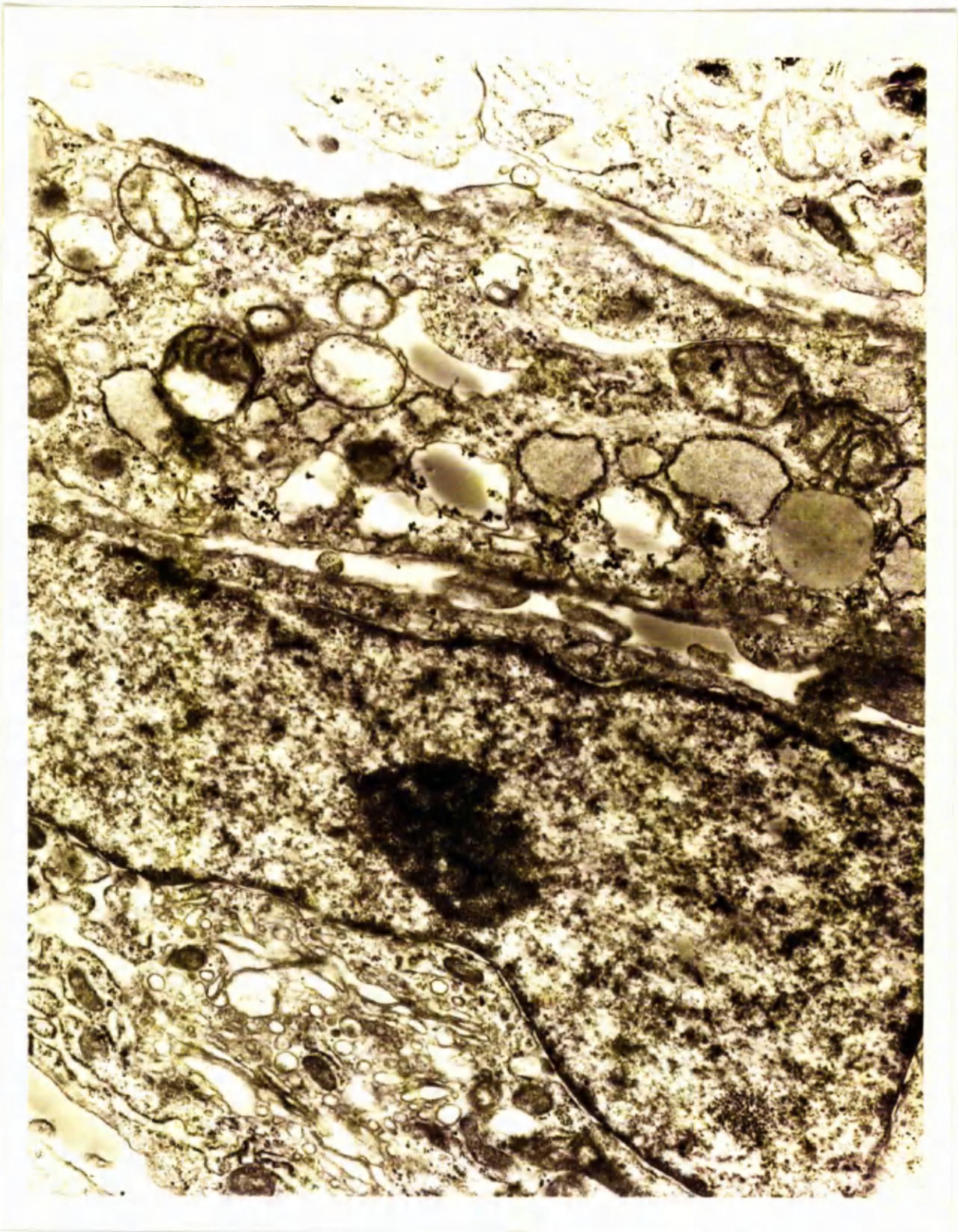


Figure 7.22 - Electron Micrograph of Cultured Breast Tumour Cells

Cells from an oestrogen receptor positive tumour (B-TMC) were cultured on a monolayer of FHI-4 cells. The cells were prepared and sectioned as described in Section 6.3.9. Thin sections were cut at right angles to the plastic substrate (Magnification X 49,500).

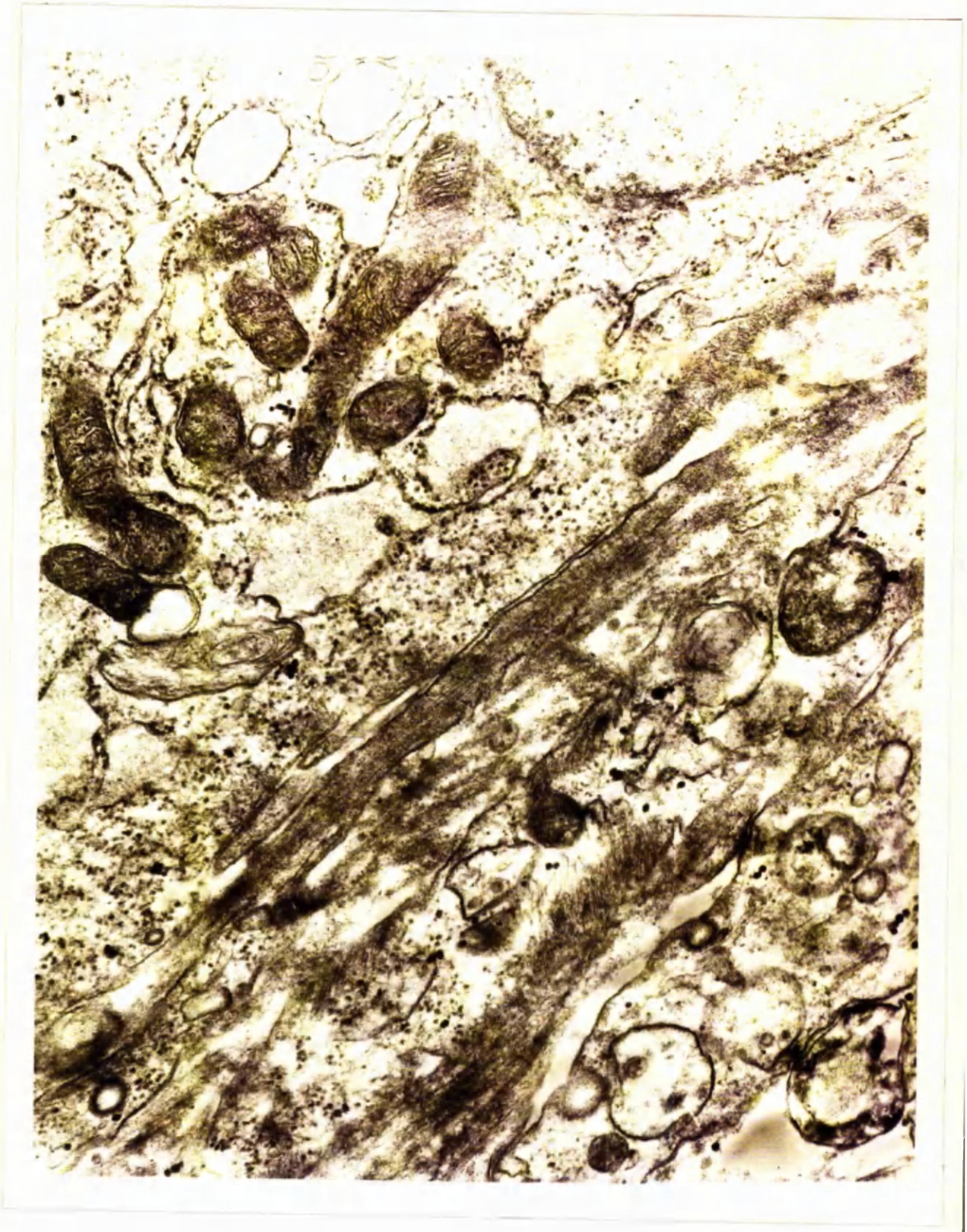
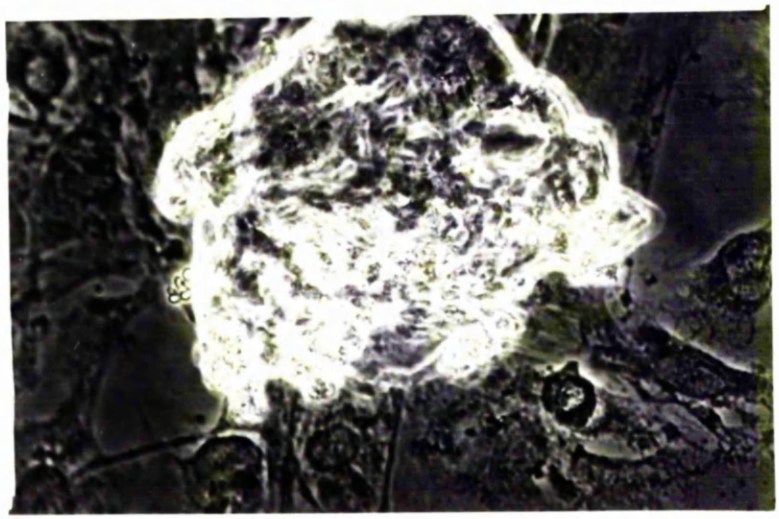


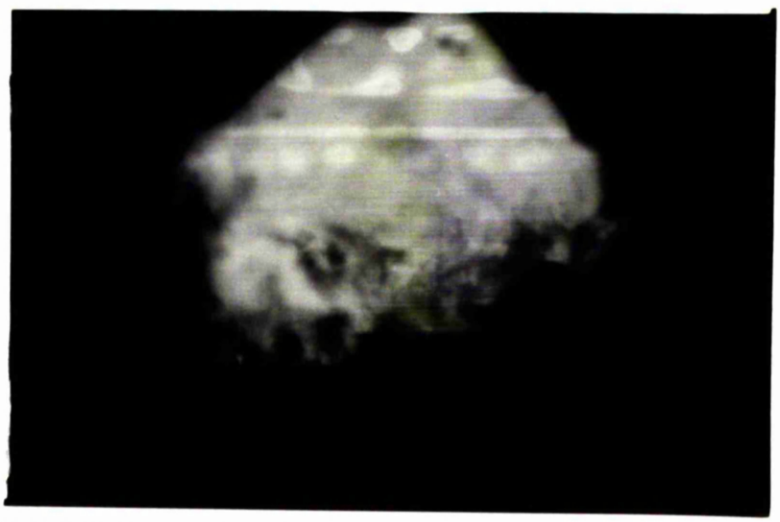
Figure 7.23 - Cultured Breast Tumour Cells

A ring colony from the breast tumour, B-MLI.
The cells were prepared as described in Section 6.3.1.2.
The cells were fixed with acetone and photographed under phase contrast microscopy as described in Section 6.3.12. (Magnification X 540).

Figure 7.24 - Immunofluorescence of Breast Tumour Cells

A ring colony from the breast tumour, B-MLI.
The cells were prepared as described in Section 6.3.1.2.
The cells were fixed with acetone and labelled with monoclonal antibody LE65 (against a component of tonofilaments) as described in Section 6.3.12.
Cells were photographed using ultra violet illumination (Magnification X 540).





the monoclonal antibody. Antibody binding was visualised using a fluorescein conjugated rabbit anti mouse IgG. Figs. 7.23 and 7.24 demonstrate that the fluorescence is localised over the ring colony. All ring colonies from three tumour cell lines tested (B-SUS, B-MLI and B-BOO) reacted with the monoclonal antibody. The low molecular weight component of tonofilaments is thought not to be expressed in established cell lines which explains why the FHI cells of the feeder layer failed to react with the monoclonal antibody.

7.8.B Evidence of Malignant Nature

Originally it was planned to demonstrate the malignancy of these cells by showing the presence of plasminogen activator, but the feeder layer of FHI-4 cells or 3T3 cells both produce plasminogen activator in large quantities (I. Freshney, personal communication) and so this method was discounted.

Nude or athymic mice were used in an effort to show that tumours could be raised after injection of the cultured cells. Six samples of cells were taken from 3 tumours, 1 pleural effusion (B-PES) and 2 breast tumour biopsies (B-ALO and B-PAC), after the cells had been in culture for 3 weeks. The cells from the pleural effusion were injected in 0.5 ml. medium containing 5×10^5 cells. The cells from the ring colonies were injected with a feeder layer of FHI-4 cells so that the exact number of tumour cells is unknown. The mice were then maintained under asceptic conditions for 6 months with no evidence of tumour growth. The animals were then sacrificed

Table 7.2 Measurement of Carcinoembryonic Antigen (CEA)

Carcinoembryonic Antigen levels were measured using an enzyme-linked immunoassay kit produced by Abbot Diagnostics. The assay was kindly performed by Mrs. Marion Meiklejohn on a suspension of cells in spent medium from a $25cm^2$ flask. Range of the assay 0 - 60 ng/ml. Results are expressed as mean $\pm$ the standard deviation.

Cells	Concentration (ng/ml)
FHI - 4/9	0
B - TMC on FHI - 4/7	0
B - MLI on FHI - 4/9	0
B - SUS on FHI - 4/9	5.25 $\pm$ 0.1

and no evidence of any cancerous growth was found at post mortem inspection. However, the failure to produce tumours does not indicate that the cells are not malignant since there is normally only a low rate of success in causing a tumour and the rate of success is lowest with breast tumours.

Table 7.2 shows the levels of carcinoembryonic (CEA) antigen in spent medium from three cultures of breast tumour and a feeder layer control. Only one tumour, B-SUS, produced CEA. The concentration is just greater than the range, detected by this assay in serum samples from normal specimens, (98% of which are below 5ng/ml). However, since this concentration was achieved by a very low number of cells it demonstrates that at least a portion of the cells in ring colonies are, by this definition, malignant. This result also implies that the tumour cells are fully viable after 3 weeks in culture since they are able to produce a high level of tumour marker.

7.9 HORMONAL MODULATION OF COLONY GROWTH

The level of several steroid hormones was measured by radioimmunoassay in the batch of foetal calf serum used in the following experiments. Table 7.3 shows that oestradiol is the only steroid hormone present in detectable amounts and that it can be reduced by 60% by charcoal stripping. The oestradiol concentration in the medium is 37pM (or 15pM after stripping) compared with 10^{-9}M (1000pM) used to stimulate growth of the ring colonies. It was, thus, decided not to use charcoal stripped serum since a) the change in oestradiol concentration was minimal with respect to the level being used to stimulate and

b) charcoal stripping may remove proteins or factors required for the ring colonies to grow (such factors have been demonstrated (Munir & Leake, 1982) for human endometrial cells in culture).

Growth was measured in $25cm^2$ flasks by both counting individual colonies and measuring the areas they covered. The cell density was measured in both control and hormone treated flasks and was found to be identical and therefore an increase in colony area should reflect an increase in cell number. Treatment of a control feeder layer flask with hormones or growth factors had no effect on the FHI-4 cells and which always remains as a monolayer.

Unfortunately the size of the breast tumour biopsies meant that only one or two measurements could be performed on each tumour. It was therefore not possible to construct a dose response curve and so the physiological concentration of the hormone and the concentrations of growth factors previously reported to cause a stimulation in growth of breast cells were used.

Table 7.4 shows the effect of a number of hormones and growth factors on the growth of ring colonies. Cortisol ($10^{-7}M$) but not oestradiol ($10^{-9}M$) caused an increase in growth of the breast tumour cells. However, oestrogen ($10^{-9}M$) and cortisol ($10^{-7}M$) seem able to act synergistically to cause a marked stimulation of colony growth. Cholera toxin (0.1ng/ml) on average also caused an increase in growth although the effects were variable between tumours.

Prolactin (1µg/ml) appeared to cause an increase in the colony number (possibly reflecting an increase in plating efficiency) but had no

Table 7.3 Steroid Hormone Levels in Foetal Calf Serum

The concentration of steroid hormones in the batch of foetal calf serum used in all experiments was measured by radioimmunoassay. This assay was kindly performed by Dr. G. Beastall (Royal Infirmary, Glasgow).

Hormone		Foetal Calf Serum	Charcoal Stripped Foetal Calf Serum
Cortisol	(nM)	< 28	< 28
Testosterone	(nM)	< 0.7	< 0.7
Oestradiol	(pM)	185	77
Progesterone	(nM)	< 2.0	< 2.0

Table 7.4 Hormonal Modulation of Colony Growth

This table shows the effect of various hormones and growth factors on the growth of treated epithelial ring colonies. Results are expressed as a percentage increase with respect to the control flask (probability that control and hormone treated populations are the same as calculated by a paired 't' test. N.S. = no significant difference, i.e. $P > 0.1$). Cells were prepared as described in Section 6.3.1.2 and growth measured as described in Section 6.3.7.1.

Hormone	Concentration	No. of Experiments	Colony Number (% increase w.r.t. control)	Area (% increase w.r.t. control)
Oestradiol (E2)	10^{-9}M	14	5.5 (N.S)	4.9 (N.S)
Cortisol	10^{-7}M	17	29.4 (P<0.005)	32.8 (P<0.005)
Oestradiol + Cortisol	10^{-9}M 10^{-7}M	14	46.3 (P<0.05)	74.8 (P<0.005)
Prolactin	1µg/ml	8	35.8 (P<0.005)	-4.7 (N.S)
Cholera Toxin	0.1ng/ml	6	37.2 (P<0.1)	28.5 (N.S)
Epidermal Growth Factor (EGF)	1ng/ml	17	-4.6 (N.S)	-2.0 (N.S)
EGF + Oestradiol + Cortisol	1ng/ml 10^{-9}M 10^{-7}M	5	-39.9 (P<0.025)	4.4

effect on the total number of cells. On average, there was no effect of epidermal growth factor (EGF) (lng/ml) treatment. When EGF was added with oestrogen and cortisol it appeared to prevent the stimulation caused by the steroids.

Table 7.5 illustrates that biopsies from older postmenopausal patients grew better in culture than cells from menopausal or premenopausal patients. This suggests that well differentiated tumours are more successful in culture than poorly differentiated tumours, since younger patients tend to have a more aggresive and hence pathologically poorly differentiated disease.

This suggestion is reinforced by Table 7.6 which demonstrates that oestrogen receptor positive tumours (well differentiated) are,on average,more successful in culture than tumours without receptor or with abnormal receptor. This implied that different classes of tumour, according to receptor status, might respond differently to hormonal stimulation. Table 7.7 shows the response to hormone treatment related to the oestrogen receptor status of the original tumour. Oestradiol receptor positive tumours show more of a response to oestradiol while cortisol exhibits a greater stimulation of receptor negative tumours. Both classes of tumour are stimulated by oestradiol plus cortisol. However the response of the receptor negative tumours is greater. Receptor negative tumours also show a greater tendency to respond to stimulation by EGF.

In order to demonstrate that the feeder layer was not responsible for effects of hormones, ring colonies were stimulated with oestradiol and cortisol. Table 7.8 shows that the response of the ring colonies

Table 7.5 Effect of Menopausal Status on Colony Growth

This table shows the effect of menopausal status on the growth of epithelial ring colonies in the absence of added growth factors. Menopause was defined as occurring between 48 and 52 years of age (unless a patient's form reported otherwise). Cells were prepared as described in Section 6.3.1.2. and growth measured as described in Section 6.3.7.1. Results are expressed as the mean ($\pm$ the standard deviation) of each individual experiment conducted. The standard deviations are large because of differences in the size of biopsies and, hence, the number of cells seeded into a flask. However growth of cells from postmenopausal samples is significantly different from that of both premenopausal (colony number $P < 0.05$ and area $P < 0.005$) and perimenopausa (colony number $P < 0.05$ and area $P < 0.005$) samples as calculated by a paired 't' test.

Status	Age	No. of Samples	Average Growth	
			No. of Colonies	Area (mm^2)
Premenopausal	48	8	23.7 (± 35.9)	27.1 (± 34.3)
Perimenopausal	48-52	7	28.2 (± 18.6)	30.8 (± 23.1)
Postmenopausal	52	36	44.8 (± 45.6)	99.9 (± 82.3)

Oestrogen Receptor Status	No. of Samples	Average Growth	
		No. of Colonies	Area (mm^2)
Receptor Positive (+/+)	25	47.9 (± 33.0)	105.6 (± 80.9)
Receptor Negative (o/o)	21	26.8 (± 23.1)	55.3 (± 48.8)
Abnormal Receptor (o/+ , +/o)	6	29.8 (± 17.3)	53.0 (± 40.0)

Table 7.7 Effect of Hormones on the Growth of Colonies with Respect to the Oestrogen Receptor Status of the Original Tumour

This table shows the effect of hormones on the growth of ring colonies in relation to receptor status of the original tumour biopsy, from which the cells were derived. Oestrogen receptor positive tumours contained more than 10 fmol/mg protein in the cytosol and more than 1000 fmol/mg DNA in the nucleus. Cells were prepared as described in Section 6.3.1.2. and growth measured as described in Section 6.3.7.1. Individual results represent the mean of all experiments conducted (probabilit that the effect of hormones on the growth of cells from receptor positive tumours is the same as the effect on cells from receptor negative tumours as calculated by a paired 't' test. N.S. = no significant difference, i.e. $P > 0.1$).

Hormone	Concentration	Receptor Status	Number of Experiments	% Increase in Colony Number	% Increase in area
Oestradiol	10^{-9}M	(+/+)	9	18.1	26.2
		(o/o)	5	-13.6	-24.7
				(P<0.005)	(P<0.005)
Cortisol	10^{-7}M	(+/+)	8	18.3	17.3
		(o/o)	9	42.1	48.6
				(P<0.025)	(P<0.01)
Oestradiol + Cortisol	10^{-9}M 10^{-7}M	(+/+)	8	51.2	39.6
		(o/o)	12	50.2	125.0
				(N.S)	(P<0.005)
Epidermal Growth Factor (EGF)	1ng/ml	(+/+)	11	-12.2	-9.5
		(o/o)	6	18.9	13.6
				(P<0.05)	(N.S)

Table 7.8 Effect of Oestradiol Plus Cortisol on the Growth of Ring Colonies on Different Substrates

This table illustrates the effect of different feeder layers on the stimulation in colony growth caused by oestradiol (10^{-9}M) and cortisol (10^{-7}M). Cells were prepared as described in Section 6.3.1.2. and growth measured as described in Section 6.3.7.1. Results are expressed as the mean (± the standard deviation) of each individual experiment conducted.

Substrate	Number of Experiments	% Increase in Colony Number	% Increase in Area
FHI-4 (Primary passage of tumour cells)	14	46.3 (±21.5)	74.8 (±24.6)
FHI-4 (Second passage of tumour cells)	8	68.8 (±37.8)	117.6 (±42.2)
FHI-F (Mitomycin C treated)	8	44.1 (±16.4	54.8 (±16.6)
3T3 (Mitomycin C treated)	7	43.3 (±18.4)	63.8 (±16.4)

to the steroids occurred at a constant level irrespective of the type of feeder layer. The fact that passaged tumour cells are more responsive to stimulation suggests that the more responsive cells are being selected as time in culture increases.

7.10 AUTORADIOGRAPHY OF RING COLONIES

Autoradiographs of hormone treated (figs. 7.27 - 7.29) and untreated (figs. 7.25 and 7.26) ring colonies show that there are both more cells and a higher proportion of more heavily labelled cells in hormone treated ring colonies. There appears to be some secondary structure in the arrangements of labelling since it also appears to form rings (of labelled nuclei) within the colony. Control coverslips showed that there was no labelling of the feeder layer irrespective of the hormone treatment. A small number of widely dispersed fibroblasts showed some labelling but these were easily recognisable by the spindle shape of the nucleus. The autoradiographs demonstrated that there is a mixed population of ring colonies (at least three types - unlabelled, partially labelled and totally labelled) as illustrated in fig. 7.29.

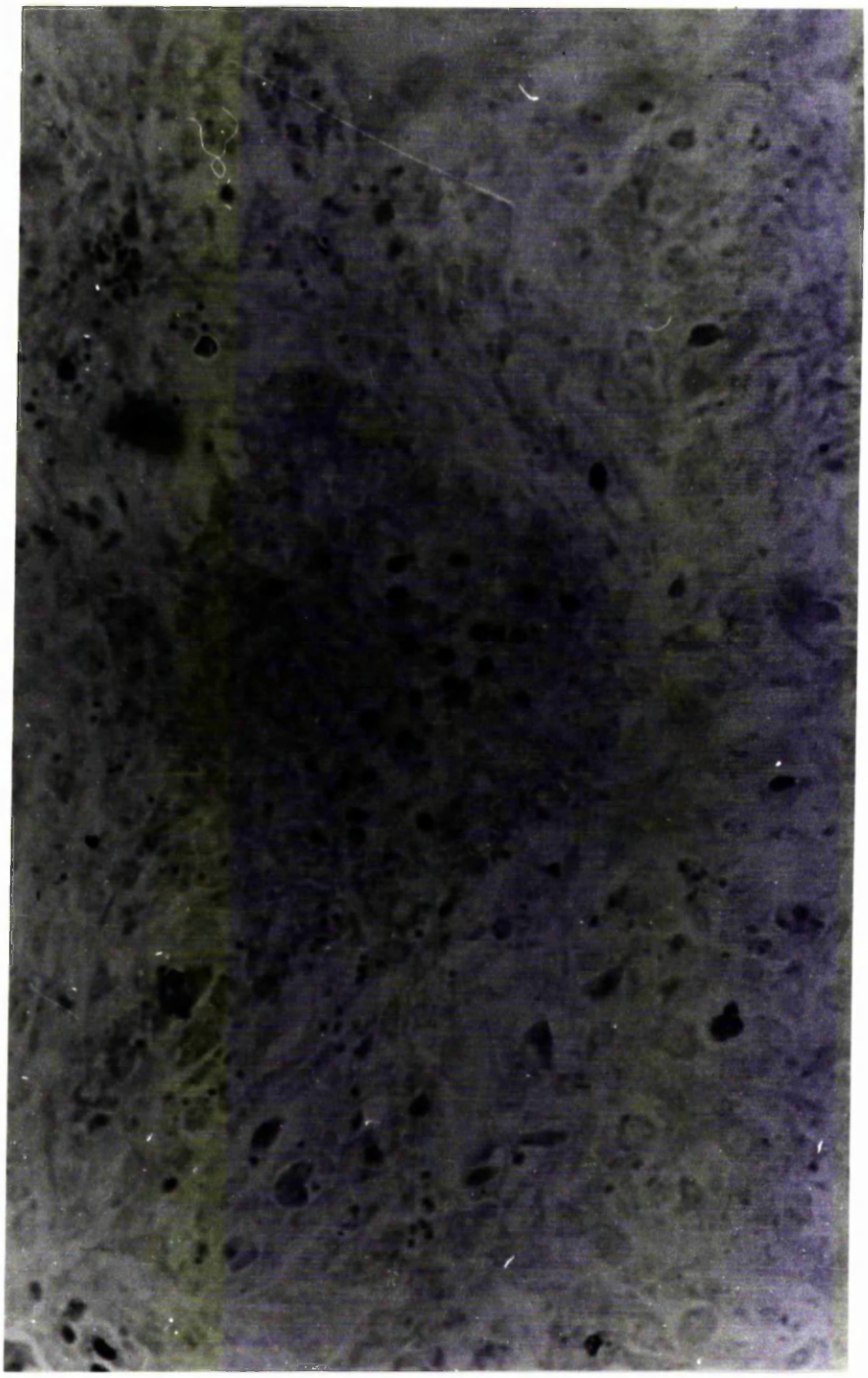
Table 7.9 shows that the number of cells labelled or the average number of grains over the nuclei (or radioactive incorporation) can be used to measure hormone stimulation. As it was not possible to measure TCA soluble counts the increase in grains could be due to an increased uptake of labelled precursor.

The use of autoradiographs would mean that more parameters could be studied and this would allow construction of a dose response curve

for drugs or mitogens for each biopsy because stimulation or inhibition can be detected in small amounts of tissue. This is demonstrated in Table 7.10 where the effect of three hormones was determined on cells from one small biopsy which would normally have allowed only one $25cm^2$ flask to be seeded. The stimulation achieved is in agreement with the results obtained in Table 7.4.

Figure 7.25 - Autoradiograph of Cultured Breast Tumour Cells

Autoradiograph (Magnification X 300) of an untreated ring colony after incubation with (^{3}H) thymidine as described i Section 6.3.7.2. Cells were derived from a biopsy of breast cancer (B-BOO) as described in Section 6.3.1.2. and had been in culture for 3 weeks.



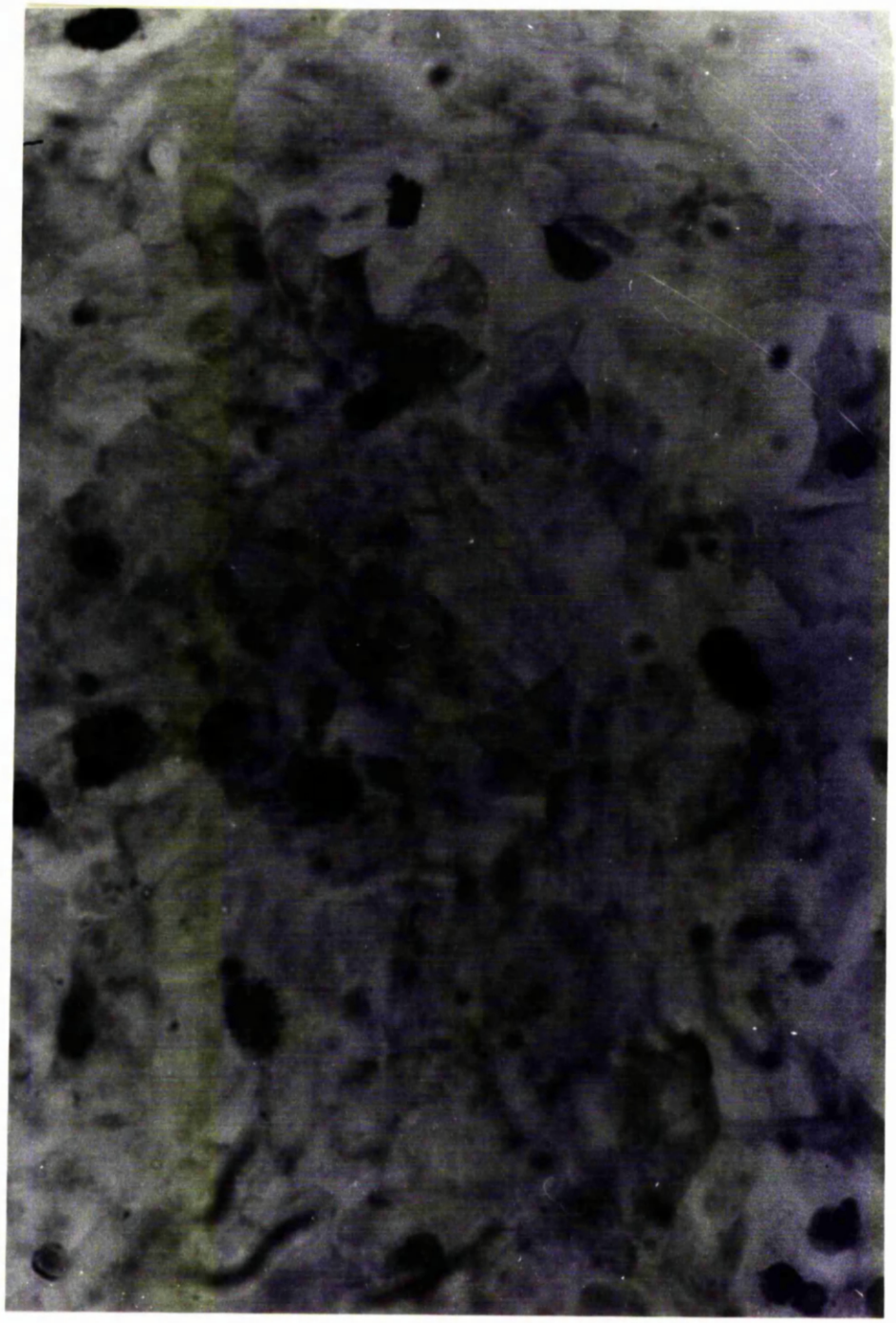


Figure 7.27 - Autoradiograph of Cultured Breast Tumour Cells

Autoradiograph (Magnification X 300) of a hormone treated (oestradiol and cortisol) ring colony, after incubation with (^{3}H) thymidine as described in Section 6.3.7.2. Cells were derived from a biopsy of breast cancer (B-BOO) as described in Section 6.3.1.2, and had been in culture for 3 weeks.

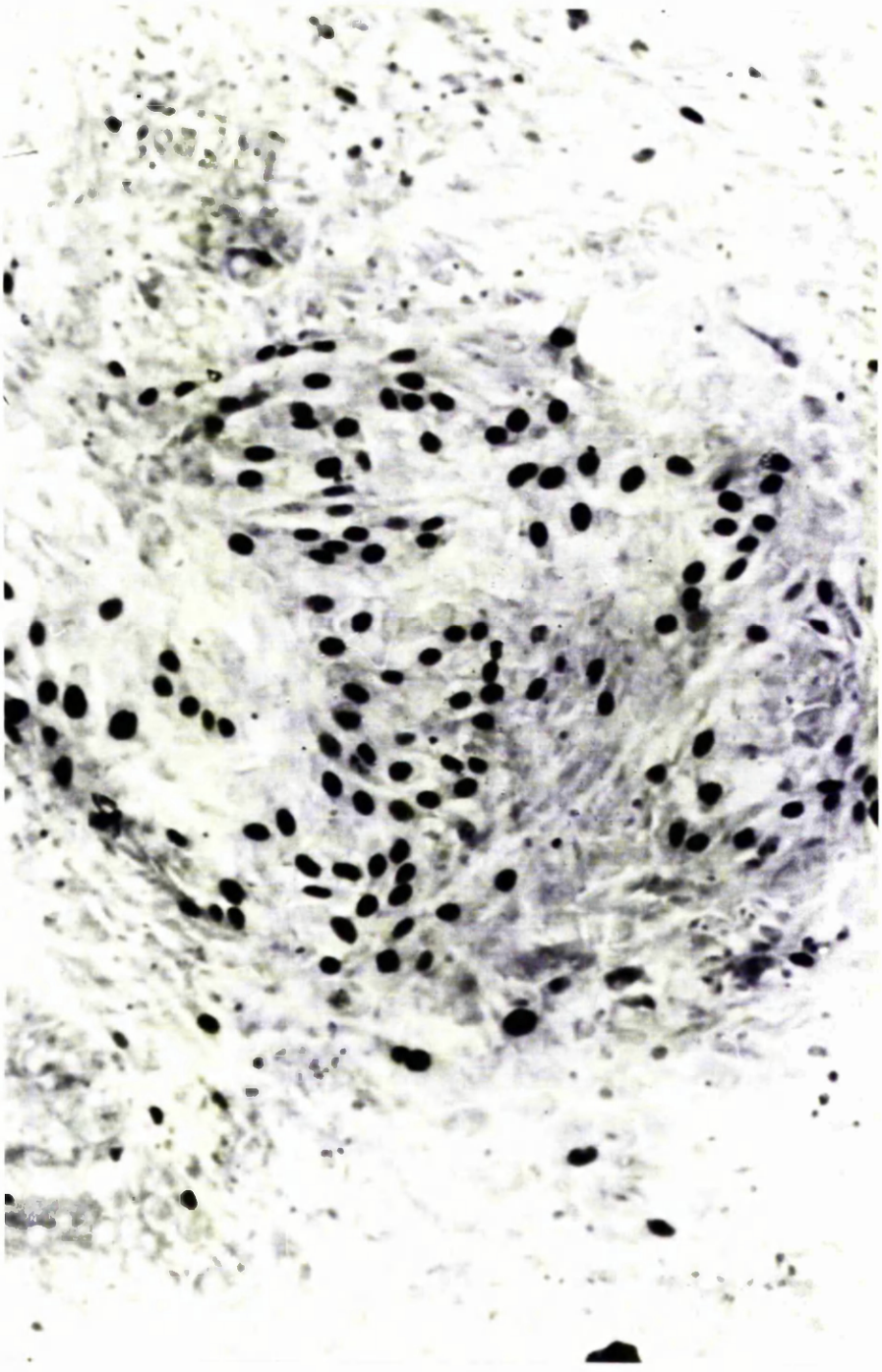


Figure 7.28 - Autoradiograph of Cultured Breast Tumour Cells

Autoradiograph (Magnification X 960) of a hormone treate (oestradiol and cortisol) ring colony after incubation with (^{3}H) thymidine as descçribed in Section 6.3.7.2. Cells were derived from a biopsy of breast cancer (B-BOO) as described in Section 6.3.1.2. and had been in culture for 3 weeks.

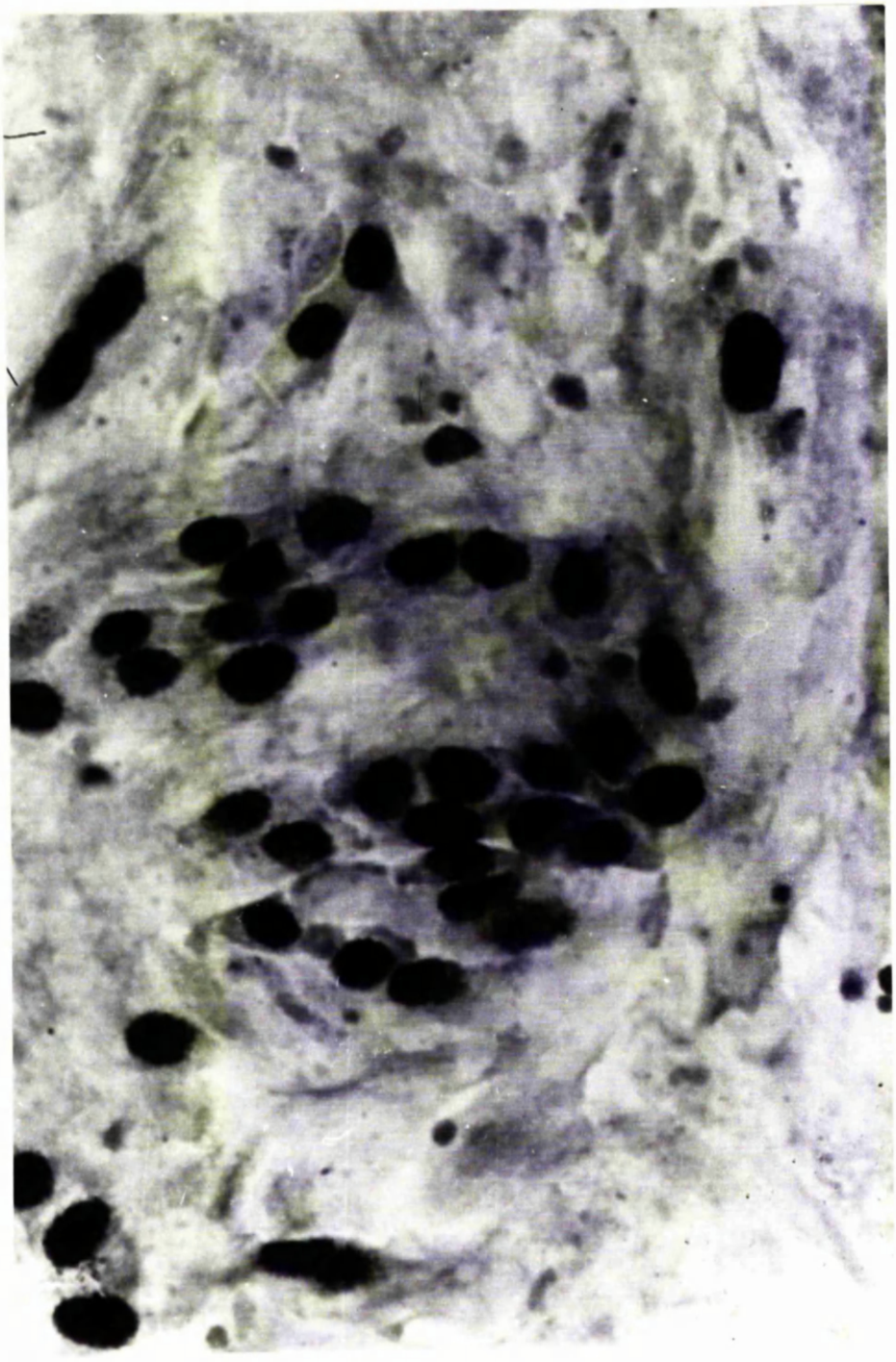


Figure 7.29. - Autoradiograph of Cultured Breast Tumour Cells

Autoradiograph (Magnification X 120) of hormone treated (oestradiol and cortisol) ring colonies after incubation with (^{3}H) thymidine as described in Section 6.3.7.2. Cells were derived from a biopsy of breast cancer (B-BOO) as described in Section 6.3.1.2. and had been in culture for 3 weeks.

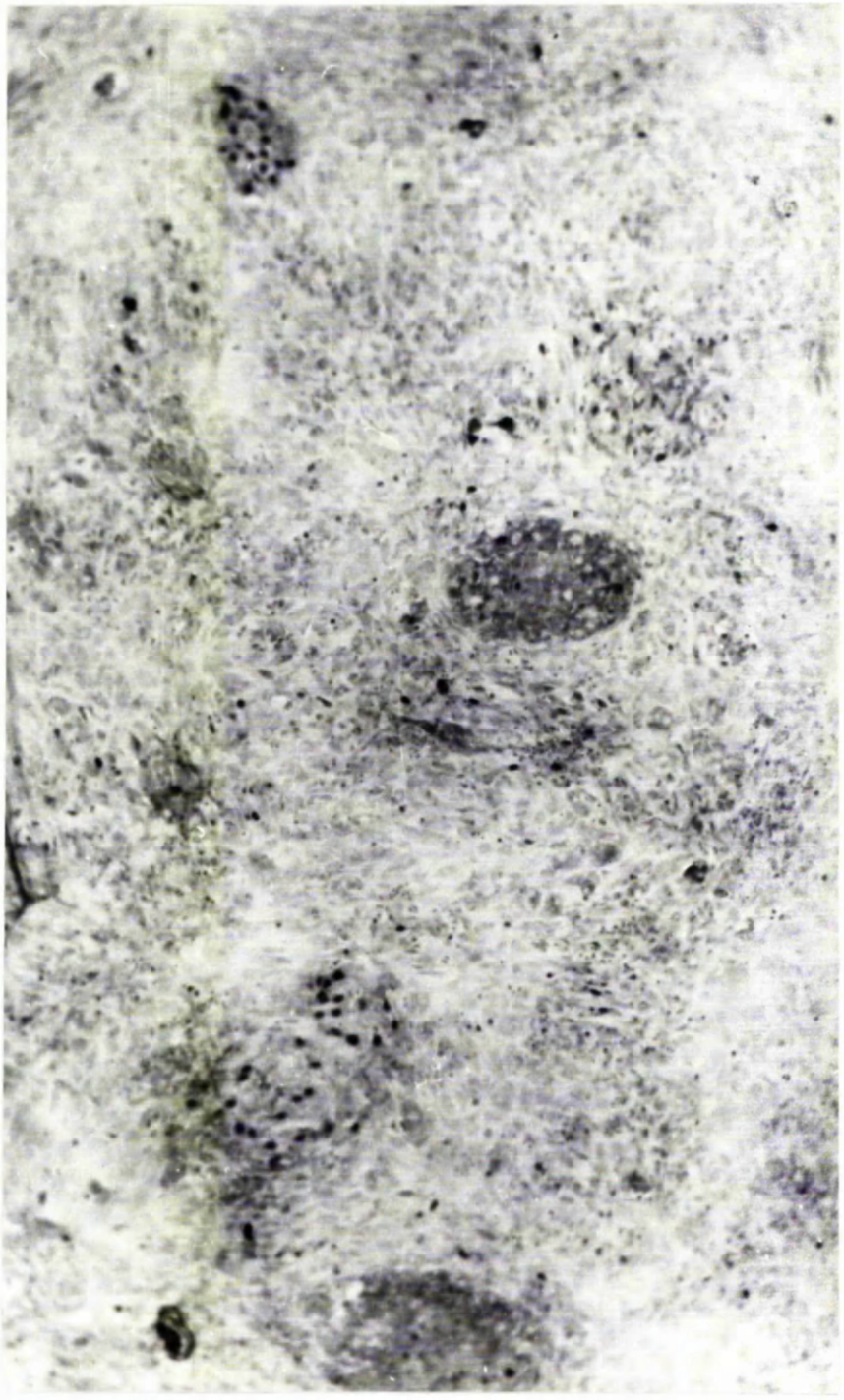


Table 7.9 Measurement of Hormonal Stimulation in Autoradiographs

The number of ring colonies and the area covered by ring colonies were counted as described in Section 6.3.7.1. The number of labelled cells per coverslip was counted on a Leitz inverted microscope. The number of grains per nuclei was counted in twenty nuclei per coverslip on a Leitz inverted microscope under oil immersion. All measurements were performed on ring colonies derived from the breast tumour B-BOO prepared as described in Section 6.3.1.2. Results are expressed as mean $\pm$ the standard deviation.

Method	Number of Experiments	Control ring colonies	Hormone treated ring colonies	% Increase
Number of ring colonies/coverslip	12	11.6 ± 4.8	19.7 ± 4.4	69.8
Area covered by ring colonies (mm^2/coverslip	12	1.13 ± 0.39	1.86 ± 0.41	64.6
Number of labelled epithelial cells/coverslip	6	606.5 ± 150.0	968.8 ± 153.0	62.7
Number of grains/ nuclei	6	45.8 ± 6.4	75 ± 17.1	63.7

Table 7.10 Effect of Hormones on (^{3}H) Thymidine Incorporation Into the Nuclei of Ring Colony Cells

Autoradiographic measurements were performed on ring colonies derived from the breast tumour, B-MLI. The number of grains per nuclei was counted in twenty cells per coverslip and averaged for 3 coverslips. Autoradiographs were prepared as described in Section 6.3.7.2. Grains were counted on a Leitz inverted microscope under oil immersion. Results are expressed as mean $\pm$ the standard deviation.

Addition	Concentration	Grains/Nuclei	% Increase over control
Control	-	63.2 ± 17.7	-
Cholera toxin	0.1 ng/ml	70.0 ± 25.0	+ 10.8
EGF	1 ng/ml	62.7 ± 18.2	- 1.0
Oestradiol	10^{-9}M	86.8 ± 23.5	+ 37.2

8. DISCUSSION

8.1 STROMAL GROWTH

The rapid growth of fibroblast cells from breast tissue has been widely reported (Lasfargue & Ozzello,1958; Owens *et al*,1976). This is one of the major reasons for the failure of most early attempts to culture breast epithelial cells (Orr & McSwain 1955; Lasfargue & Ozzello 1958; Whitescarver 1974). Despite the failure to detect steroid hormone induced stimulation of growth, a number of other groups have reported the presence of steroid hormone receptors in fibroblasts both *in vitro* (Fleming *et al*,1980; Ozasa *et al*,1980; Eil & Marx,1981) and *in vivo* (Pertschuk *et al*,1980). As yet there have been no reports of cultured breast fibroblasts containing steroid hormone receptors. This may be due to receptors being lost as the cells adapt to, or are selected for, rapid growth in culture. It is worthwhile continuing to search for a breast fibroblast cell line which responds to steroid hormone stimulation since the ease and speed with which these cells grow would make them an attractive system in which to study the mechanism of steroid hormone action. In particular, Skarda (1983) has recently suggested that stroma secrete EGF in response to oestrogen stimulation which in turn induces growth of breast epithelial cells. Hence stroma may play an important part in the effects of oestrogens on breast tissue.

8.2 FEEDER LAYER

The main purpose of a feeder layer is to prevent stromal attachment (Rheinwald & Green 1975 a:b). All four feeder layers tested were

successful at inhibiting but not completely preventing stromal growth. The least effective method tested was coating the flask with collagen. It may be that the coating used was too thin since Yang et al (1981) have reported that the thickness of the collagen layer is important in regulating which cells types can attach and grow on the surface. The source of the collagen may also be critical, since a higher rate of success has been reported using collagen prepared from rat tail (Yang et al, 1981; Furmanski et al, 1981). The second possible role for the feeder layer is that cells in the monolayer (even mitomycin C treated) are able to condition the medium by releasing factor(s) which aid the plating and growth of malignant epithelial cells. There was little difference between the growth of breast cells on a feeder layer of mitomycin C treated, 3T3 or FHI-F fibroblast cells. Namba et al (1981) and Smith et al (1981a) also found that the type of fibroblast line used as a feeder layer did not have an effect on cell growth. This means that either fibroblasts do not condition the medium or that this property is common to all fibroblast cell lines, and that no specificity is exhibited.

However, the greatly increased plating efficiency and growth, achieved when using the epithelial cell line, FHI-4, as a feeder layer suggests that some of the factors which are able to stimulate breast tumour cells are released only by specific cell types. This capacity of some cell types to condition medium was first proposed by Armstrong & Roseneau (1978). The idea has been extended by some groups (Stampfer et al, 1980; Smith et al, 1981a) which now use "spent" or conditioned medium from certain cell types to stimulate growth of both normal and malignant cells in culture. These growth stimulating factors appear to be specific, since the cell lines used to provide

the conditioned medium are all human and epithelial. The specificity of the growth factors secreted by the FHI-4 cells would explain why these cells were found to provide the most effective feeder layer since, like breast tumour cells, FHI-4 cells are undifferentiated human epithelial cells. Therefore it may be possible to improve the growth of breast tumour cells on collagen layers by adding conditioned medium from FHI-4 cells.

During growth of the cells from the pleural effusion BP-ES, the cells exhibited differences in morphology when grown on a plastic substrate (Fig. 7.9) or a feeder layer of FHI-4 (Fig.7.8). Similarly, a substrate dependent change in morphology has been reported for growth of MCF-7 cells on plastic or collagen (Leung & Shiu, 1982). This change in morphology was accompanied by a change in growth rates in response to serum or hormonal stimulation. However Table 7.10 shows that, despite growth on different feeder layers, the breast cells continued to respond to approximately the same relative degree to hormonal stimuli.

8.3 BREAST TUMOUR RING COLONIES

The success rate of 80% in establishing ring colonies from breast tumour biopsies is higher than previously reported. Green (1982) reported that only 30-40% of human tumours (depending on the site of origin) gave rise to significant colony growth. The difference may be due to the fact that Green reported the success rate in cloning tumour cells, and it is possible that clumps of tumour cells (as prepared by collagenase treatment) grow better than single cells. A second possible reason for the differences may be that the feeder layer

of FHI-4 cells, is particularly suited for culturing human breast tumour cells.

Both the mechanical (Lasfargue 'spillage' technique) and enzymatic (collagenase digestion) methods of preparing cells from breast tumour biopsies gave rise to similar cell growth. The cells exhibited a similar morphology under the light microscope and a similar response to stimulation by hormones on growth factors (Section7.7). The only difference was the ability of the cells prepared by the Lasfargue method to grow on a plastic substrate without rapid overgrowth by stromal cells. This suggests, in agreement with the original report (Lasfargue & Ozzello, 1958), that the cells prepared by the Lasfargue 'spillage' technique contain a very low proportion of stromal cells. The yield of viable cells is higher using the enzymatic method, with a large percentage (> 75%) of the cells, prepared by the mechanical method, damaged or lysed. Hamburger et al (1982) have reached similar conclusions, that the enzymatic and mechanical methods of preparing cells from tumours produced the same type of cells and that the yield of viable cells was much higher when using an enzymatic method.

A number of groups have reported either no success at all or, at least, difficulties in passaging primary cultures of breast tumours (Kirkland et al,1979; Stempfer et al,1979; Yang et al,1981). Trypsin treatment, used to passage breast tumour cell ring colonies growing on an FHI-4 feeder layer, appears to have an average success rate (~ 70%). This rate is higher than reported by Kirkland et al (1979) and Stampfer et al (1979) but less successful than that for cells grown on collagen gels prepared from rats tails (Yang et al 1981).

8.4 CHARACTERISATION OF BREAST RING COLONIES

8.4.1 EPITHELIAL NATURE

Several groups have relied heavily on the morphology, under light microscopy, of cells cultured from breast biopsies to prove that the cells were epithelial (Gaffney & Pigott, 1978; Klevjer-Anderson & Buehring, 1978; Yang et al, 1981; Poulson et al, 1982). Some groups have then assumed that the epithelial growth is all, or at least partly, due to malignant cell growth (Klevjer-Anderson & Buehring, 1978; Poulson et al, 1982) and not to the normal epithelial cells that must remain from the original infiltrated tissue.

Most groups reported that epithelial cells within individual islands of either normal or malignant cultured cells generally do not overlap each other and that growth is restricted to a monolayer (Buehring & Williams, 1976; Kirkland et al, 1979). They therefore conclude that growth patten (e.g. monolayer versus multilayered growth) cannot be used to distinguish between normal and malignant cells. The growth of the cells as a ring colony can also occur with tumour or normal cells. Growth is not necessarily invasive and occurs largely as a monolayer on top of the feeder layer. There have been reports that some of the cells prepared from breast tumour biopsies exhibit outgrowths capable of penetrative growth into collagen gels (Yang et al, 1981) or feeder layer (Smith et al, 1981b). This may be related to the portion of ring colonies which appear to push aside or kill the feeder layer (Fig. 7.12). In the future it may be possible to relate these properties to the invasive potential of the tumour.

Electron microscopy is the most popular method to demonstrate the epithelial origin of primary cultures derived from solid breast biopsies (Stampfer et al, 1980; Smith et al, 1981 a;b). However these studies have been limited to showing the presence of desmosomes, tonofilaments, microvilli, etc. in order to prove the cells to be epithelial. In this study (Figs. 7.20 - 7.22) all these characteristic features of epithelial cells were seen. In particular, active golgi bodies, rough endoplasmic reticulum, and mitochondria suggest that the cells are involved in active protein synthesis and secretion. This indicates that the majority of the cells remain healthy and viable after two weeks in culture.

There have been no studies which compared the morphology with that of the original tumour or studied the changes in morphology in the cells after hormonal stimulation or at different periods of time in culture. There have been a number of studies of the ultrastructure of established cell lines from pleural effusions (Russo et al, 1976; 1977; Osborne & Lippman, 1978) and solid tumours (Buehring & Hackett, 1974). Buehring & Hackett (1974) found a great variation in the ultrastructure of cells in lines established from solid tumours. This ranged from cell lines which resembled cells in primary biopsies through to cells which contained either a variable amount of, or no, desmosomes and tonofilaments.

Ultrastructur al studies of the cell line MCF-7 have shown that under certain conditions it closely resembles the original tumour from which the line was derived (Russo et al, 1976; 1977) and that changes occur on hormonal stimulation (Vic et al, 1982). Recently Stegner (1982) has reported that ultrastructural differentiation (such as the presence of golgi vesicles, desmosomes, interdigitations, microvilli, cytoplasmic

inclusions) can be used to predict the receptor status of the tumour. This is further evidence that the degree of differentiation can be used to predict the presence of oestrogen receptors and hence hormone dependence. The one sample studied was consistent with this report since the cells were derived from a receptor positive tumour and were found to be well differentiated.

The use of immunofluorescence to label tonofilaments in the ring colonies while providing further evidence that the cells are epithelial also conforms with the predictions of Lane (1982). This report predicts that antigen is only present in epithelial cells which have been in culture for a short period (i.e. the ring colonies) and not in long term cultured cells (i.e. FHI-4). This technique is potentially very useful since in the future it could be used to demonstrate the localization, over ring colonies, of a number of other human, breast or tumour markers such as the milk proteins lactalbumin or casein. Sasaki et al (1981) have raised antibodies against a number of surface proteins on breast cells. The antibodies have been used to study the origins of a number of primary and established breast cell cultures. This technique could be adopted to study the binding of plant lectins to the breast ring colonies. The extent of binding to cultured breast cells of the lectin Concanavalin A (Furmanski et al, 1981) has been related to the prognosis of the patient and early recurrence of disease, while the binding of the peanut agglutin to the carbohydrate moiety of milk fat protein on the surface of breast epithelial cells has been proposed as a marker of the presence of the oestrogen receptor (Klein, 1982). The binding, and extent of binding, of these antibodies and lectins to ring colonies could be used to highlight differences in

morphology between colonies, and hence cell types, derived from the same tumour. This would, hopefully, confirm that the cells within the ring colony are typical of the parent tumour, rather than highly selected.

8.4.2 MALIGNANT ORIGIN OF CELLS

There is a great deal of variation in reports of the rate of success in establishing tumours in nude mice. However, it is generally accepted that breast tumours are least likely to give rise to tumours in nude mice (Shimosata *et al*, 1976; Freshney, personal communication; Taylor-Papadimitrou, personal communication). The reasons for the low success may be related to the difficulties in establishing cultures. Giovanella *et al* (1974) reported a higher rate of success using cultured cells from established cell lines. However, the number of cells injected was very important. It is therefore probably not surprising that the three attempts to generate tumours from primary cultured cells did not succeed. It is generally accepted that failure to generate a tumour in nude mice does not prove that the cells are not malignant. Different cell numbers in the innoculate and cells from different passage numbers should be tested.

The secretion of carcinoembryonic antigen (CEA) by one of the primary cell lines indicates that at least some of the cells cultured in ring colonies are malignant. Not all breast tumours secrete CEA, Walker (1980) reported that 50% (45/90) of primary breast cancers produced CEA, resulting in significant serum levels. The percentage appears to be lower in established cell lines with only 1 out of 26 secreting CEA (Lippman *et al*, 1976).

Plasminogen activator has been proposed as a marker of malignancy but it is produced by both normal and malignant breast cells in culture (Kirkland et al, 1979; Yang et al, 1980b) and by feeder layers of FHI and 3T3 cells (Freshney, personal communication). Therefore any attempt to use plasminogen activator as a marker would require determination of the molecular weight. This would be justified if reports that secretion of plasminogen activator is related to invasive potential or hormone dependence are substantiated (Sherman et al, 1980; Thorsen, 1982).

In the future it may be possible to use immunofluorescence to localize tumour markers on ring colonies to determine if all the ring colonies are derived from malignant cells.

8.5 STIMULATION OF RING COLONY GROWTH

8.5.1 HORMONAL STIMULATION

A number of systems used as models of breast cancer, such as MCF-7 cells (Osborne et al, 1976; Butler et al, 1982) and DMBA - induced tumours in rats (Cohen & Hilf, 1974) have been shown to be very sensitive to insulin. It was not possible to grow ring colony cells in the absence of insulin (Laing, 1980).

The glucocorticoid receptor has been detected in a large proportion of human breast tumours with estimates varying from 33% (Teulings & Gilse, 1977) to 50% (Osborne & Lippman, 1978). Some caution is required in interpretation of this data, in that most of the supporting stroma (fibroblasts, leukocytes and adipocytes) are glucocorticoid target

tissues and contain glucocorticoid receptors. Osborne & Lippman (1978) also found that 3 out of 4 established cell lines from pleural effusions contained glucocorticoid receptors but responded to glucocorticoid treatment with a decrease in both growth and DNA synthesis. However, glucocorticoids have been widely reported as stimulating the growth of primary cultures from both normal and malignant breast cells (Gaffney & Pigott, 1978; Stampfer et al, 1980; Yang et al, 1981).

Cortisol was found to stimulate the growth of cells in the ring colonies in agreement with these earlier reports. However, there have been no reports of the extent of response to cortisol in vitro being related to the original tumour, in terms of, say, receptor status. However, Klevjer - Anderson & Buehring (1980) did report that hydrocortisone stimulated growth of malignant cells more frequently than growth of normal cells.

Reports of the response of primary cultured breast cells to oestrogen treatment have varied from no response (Kirkland et al, 1979) to a variable stimulation (Klevjer - Anderon & Buehring, 1980; Stampfer et al, 1980). Part of the explanation may lie in the fact that the response to oestradiol was more distinct when tumours were divided with respect to the receptor status of the original tumour tissue. However, the response to oestrogen treatment, both in ring colonies (Table 7.4) and in published reports of primary cultures of breast cells, is much lower than that originally reported for MCF-7 cells (i.e. a doubling in the number of cells in 8 days relative to a control), although MCF-7 cell lines no longer respond to oestrogen stimulation to this extent (Page et al, 1982).

There have been reports of hormones or growth factors acting synergistically in the stimulation of breast cells. There have been no previous reports of oestrogen and cortisol acting synergistically to cause an increase (74.8%) in cell number. It is unlikely that this is caused by oestradiol inducing glucocorticoid receptor since this stimulation was greater (125%) in cells derived from oestrogen receptor negative tumours than in similar cells from receptor positive tumours (39.6%). Similarly there have been no reports of cortisol causing induction of the oestrogen receptor.

The effects of prolactin on cultured breast tumour cells are controversial. There have been no receptors for, or response to, prolactin detected in cell-lines derived from pleural effusions (Osborne & Lippman, 1978), but DMBA - induced breast tumours in rats are very sensitive to prolactin (Asselin & Labrie, 1978). Prolactin is also known to play an important part in growth and development of rodent mammary glands (Turkington, 1972). Klevjer - Anderson & Buehring (1980) found that prolactin stimulated the growth of normal cells more frequently than malignant cells. Treatment of the ring colonies caused an increase in the number of colonies but no corresponding increase in the number of cells. This suggests that prolactin increases the plating efficiency but not the rate of growth.

8.5.2 STIMULATION BY GROWTH FACTORS

Cholera toxin and other factors which increase intracellular cAMP have been widely reported as stimulating the growth of primary cultures of both normal and malignant breast cells (Taylor - Papadimitriou *et al*, 1980; Yang *et al*, 1980; Yang *et al*, 1981; Stampfer, 1982). There

have been reports of a more variable response to cholera toxin in malignant breast cells, the response in the ring colonies was also found to be variable. This may be due to the already elevated levels of cAMP in some breast tumours (Elliot et al, 1981).

Epidermal growth factor (EGF) has been reported as stimulating normal breast cells in culture (Kirkland et al, 1979; Stampfer et al, 1980; Yang et al, 1980) but EGF treatment of cells derived from malignant tissue has been reported as causing occasional stimulation (Yang et al, 1980) or no response (Kirkland et al, 1979). EGF treatment of cells in ring colonies, while variable, had on average no net effect. The failure of EGF to stimulate growth of the ring colonies could be explained if one of the factors secreted by the FHI-4 feeder layer was EGF. But when oestradiol, cortisol and EGF were administered simultaneously then there was no stimulation, suggesting that EGF could block the stimulation by oestradiol and cortisol and hence could not be one of the factors released by the feeder layer.

In the future it may be possible to use immunofluorescence to determine if hormones and growth factors can cause changes in protein induction and synthesis in cultured cells. It may also be used to detect different cell types by their differing responses to hormonal stimulation.

A number of groups (Kirkland et al, 1979; Klevjer - Anderson & Buehring, 1980; Yang et al, 1981) have reported culturing epithelial cells with different types of morphology in primary cultures. The primary cultures giving rise to ring colonies also contain a mixed population of epithelial cells (possibly up to three types). This is best demonstrated by

autoradiography by the differing patterns of labelling (Fig. 7.29). It is widely accepted that breast tumours *in vivo* contain a mixture of different malignant epithelial cells.

8.6 POTENTIAL USES OF AN IN-VITRO SYSTEM

The fact that the growth pattern of primary cultures from breast biopsies can be related to the menopausal status or oestrogen receptor status of the original tumours (Tables 7.5 - 7.7) indicates that some of the properties of the primary tumours are retained in culture and reflected in the growth pattern. This suggests that cells cultured from the breast tumour biopsies could be typical of the original tumour and, therefore, used to predict such things as hormone responsiveness or drug sensitivity of the tumour within the patient. However, Von Hoff has shown that the *in vitro* - *in vivo* correlation remains 60% for sensitivity and 90% for resistance over a wide range of tumour types from a number of laboratories (Green, 1982). These figures are comparable with the accuracy of oestrogen receptor determination on a fresh biopsy in predicting hormone dependence. Therefore, the correlation between response of the cultured breast cells and growth of the tumour *in vivo* would have to be shown to be an improvement over the oestrogen receptor values, currently used to predict hormone dependence, in order to justify the extra cost involved in cell culture. This correlation between cultured cells and the primary biopsy would most easily be tested by measuring the oestrogen receptor content of the ring colonies, (possibly by thaw - mount autoradiography (Stumpf *et al* 1981)) and comparing the receptor content with that of the original tumour.

The close correlation between the two methods of measuring hormonal stimulation means that (^{3}H) thymidine incorporation can be confidently related to cell growth. The advantage of autoradiography is that an increased number of experiments could be performed on each biopsy (such as Table 7.10). This would allow the construction of dose response curves for a number of drugs and hormones, permitting the comparison of different drugs and drug concentrations. In general terms, the system could be used to study in detail the effects of different hormones and growth factors, under controlled conditions, to provide information on the factors which control growth of breast tumours. In clinical terms this system could be used to predict the aggressiveness of individual tumours and thereby to stratify patients for adjuvant therapy. Cell culture could also be used to select the most suitable type of drug therapy for each individual patient by studying the growth inhibition of cells cultured from the primary tumour.

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