PROGESTIN RECEPTOR HETEROGENEITY IN A BREAST CANCER CELL LINE

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This dissertation is submitted to the Faculty of Science, Rhodes University, Grahamstown, in fulfillment of the requirements for the degree of Master of Science in Biochemistry.

Johannesburg 1995

DECLARATION

I declare that this dissertation entitled "Progestin Receptor Heterogeneity in a Breast Cancer Cell Line" is my own unaided work. It is being submitted to Rhodes University in fulfillment of the requirements of the Degree of Master of Science in Biochemistry and has not been submitted for any degree or examination at any other University.

Anita Levy

1995

DEDICATION

TO JONATHAN, MARC AND NEIL

ACKNOWLEDGEMENTS

It was a priviledge to have Prof. L.A. van der Walt as my mentor and supervisor. I greatly appreciate Prof. van der Walts' faith, encouragement and support which continually spurred me on to complete this task.

I wish to acknowledge the following:

The South African Insitute for Medical Research for providing the necessary facilities to carry out this research.

Dr. A. Davidoff for giving of her very valuable time to instruct me in the insights of flow cytometry.

Prof. P. Gray for giving me the time to see this thesis through to the end.

The Chemical Pathology department for their friendship, encouragement and for making me feel so much part of the team.

My beloved mother who never lived to see the end of this chapter in my life.

LIST OF ABREVIATIONS

ACTH	adrenocorticotropic hormone
ALD	aldolase
AR	androgen receptor
BrDU	bromodeoxyuridase
BSA	bovine serum albumin
CBG	corticosteroid binding globulin
DCC	dextran coated charcoal
DES	diethylstilbestrol
DNA	deoxyribonucleic acid
DPM	disintergrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
ER	oestrogen receptor
ERE	oestrogen response element
FCS	foetal calf serum
fmol/mcp	femtomoles per milligram cytosol protein
^3H E_2	[2,4,6,7-^{3}H]-oestradiol-17 *B*
^{3}H-org	^{3}H-organon 2058
GR	glucocorticoid receptor
SDG	sucrose density gradient
HPLC	high performance liquid chromatography

HPIEC	high performance ion exchange chromatography
Hsp	heat shock protein
IGF	insulin growth factor
kd	disassociation constant
kDA	kilodaltons
MW	molecular weight
NSB	non specific-binding
PBS	phosphate buffered saline
PI	propidium iodide
PRE	progesterone response element
PR	progestin receptor
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
SDG	sucrose density gradient
SHBG	sex hormone-binding globulin
S	Svedberg/sedimentation unit
TGF	transforming growth factor
TAM	tamoxifen
OHTAM	4-hydroxytamoxifen

ABSTRACT

Anti-oestrogens act via the oestrogen receptor whether they compete with the hormone for binding to the receptor and therefore interfere with DNA binding or inhibit transcriptional activity. These receptors exist as a large 8S complex and/or a small 4S form on sucrose density gradients. High performance ion-exchange chromatography has confirmed that the oestrogen and progestin complex is present in various isoforms. Progestin receptor heterogeneity could be influenced by the presence of oestrogens and anti-oestrogens in the culture media of hormone-dependent neoplastic cells.

Cell culture methods offer the opportunity to test effects of specified components in repeated experiments on a homogeneous population of cells. MCF-7 and T47-D human breast cancer cell lines were conditioned to grow in a serum-free environment. There was no difference in cell proliferation rates, nor in their oestrogen or progestin receptor levels when compared to the same cells grown in conventional media. Receptors were present mainly in the large molecular 8S form.

Both the MCF-7 and T47-D breast cancer cells showed an increase in proliferation rate with the addition of oestrogen or diethylstilbestrol. There was a corresponding loss of progestin receptor levels and an

alteration in the high performance ion-exchange isoforms. Flow cytometry confirmed differences in the S-phase components of the cells following exposure to oestrogens.

The proliferation rates of the cell lines as well as their progestin receptor levels decreased when treated with tamoxifen or the hydroxylated tamoxifen. There were marked changes on high performance ion-exchange chromatography profiles. DNA ploidy and S-phase showed signs of toxicity and there was an increase in cellular debris. The MCF-7 and T47-D human breast cancer cell line retained response to anti-oestrogen saturation.

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INTRODUCTION

1. Overview

Breast cancer is one of the most prevalent forms of cancer among Western european women and after lung cancer the most lethal. This includes white woman in South Africa. The lifelong odds of protracting breast cancer now stands at 1 in 8 which is double the risk reported in 1940 (1). The mystery of what lies behind this rise in risk over the past half century seems to have deepened and grown more complex. Family history of breast cancer appears to be one of the genetic risk factors (table 1). Epidimiologists have, however, shown that other factors besides inherited vulnerability is responsible for more than 60% of breast cancers observed (1). A popular theory, that of a high fat diet, is receiving great attention. Other possible contributing factors such as: exposure to toxic chemicals, alcohol consumption, stress and even exposure to electromagnetic fields are being investigated.

The large number of woman succumbing to this disease and eventually developing metastatic disease requires both the improvement of therapy and necessitates that the selection of appropriate therapies be made more accurately. Investigations during the past half decade have elucidated the mechanisms by which steroid hormones influence the

TABLE I: RISK FACTORS FOR BREAST CANCER IN FEMALES

Factor	High Risk	Low Risk	MRD
Age	Old	Young	***
Country of Birth	North America	Asia,Africa	**
Socioeconomic class	Upper	Lower	**
Marital Status	Never married	Ever married	*
Area of residence	Urban	Rural	*
Place of residence	Northern US	Southern US	*
Race	White	Black	*
Age at first full term pregnancy	Older than 30	Younger than 20	**
Oophorectomy	No	Yes	**
Body build, postmenopausal	Obese	Thin	**
Age at menarche	Early	Late	*
Age at menopause	Late	Early	*
Family history of premenopausal bilateral breast cancer	Yes	No	***
History of cancer in one breast	Yes	No	***
History of fibro-cystic disease	Yes	No	**
Any first-degree relative with breast cancer	Yes	No	**
History of primary cancer in ovary or endometrium	Yes	No	**
Radiation to chest	Large doses	Minimal exposure	**

MRD = Magnitude of differential

*** = Relative risk of greater than 4.0.
** = Relative risk of between 2.0 and 4.0.
* = Ralative risk of between 1.1 and 1.9.

differentiation and development of target organs.

It has long been known that some human breast cancers are hormone dependent in that they undergo striking regression when deprived of supporting hormones by the removal of ovaries (2,3,4), adrenals (3,5) or pituitary (6,7) or on altering the hormonal millieu by the administration of androgens (7), large doses of oestrogens (8) or anti-oestrogens such as tamoxifen (9,10). For these patients who respond to endocrine manipulation by either ablative or additive means represents the best treatment available at this time for breast cancer. In a worldwide multi-trial collaborative study including 75,000 women (12a,12b), it was found that significant reduction in the rates of both recurrences and death are achieved by use of the anti-oestrogen tamoxifen and that tamoxifen reduces the risk of developing contralateral breast cancer by some 39% (12a,12b). Unfortunately only approximately 30% of human breast cancers are of the hormone-dependent type and thereby responsive to endocrine manipulation. There has thus been a need for some means to accurately distinguish those women with hormone dependent cancers, who are favourable candidates for endocrine therapy, from those patients whose tumours are unresponsive to hormonal treatment and should be placed directly on alternative regimes eg. chemotherapy (13,14).

Ever since the demonstration by Beatson (2) almost a century ago that oophorectomy caused the regression of malignant breast disease,

oestrogens have been implicated. Beatson's theories were taken up in the 1950's by Huggins and Dao (3) who obtained 38/100 remissions in patients with metastatic breast cancer who had undergone oophorectomy and adrenalectomy.

Jensen and Jacobson (15) made an invaluable contribution when they synthesised ^{3}H oestradiol with a high specific activity, which made it possible to observe the differential accumulation and retention of ^{3}H-oestradiol by the uterus and other target organs. Noteboom and Gorski (16) also found that after the administration of low levels of [6,7-^{3}H] oestradiol-17*B* to immature rats, the isotope was found to be incorporated in all subcellular fractions of the rat uterus. The incorporation of ^{3}H oestradiol could be inhibited by steroid and non-steroid oestrogens, but not by non- oestrogenic steroids. The inhibition appeared to be competitive and they suggested that the uterus contained a binding site which appeared to be stereospecific for oestrogenic molecules and hypothesized that it probably was of protein origin.

A major breakthrough in our understanding of steroid hormone action came about with the discovery of receptors for oestrogens in the 1960's (17,18) and this work led to the model, which was based on cell homogenization and fractionation studies, for the mechanism by which steroid hormones regulate gene expression (figure 1).

In the 1980's (19) exclusive nuclear localization of the oestrogen

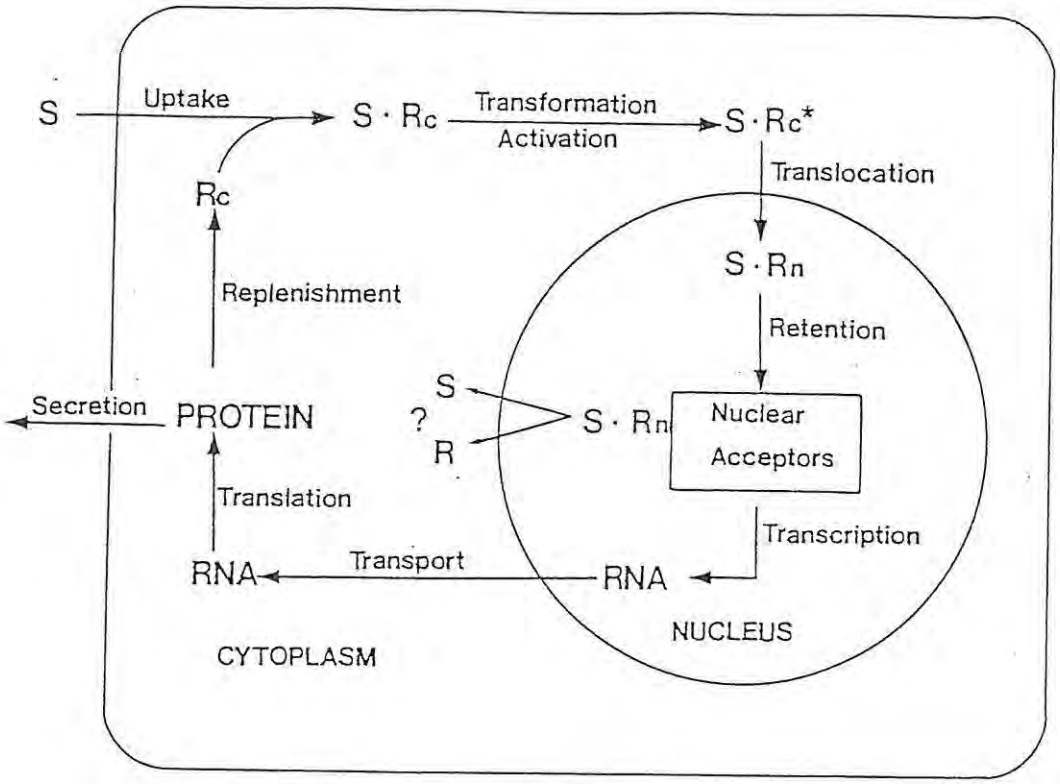


FIGURE 1: Classical "two step mechanism" of intracellular events following the interaction of a steroid hormone with a target cell. Cytoplasmic form of receptor is designated Rc, nuclear form as Rn, and steroid hormone as S. Adapted from literature sources (17,18).

receptor (ER) was reported even in the absence of endogenous hormone. It was suggested that unoccupied oestradiol receptors are loosely bound to the nucleus and released only in the cytosolic fraction on homogenization. This was later confirmed (20,21,22). This hypothesis was also confirmed with work done on the progesterone receptor (PR) and androgen receptor (AR) the exclusive nuclear localization of these receptors even in the absence of endogenous hormone (figure 2).

Steroid hormone receptors are responsible for the interaction between hormone and cell, and functions to trigger a biochemical chain of events characteristic for a particular hormone (23). Receptor proteins are found in concentrations ranging from 50 to 50,000 sites in target cells, but are virtually absent in non-target tissues. A biologically important property is the association of the steroid hormone with its characteristic receptor protein in a manner exhibiting high affinity and ligand specifity.

The most important observation concerning oestrogen and progestin receptors in human breast cancer was made by Jensen and co workers in 1971 (24) when they correlated the presence or absence of specific oestrogen binding with the outcome of endocrine therapy in breast cancers. In the late 1970's more evidence accumulated in favour of this hypothesis (23,25,26). It has consequently been proposed (27) that when malignant transformation occurs, the cell may retain all or only part of the normal population of receptor sites. If the cell retains receptor

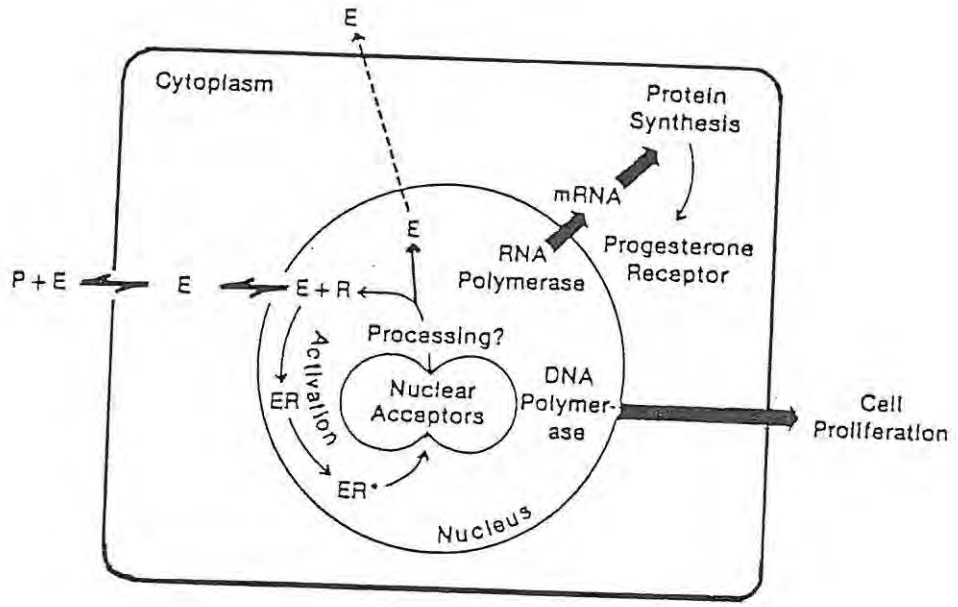


FIGURE 2: Revised subcellular model of steroid hormone interaction with a target cell. Plasma oestrogen [E] diffuses directly into the nucleus where it binds with unoccupied Oestrogen receptor [R], and initiates protein synthesis and cell proliferation. Adapted from literature sources (20, 21, 22).

sites, its growth and function is as that of the normal cell, and it is potentially capable of being regulated by its hormonal environment. Were the cell to lose its receptors as a consequence of malignant transformation, it may no longer be recognised as a target cell by the circulating hormones and endocrine control would thus be abolished (23).

Early methods and clinical studies of oestrogen receptors (ER) were summarised at a 1974 International Symposium (28). Approximately 50-60% of breast tumours exhibited ER. Lack of ER generally denoted the inability of a cell to respond to hormonal manipulation. The presence of ER was thus found to be a prerequisite for normal cellular responses. The use of this criterion has increased the accuracy of selecting endocrine manipulation likely to produce an objective remission in the patient with advanced breast cancer. Data on clinical trials contributed from centres around the world (12a,12b) clearly indicate that if a patient's tumour does not contain ER ,then there is only a slim chance of tumour regression following anti-oestrogen therapy. However, of those patients whose tumours contain ER and PR there are still 20% of whom will not respond to anti-oestrogen therapy. Only 60% of those tumours that are only ER positive. will respond to anti-oestrogen manipulation.

2. Mechanisms of steroid hormone action

The observation of Jensen (29,30) and Gorski (31) led to the proposal of the "two step mechanism" for the hormone induced transfer of the

receptor from the cytoplasm to the nucleus. The unbound steroid enters the cell apparently by passive diffusion, but in some cases active uptake may be involved. In target cells (cells sensitive to the hormone), the steroid combines with its specific receptor protein in a reaction termed uptake. These receptors are relatively large protein molecules that have specific binding sites for the hormone and are found in both the cytoplasm and nucleus of the cell. The binding of steroid to its receptor results in the formation of an "activated" or "transformed" receptor-steroid complex that has affinity for various nuclear binding sites. Prior to translocation into the nucleus, the steroid receptor complex must, therefore, undergo an activation step. After it enters the nucleus, the steroid-receptor complex associates with chromatin in an event called retention.

The binding of the receptor-steroid complex to specific acceptor sites located in the nucleus is thought to alter gene expression. Presumably, these acceptor sites whose transcription is to be induced by the hormone, are located at or near the DNA sequences. Second site cascade effects may also be conceivable. The biosynthetic events that result from receptor-steroid interactions include precursor mRNA transcription, processing, and translation into specific proteins that alter cell function, growth, or differentiation (32).

Once the receptor-steroid complex has interacted with acceptor sites, it

appears to undergo reactions that are not well understood but that eventually result in the re-establishment of unoccupied receptor (recycling) and elimination of the steroid from the cell. These reactions probably involve dissociation of the steroid from the receptor and the conversion of the receptor to a form that can now rebind hormones. The steroid may be metabolised to a derivative that does not bind tightly to the receptor and hence diffuses out of the cell.

Our understanding of steroid receptor action has increased in recent years by the cloning of c-DNAs/genes for the receptors (33-37). In terms of steroid receptor structure, various functional domains have been characterized for steroid binding, DNA binding and transactivation (36,37). Such regions have been distinguished in receptors for oestrogen biochemically and immunologically (38,39). From recombitant cDNA clones it has been possible to predict the complete amino-acid sequence of the receptors for oestrogen (40,41) and progesterone (42-44). Comparison of the sequences for the different receptors has led to the fact that receptor structure can be divided into 6 domains, A-F (45) of which region C is conserved throughout all receptors. By alignment of region C, it would appear that receptors vary in size at their amino-terminal ends (regions A,B) but the carboxy-terminal portions (regions C-F) remain similar in size. It has been found that region C holds the DNA binding domain and region E the steroid binding domain for oestrogen (46). Further studies of region C have suggested that DNA binding of

receptors may be by "zinc binding fingers" akin to those of transcription factors (47). Region C appears to hold only a small fraction of total activity and additional transactivation domains are located in both amino- and carboxy-terminal regions (36,37).

3. Criteria for receptor identification

A complete understanding of the relationship between steroid receptor binding and the mechanism of hormone action depends on the valid characterization and accurate measurement of steroid receptors. The following criteria should be met in order to positively identify a protein as being a specific receptor of that ligand (48):

a. Finite binding capacity: The biological response to steroid hormones is a saturable phenomenon. Assuming that the formation of receptor hormone complexes is obligatory for the production of biological responses the number of receptors per unit mass of tissue should be limited and hence a finite number of receptor sites should exist.

b. High affinity: Steroid receptors should possess a high affinity for the respective hormones. This is expected because the circulatory levels of steroid are usually 10^{-10}m to 10^{-8}m. The existence of receptor-mediated responses of physiological importance thus requires that the receptor have an affinity for the respective hormone that falls in the range of concentrations found in the plasma, otherwise the response would not occur.

c. Steroid specificity: Receptors are expected to display high affinities for

a specific hormone or class of hormones. This specificity enables a given target to respond to a hormonal signal without interference from other signals/hormones of the same class. Their agonists and antagonists should thus compete effectively for a given class of receptor while not affecting other receptor systems. Receptor sites do not however, display absolute stereo-specificity, i.e. the binding site on the receptor has a limited capacity for the recognition and differentiation of ligands other than it's primary hormone.

d. Tissue or cellular specificity: Specific cell types or tissue respond to certain steroid hormones. Since the response is thought to be mediated via receptors, the latter should exist in these cell types and not be in significant numbers in non-responsive cells. This criterion has been applied very successfully to receptor systems for hormones. Only certain tumours, for instance, are assimulated by sex steroids (eg., the uterus, vagina and mammary gland in the case of ER and PR) and the density of ER and PR is much higher per unit mass of target tissue in these organs than in the spleen,lung or other non-target tissues.

e. Correlation with biological response: Implicit in all studies of macromolecules that bind steroid hormones is the assumption that binding results in a biological response. Binding of the hormone to the receptors must thus precede or accompany tissue responses, and the extent of the response should relate to some function of receptor occupancy.

4. Measurement of steroid receptors

It was first shown in 1966 (49) that the receptor complex sedimented as an 8S component in low salt sucrose density gradients and that the complex could be dissociated into smaller 4S units in the presence of 400mM potassium chloride. They also found that the receptor binding molecule was heat labile, non-dialysable, precipitable with ammonium sulphate and was destroyed by proteolytic enzymes, but not by ribonuclease or deoxyribonuclease. Based on these observations, steroid hormone receptors were defined as those protein molecules that bind steroids with high affinity, specificity and with finite binding capacity (50).

The development of new methodologies were essential before a more detailed molecular description could emerge. The steroid receptors were found to be in low concentrations in endocrine target tissue and were also very unstable and extremely labile (51). It was these properties that hampered progress and delayed basic information on receptor protein structure. Furthermore, the presence of contaminating proteins in cell extracts able to interact in a non-specific fashion with radiolabelled steroids complicated the situation.

The appearance of certain synthetic steroid ligands such as the synthetic progestins R5020 (17-alpha,21-dimethyl-19-nor-pregn-4,9,diene-3,20-dione) (52) and Organon 2058 (17-alpha,21-dimethyl-19-nor-pregn-

4,ene-3,20-dione) (53) helped to alleviate the problems of rapid receptor deterioration. These synthetic ligands bind more strongly and have the added advantage of not binding non-specifically to endogenous proteins. These compounds are now used routinely with wide acceptance in receptor studies.

The multipoint titration procedure for receptors using subsaturating and saturating concentrations of radioactive hormones was introduced (54). Cytosol prepared by homogenization should be used immediately as receptor properties deteriorate rapidly. Non-specific binding is also measured by the addition of a 200-fold excess of unlabelled hormone. The protein-bound hormone is then separated from the unbound by adsorption to hydroxyl-apatite, DEAE, filtration, protamine precipitation or most commonly dextran-coated charcoal,followed by centrifugation. All reactions are equilibrated at 5°C and the reactions are then allowed to equilibrate. Specific receptor binding is defined as the total binding minus the non-specific binding. The data is manipulated according to the single component (one steroid molecule per binding site) method of Scatchard (55).

Sucrose density gradients have become a basic research method for the characterization of steroid binding proteins in that this method separates these macromolecules based on size, shape and buoyant density. This is a relatively gentle method and can be applied to small quantities of

unpurified material. A wide variety of other techniques have developed over the years in order to characterize the steroid-receptor complexes. Adsorption to DEAE-cellulose (56), precipitation with ammonium sulfate and phosphocellulose (57), hydroxyl-apatite (58), gel-filtration chromatography (59,60), and affinity chromatography using steroids that were covalently linked to an insoluble matrix (61,62) represent some described approaches.

An accurate definition of the native structure of steroid receptors in the cell either before or subsequent to binding to DNA was essential. Molybdate, originally added to inhibit phosphatases, was found to have stabilizing effects on the 8-10S forms of progestin receptor (PR) (63,64) and oestradiol receptors (ER) (65,66). In working with these preparations by the above classical separation techniques and with newer methods such as photoaffinity-labelling with tritiated R5020 (67,68), digestion with proteases (69,70) and the use of receptor antibodies for oestrogen (71) and progesterone (72), it soon became apparent that the steroid receptor complex was more complicated than originally assumed (73).

The development of receptor-specific antibodies represented a major advance in receptor studies. Monoclonal antibodies were developed for the detection, localisation and quantitation of steroid receptors (74). Since the antibodies bind to specific antigenic sites on the receptor which are thought to be distinct from the ligand binding site, monoclonal

antibodies are able to detect receptors whose ligand binding sites may be saturated with endogenous hormone. Monoclonal antibodies may also be able to recognise denatured or transformed receptors that would not bind steroid (75). Immunohistochemical methods have been developed to routinely assess the oestrogen receptor status of breast tumor biopsies at the time of surgery (76,77).

The development of high-performance liquid chromatographic techniques (HPLC) alleviated to some extent the need to circumvent the problems of prolonged manipulation in receptor preparation. HPLC in the ion-exchange (78,79), chromatofocusing (80) and hydrophobic interaction modes (81) were developed for rapid, effective separation of receptor isoforms. These techniques also confirmed that steroid receptors exhibited molecular heterogeneity. Steroid receptors are dynamic proteins whose properties of size, shape, surface charge and hydrophobicity vary depending on the conditions of their environment. Using various types of chromatography one can exploit these properties so that the various species of the receptors may be separated and studied (82).

5. Progesterone receptor

The mechanisms by which oestradiol and progesterone regulate the proliferation and differentiation of uterine epithelial cells most probably do not apply to the breast (83-85). In the uterus oestrogens are clearly mitogenic and the addition of progesterone to the oestrogenized

endometrium leads to the appearance of a secretory pattern characterized by cells engaged in protein synthesis rather than cell division (86). In the uterus oestradiol may thus function as a proliferative hormone and progesterone as a differentiating hormone. Hence the unopposed actions of oestradiol are considered to be tumourigenic in the uterus while the risk of endometrial hyperplasia and cancer is lowered when oestrogens are combined with progestins. In fact, the combined regime may even be protective since a decrease in endometrial cancers has been reported in women prescribed combined oestrogens and progestins compared with women receiving no treatment (87). However, considerable evidence has now accrued to suggest that in the epithelium of the breast, progesterone has a different influence. Progesterone in the breast appears to exhibit a strong proliferative effect. Studies in support of this come both from experimental models (88) and from normal cycling women (89).

Fundamental differences in the actions of oestradiol and progesterone in the breast is that the latter stimulates DNA synthesis, not only in the epithelium of the terminal bud, but also in the ductal epithelium. Compared to the increase caused by oestradiol treatment (11.3 fold), progestin treatment only marginally increases the mitotic index of normal human breast ductal epithelium two fold. It is difficult to sustain the hypothesis that progestins are protective in the breast (84). It would seem that more research must be done to understand the actions of

progestins in the normal breast, and that clinical decisions based on the inappropriate uterine model system is unjustified (90).

Like all steroid hormones, progesterone and the synthetic progestins function by binding to intracellular proteins, the progesterone receptor, the presence of which specifies a progesterone target tissue and target cell. Progesterone receptor (PR), when bound to ligand, control the transcription of genes for ER (91), for insulin receptors (92), for epidermal growth factor receptors (EGF) (93), as well as the genes transforming growth factor (TGF) (94,95). Thus, the proliferative effects of progesterone may in part be a reflection of its ability to modulate the levels of growth factors and their cognate receptors. The mechanism by which this regulation is achieved and the structure of the PR proteins are complex (36,37,40,96).

Complementary DNA'S for the chicken PR were cloned independently in 1986 (43) and by Coneelly also in 1986 (42), and for the human PR in 1987 (97). PR belongs to the steroid/thyroid hormone receptor superfamily of ligand-activated DNA binding proteins (36,37) which are composed of a number of independently functioning domains required for nuclear localization, ligand binding, DNA binding, dimerization and transcriptional activation. Progesterone receptor, like ER, is distinctly a nuclear protein (98). In the absence of hormone the receptor is hetero-oligomers loosely bound in the nuclear structure and extracted into the

cytoplasm during homogenization (99), whereas after *in vivo* hormone binding, all receptors are tightly attached to nuclear elements. Additional sites on the receptors are responsible for their interaction with one or more accessory proteins including heat shock proteins and several of the serine residues are covalently modified by phosphorolation. Not all of these domains have been definitely mapped. In general the N-terminus is involved in transactivation and contains several phosphorolation sites; the DNA-binding domain is centrally positioned and may have a weak dimerization function. The ligand-binding activity is restricted to the C-terminus, which also contains structural features required for protein-protein interactions plus a second transcriptional activation domain (36,37,96,100,101).

The two major protein species, the A and B receptors, were originally described by O'Malley and co-workers in 1970 (59) and 1972 (102) in the chick oviduct. Subsequent studies using the human breast cancer cell line T47D showed that PR exists as two isoforms, the N-terminally truncated 94 kDa A receptor and the 116 kDa B receptor (103-106). The amino-truncated receptors are a naturally synthesized form (103,104). Originally, the unliganded A and B isoforms were thought to be subunits of a larger heteromeric 8S receptor (107). This, however, is not the case. Instead, each receptor species forms independent 8S heteromeric complex on sucrose gradients (108-110), by binding to as many as five non-hormone-binding proteins including two molecules of 90 kDa heat

shock protein (hsp) and one molecule of hsp 70 (111-113). In this oligmeric state, the receptor proteins are basally phosphorolated and unable to bind DNA, but they are maintained in a stable conformation that allows them to bind progesterone or progestins.

Hormone binding to the 8S receptor form activates a rapid series of changes, the exact sequential order of which is still unknown. Within seconds after hormone binding, the receptors become hyperphosphorylated (114-116) and several of the associated proteins (but not hsp 70) dissociate, leading to generation of a faster sedimenting 4S receptor form (113-118). Two 4S receptor molecules dimerize (118) and then bind to DNA at specific transcription enhancer sites called progesterone response elements (PRE) (36,27,41,96). Since both homo and heterodimers can form between the A and B isoforms, three possible classes of receptor dimers (A:A,A:B,B:B) can bind to a PRE, each having a potentially different transcription regulatory capacity (101,118). This diversity in the repertoire of responses to one hormone sets progesterone apart from the regulatory information carried by other sex steroid hormones studied to date. However, no formal proof exists that both isoforms are in the same cell.

That this molecular heterogeneity is indeed translated into functional heterogeneity was first demonstrated in 1988 (119) that assessed the cell specific transcriptional activation of two different target genes by

the chicken A and B receptors. It seems that the N-terminal region of PR, present in B but missing in A receptors, plays a specific role in transcription that differs depending both on the gene being modulated and the cell being analysed. Thus, the A receptor can be stimulatory in a setting where the B receptor is inhibitory. It suggests that the N-terminal of the B receptor confers specific transcription regulatory functions to PR. These data suggest that, as we learn about the tissue specific effects of the two PR isoforms it may be important not only to measure PR levels, but also to identify the particular PR isoform present in a breast tumour. While equimolar amounts of A and B receptors have been demonstrated in breast cancer cell lines, it is not known whether this stoichiometry applies to PR-positive breast cancer.

6. ER and PR in human breast cancer.

Analysis of oestrogen and progesterone receptors in biopsies of breast carcinomas continues to play an important role in the selection of patients likely to respond to hormonal therapy. Reports from many laboratories over the years have been consistent with their results. Seventy to 80% of ER and PR positive patients, 50-60% containing ER positve and PR negative patients, 30% of ER negative and PR positive patients and only about 5% of ER negative and PR negative patients exhibit objective remissions to additive or ablative endocrine therapy (23,24,26,73,120-127). These receptors may thus also be considered prognostic variables with regard to recurrence-free and overall survival

(123,126,127,).

The molecular basis of successful endocrine therapy appears to be related to the fact that certain tumors have retained the cellular mechanism to respond to the same hormonal stimuli as their progenitor cells (128). When malignant transformation occurs, the cell may retain all or part of the normal population of receptor sites. If the malignant cell retains receptors, its growth and function are potentially capable of being regulated by the hormonal environment, as in a normal cell. Such tumours would be responsive to endocrine therapy. If specific receptors are lost from the tumour, this may indicate that the tumour is endocrine resistant and so would be unresponsive to endocrine manipulation (27). The presence of ER and PR in a tumour is known to provide a molecular basis for distinguishing hormonally-responsive breast carcinomas from those that are not (129).

Although ER negative and PR negative patients rarely respond to endocrine manipulation there is nevertheless a small percentage of about 5% that do respond. There are several possible explanations for this response. Steroid receptors are extremely labile proteins and failure to detect receptor activity may be due to methodological artifacts. The most common problems include the incorrect handling and storage of specimens (130), insufficient sampling of malignant cells due to inadvertant biopsy of neighbouring tissue and the breast tumour itself

may be heterogeneous and a biopsy that is ER negative may not be totally representitive of other tumour deposits. A variety of additive and ablative therapies employed in patients with breast cancer may also act via mechanisms mediated by receptors or growth factors other than ER.

A less encouraging conclusion derived from early studies was that ER positive tumours, while more likely to respond to surgical endocrine manipulation, did not show a uniform response. In fact only one half of ER positive patients underwent demonstrable clinical remission following anti-oestrogen manipulation (131). Extensive studies addressing the issues of molecular and cellular heterogeneity and their relation to progression of breast cancer and their resistance to hormonal therapy have been carried out.

Complementary DNA (cDNA) was cloned and sequenced from MCF-7, a human breast breast cancer cell line (40,41). The gene for ER was later successfully analysed in 1988 (132). The A/B domains contain regions that regulate the transcriptional function of the proteins. The C domain contains two DNA binding zinc fingers and represents the region of the protein that binds to the oestrogen response element (ERE). Mutations in this portion of the protein changes its affinity for DNA resulting in suboptimal or complete loss of binding as shown by mutagenesis analysis . The hormone binding properties of the receptor reside in region E. Since the two functions, the DNA binding and hormone binding, are

carried out by separate parts of the protein, they are to some extent independent. Thus, it is possible to have variant receptors that can bind to DNA with limited affinity without first binding hormone and vice-versa (46,133).

The nuclear localization signal, located downstream from the DNA binding domain, is a region of the protein that must be present for the receptor to remain within the nucleus in the absence of ligand (134). It has been identified in PR and is presumed to be similar in ER. The Hsp 90 appears to bind to regions in the hormone binding domain of some steroid receptors when ligand is absent, and its binding is thought to prevent receptor dimerization and DNA binding (135). Ligand activation leads to hsp 90 dissociation and monomer dimerization in solution (133).

The dimerization domain that mediates this interaction between the two ER molecules has been localized to the carboxyterminal end of the hormone binding domain (136). A weak dimerization domain may also be present in the second zinc finger of the DNA binding domain (133). Additional sites for heterologous protein interactions may also be located in the hormone binding domain (137) and covalent modifications by phosphorolation further enhance the complexity of this protein molecule (138).

An alternate oestrogen receptor messenger mRNA has been identified

(139), which appears to be be the primary transcript present in human uterus as opposed to the breast cancer cell line MCF-7. A truncated ER message specific to pituitary cells has been described (140). Probably in normal cells the regulation of gene transcription and even ER protein structure may be tissue specific. In malignant cells there is mounting evidence that in addition to silent mutations and regulatory heterogeneity, mutations in ER exons exist that would influence protein structure and protein function. (141,142).

Several wild type ER mRNA mutations have been found (141-145). The functional importance of these mutations is still under investigation but they clearly suggest mechanisms by which mutant receptor forms can subvert the activity of wild type forms, when both are expressed in the same tumour cell. The question is does message variants reflect protein variants? Foster in 1991 (146) and Scott in 1991 (147) examined the ability of tumour ER to bind an oestrogen response element (ERE) and these studies showed that some tumours containing abundant immunoreactive ER failed to demonstrate DNA binding ER or that the DNA binding forms appeared to be truncated. Based on these preliminary data, the prevalence of non-DNA binding ER forms or truncated ER forms among ER positive or PR positive tumours may exceed 50%, a significant number whose structural analysis may become a critically important prognostic tool.

A small group of tumours that are ER negative but PR positive have a higher response rate than is usually expected of ER negative tumours. Either PR synthesis in these tumours are entirely independent of ER action or either a variant or other unmeasured form of ER exists. The key to explaining steroid action lies in defining the mechanisms that are interposed between the initial ligand recognition event and the activation of the transcription of hormone-responsive genes encoding each of the steroid receptors, together with the description of specific sequences of DNA termed hormone response elememts (HRE's) that bind the hormone receptor and control the efficiency of transcription (37,148), has allowed the application of molecular genetic techniques to this problem.

7. Patient treatment

a. Endocrine therapy

Various endocrine manipulations by either ablation of the ovaries, adrenals or pituitary gland or administration of hormones or anti-hormones have demonstrated activity against breast carcinoma (149), and have been discussed previously.

i) Ablative therapies

The types of ablative therapy endocrine treatment have changed dramatically over the past 20 years; oophorectomy is still in common use but other forms of endocrine surgery, such as adrenalectomy and hypophysectomy have been superseded by drugs with few side effects

and unlike surgery, with reversible actions.

Ovarian ablation

Bilateral oophorectomy (2) was one of the first treatments available for premenopausal women with metastatic disease and is still often used, sometimes with an anti-oestrogen.

Hypophysectomy and adrenalectomy

These second line endocrine manoeuvres appear comparable but may have have largely been supplanted by the development of aminoglutethimide (5).

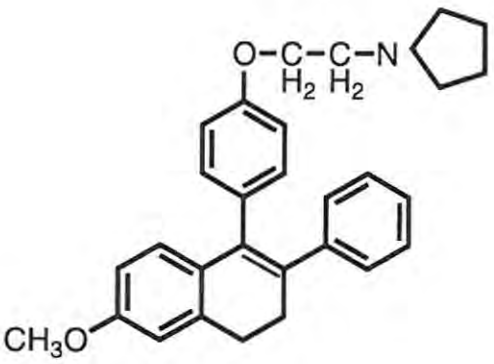
ii) Additive therapies

Oestrogens

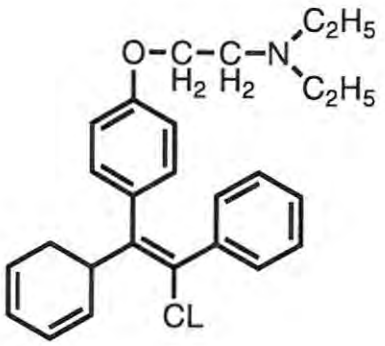
Until recently, oral oestrogen therapy was the standard treatment for postmenopausal women with metastatic breast carcinoma (9). Because of equivalent therapeutic effectiveness with much less toxicity, the anti-oestrogen tamoxifen has gradually replaced oestrogens as the first choice of endocrine treatment in postmenopausal women.

Anti-oestrogens

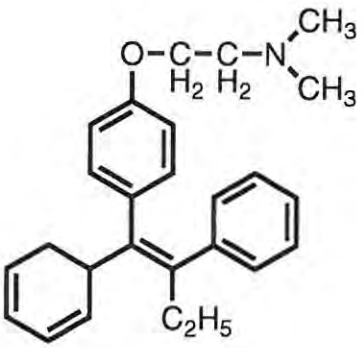
Any substance that antagonises the action of oestrogens may be termed an anti-oestrogen. All such compounds of current clinical relevance are derivatives of triphenylethelene. These include nafoxidene, clomiphene and tamoxifen (figure 3). These compounds compete with oestradiol for binding to specific oestrogen receptor binding sites. The explanation for their biological activity, however, is more complicated and cannot be explained in terms of this effect alone (150).



NAFOXIDEN



CLOMIPHENE



TAMOXIFEN

FIGURE 3: Chemical structures of anti-oestrogens nafoxidene, Clomifene and Tamoxifen.

Tamoxifen (Nolvadex ICI 46,474)

Tamoxifen is a triphenylethelene, and is a partial anti-oestrogen. Tamoxifen is a competitive inhibitor of oestradiol binding of oestrogen receptors (151). The compound inhibits the binding oestrogen to target tissues *in vivo* (152-157). It is clear however that the high affinity of tamoxifen to a variety of proteins causes ubiquitous binding of the drug *in vivo* and probably accounts for its long biological halflife (158). Oestrogens decrease cell cycle time and tamoxifen causes reversible blockade at the G_1 phase (159,160). Anti-oestrogens inhibit oestrogen stimulated increases in transforming growth factor *a* (TGF*a*), a stimulatory factor (161). There is a complementary rise in transforming growth factor *b* (TGF*b*), an inhibitory growth factor (162). Although the actual regulatory mechanisms that orchestrate the inhibitory and stimulatory actions of growth factors are unclear; this modification of the endocrinological regulation by growth factors on breast cancer cell kinetics, continues to be an area of investigation. It is also reported that there is an increase in sex hormone binding-globulin, which may decrease the availability of the free estrogens for diffusion into the cells (163). There is also a school of thought that suggests that tamoxifen administration leads to an increase of natural killer cells (164). Overall the experimental evidence points to the fact that tamoxifen is tumouristatic (165,166), rather than tumouricidal (167,168). Investigations using a variety of modules have evaluated the effect of tamoxifen on tumour promotion and have shown that when tamoxifen is given after a

carcinogen, but before the appearance of a tumour, it prevents the occurence of a palpable tumour for as long as the administration of the drug is maintained (155,156,166). Long term adjuvant tamoxifen treatment or treatment until relapse, is predicted to be the best therapeutic strategy to control the recurrence of breast cancer following mastectomy/lumpectomy (169-174).

The clinical pharmacology of tamoxifen was initially investigated in patients with advanced breast cancer (175-178). The drug is readily absorbed and accumulates to steady state levels by the end of the first month of treatment (176). The serum levels of tamoxifen at steady state are extremely variable and are not related to response (179). The principle metabolite of tamoxifen is N-desmethyltamoxifen (figure 4) which produces blood levels approximately twice as high as those of tamoxifen by the end of the first month of treatment. The serum half-lives of tamoxifen and N-desmethyltamoxifen are extremely long probably because the compounds are highly protein bound(180). Tamoxifen is extensively metabolized in patients (181,182) but no significant quantities of oestrogenic metabolites have been noted that would cause concern during long term adjuvant tamoxifen therapy. N-desmethyltamoxifen is further metabolised to metabolites which have a glycol side chain (183,184). Tamoxifen is also converted to 4-hydroxytamoxifen but this metabolite is only observed in low concentration in the serum.(178). However, this metabolite has a very

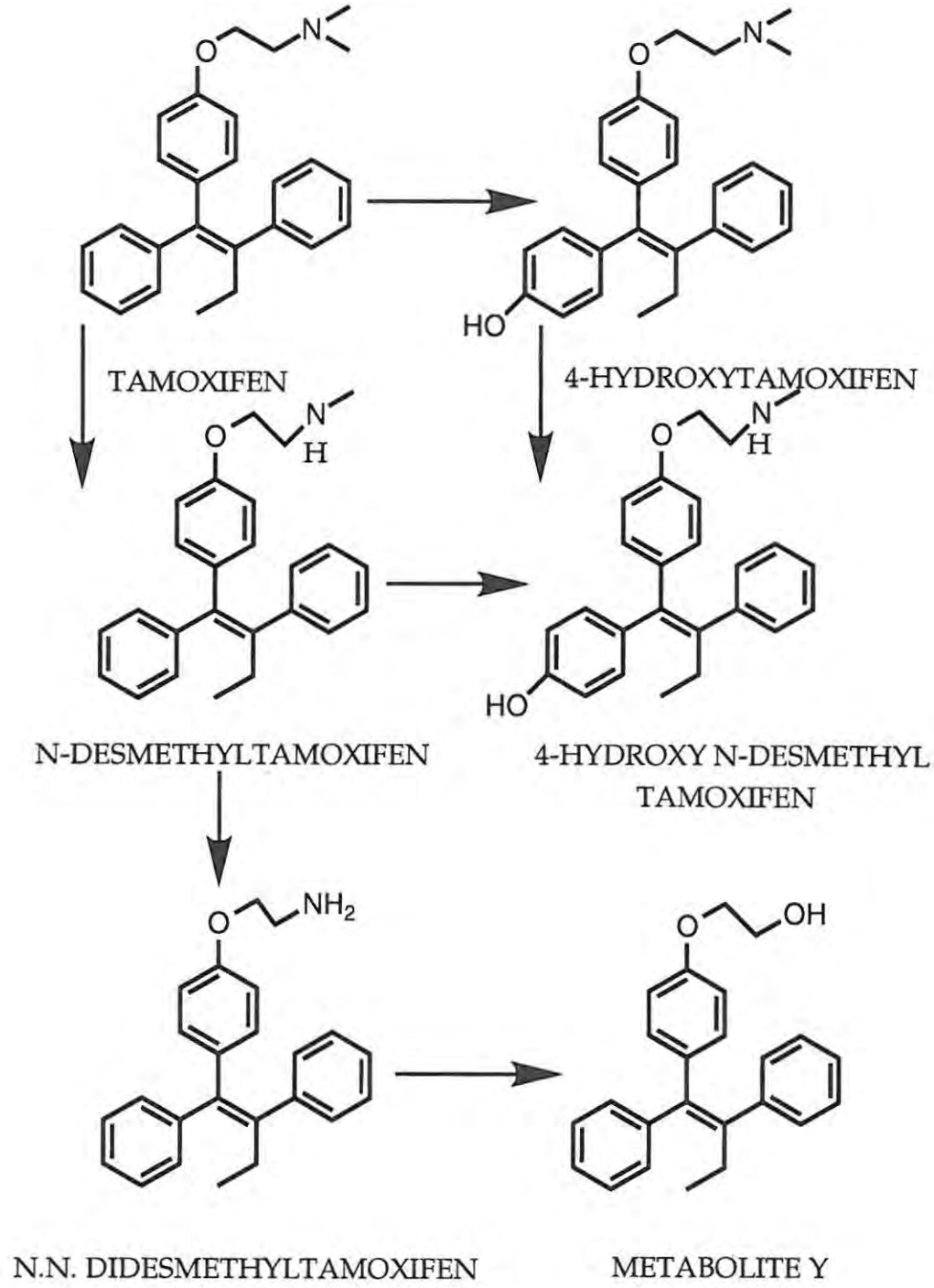


FIGURE 4: Metabolites of tamoxifen identified in patients.

high affinity for the oestrogen receptor (the same as that of oestradiol) and at the same time is a potent inhibitor of oestrogen action (185-188). Therefore, 4-hydroxytamoxifen plays a significant role in supporting the anti-tumour action of tamoxifen. 4-hydroxy N-desmethyltamoxifen has also been identified in patients (189), and has been shown in the laboratory to be formed either from 4-hydroxytamoxifen or N-desmethyltamoxifen (190). Tamoxifen has also been shown to exhibit oestrogen-like effects that appear to have target site specificity (191), as it displays oestrogen-like effects in bone (192,193).

Progestational agents

These have proved useful as second-line endocrine treatments in patients who have responded to prior endocrine therapy (194,195). The oral progestin megestrol-acetate, is easiest to use and is relatively free of side effects (196,197). Their exact mechanism of action is unknown, although possibilities include blockade of PR, interference with ER synthesis, androgen-like effects, or possibly effects on other tissues such as the immune system (198).

Androgens

These agents have also been used largely as second-line endocrine treatments (198). Response rates seem to be somewhat lower than with other endocrine treatments except for bone metastases, where androgens may actually be superior to oestrogens (199).

Glucocorticoids

Large doses of glucocorticoid can induce regression of metastatic breast cancer in 10% to 20% of patients (199). Remissions are short lived, but the rapid onset of effect of glucocorticoids makes them useful in advancing disease.

Aminoglutethimide

This oral agent has recently been introduced in the field of endocrine treatment of breast cancer as a medical equivalent of adrenalectomy (200). Aminoglutethimide has dual actions, both inhibiting the conversion of cholesterol to pregnenolone, which is an important precursor of all steroid hormones (201) and inhibiting the aromatisation reaction needed to convert adrenal steroids to oestrogens by fat and other extra-adrenal tissues (202). This inhibition of the aromatase enzymes essentially eradicates oestrogen production in postmenopausal or castrated women. Replacement doses of glucocorticoids must be given with aminoglutethimide to prevent a compensatory rise in adrenocorticotropic hormone (ACTH) which can overcome the aminoglutethimide inhibition of pregnenolone synthesis from cholesterol (200). Aminoglutethimide has compared favourably with surgical adrenalectomy with comparable response rates and remission duration, thereby appearing to be a preferable alternative (203).

Others

Several newer endocrine agents with promising preliminary reports include Leuprolide (204) an analogue of human gonadotropin-releasing

hormone, which causes an effective medical castration of premenopausal women and trilostane (205), which inhibits the actuation of steroid precursors.

b. Chemotherapy

Cytostatic chemotherapy is often used to treat patients with metastatic breast carcinoma (206). Symptomatic and objective responses (tumour shrinkage) often ensues. Whether this translates in longer patient survival is not always clear, but certainly there are many patients with aggressive metastatic disease in the liver or lungs, whose life span as well as quality of life have improved with such treatment (206). When discussing various treatment programs, most studies have tended to focus on response rate, using relatively uniform objective criteria to define response (207). Patients receiving chemotherapy are preselected (208).They are generally viewed as having too aggressive disease for endocrine therapy or have already been treated with endocrine therapy and either failed or relapsed after an initial response. Patients with more indolent disease tend to be treated initially with endocrine therapy. There are few prognostic factors predictive of response to chemotherapy. Although kinetic studies (209) suggest that the more rapidly growing carcinomas respond better to chemotherapy, patients with short disease-free intervals (presumably due to fast growing carcinomas do not show higher response rates to chemotherapy. Carcinomas that are ER poor tend to grow faster *in vitro* and clinically. It might thus be anticipated

that these malignancies would show a better response rate than oestrogen receptor-rich tumours (210). Factors that are associated with increased response rates to chemotherapy include better overall response of soft tissue tumours and lymph node metastases (211), as opposed to liver, bone and lymphangitic lung matastases (206,212). Many chemotherapeutic agents are effective against breast carcinoma, the most commonly used include cyclophosphamide, methotrexate, 5-flouracil, vincristine and adriamycin (213). Since the initial reports in 1963 (214) and especially in 1969 (215), combination chemotherapy regimes have generally been viewed as the preferred initial chemotherapy approach. Although most oncologists use combination chemotherapy regimes, the data supporting the benefits of combination chemotherapy compared to single agents used sequentially do not always clearly show a survival benefit for combination chemotherapy (216). Patients with aggressive disease may thus well require intensive combination chemotherapy. For others with indolent disease the use of one agent at a time (sequentially with disease progression) may prove just as beneficial (217).

c. Adjuvant therapy

The potential benefits using endocrine and chemotherapy together have long been investigated. Theoretically, such combination may prove to be additive, synergistic, or even antagonistic if the endocrine therapy interferes with tumour cell growth and renders the carcinoma less responsive to chemotherapy (208,218). Studies comparing

oophorectomy alone with oophorectomy and chemotherapy suggest that the combination produced better response rates and response durations (219,220). Short-term but not overall survivals were also improved. A study comparing chemotherapy and chemotherapy plus oophorectomy in premenopausal women and diethylstilbestrol(DES) in postmenopausal women demonstrated improved response rates with the combined treatment but no clear survival advantages (221). More recently, several studies have assessed the addition of tamoxifen to chemotherapy (222,223). Results have been mixed, with some reports suggesting improved response rates without survival benefits (224,225) while others showed no benefit of tamoxifen (226,227) or even inferiority of simultaneous chemotherapy and tamoxifen compared using tamoxifen after chemotherapy failure (228). At present there is no definitive evidence favouring simultaneous rather than sequential use of chemotherapy and endocrine therapy. In 1992 the Early Breast Cancer Trialists Collaborative Group reported in the Lancet (12a,12b) on the systemic treatment of early breast cancer by hormonal, cytotoxic, or immune therapy. This involved 133 randomised trials involving 31000 recurrences and 24000 deaths among 75000 women. Significant reductions in the annual rates both of recurrence and of death are produced by tamoxifen, which was also found to reduce the risk of development of contralateral breast cancer by some 39%. It was found that chemotherapy plus tamoxifen is better than chemotherapy alone for recurrence. The 30-40% proportional risk reductions that can be

produced by combined chemo-endocrine therapy in middle age are similar for node-positive and for node negative patients, but the absolute improvement in 10-year survival is about twice as great for the former (at least 12 deaths avoided per 100 women treated) as for the latter.

8. DNA ploidy and S-phase

The ultimate aim of any oncologist would be to have prognostic factors that could predict completely the clinical behaviour of primary breast cancer. Such factors could be used to separate patients who are likely to be cured by local therapy and who would not benefit from the addition of adjuvant therapy, from those who are destined to recur and die without intervention. Towards this aim attention has focused on the proliferative potential of tumour cells which may be associated with the metastatic spread of breast cancer. The most commonly used techniques to measure proliferative activity are the mitotic index, the thymidine labeling index (229,230) and more recently, the S-phase fraction determined by DNA flow cytometry (231,232).

Flow cytometry is a relatively new technology. DNA flow cytometry provides a measure of DNA content (ploidy) and a measure of proliferative activity (S-phase fraction) (233). It has been used to evaluate the nuclear DNA content of several types of human cancers. The fundamental steps in DNA flow cytometry include the isolation of intact nuclei. The incubation of nuclei with a fluorescent stain such as

propidium iodide, followed by the measurement of the fluorescence intensity of the individual stained cells. The fluorescence levels are determined by passing the nuclei through an excitation source, usually an argon-ion laser. Photomultipliers capture the fluorescence levels and convert them to digital signals that can be stored in a computer for further analysis.

The result of flow cytometric analysis is a DNA histogram from which cell cycle components can be estimated. Cell populations are often divided into three basic cell cycle compartments: the G_0/G_1 compartment consists of normal non dividing (G_1) or quiescent (G_0) cells. The S-phase fraction comprises cells undergoing replication or cell synthesis and the G_2/M compartment includes cells in the postsynthetic phase (G_2) and cells in mitosis.

Most breast tumours comprise at least 2 different populations of cells. Normal tissue consisting off lymphocytes and benign cells and tumour cells that may or may not undergo chromosomal aberrations resulting in an abnormal amount of DNA content. Tumour ploidy is often quantitated by the DNA index, defined as the ratio of G_0/G_1 peak of the tumour population to the G_0/G_1 peak of the normal population. Diploid tumours have a DNA index of 1.0 reflecting a normal amount of DNA. Most aneuploid breast cancers have an excess amount of DNA with a DNA index greater than 1.0. It is also possible to have multiple DNA indices.

The S-phase fraction determined by DNA flow cytometry has been proposed as a measure of cell proliferation. Several studies (234) have now been published describing the relationship between S-phase fraction and other prognostic factors. Tumours with increased S-phase fractions are seen in poorly differentiated, steroid receptor-negative tumours. There is a trend for larger tumours from premenopausal patients with positive axillary lymph nodes to have higher S-phase fractions. Both DNA ploidy and S-phase fraction have been correlated with the clinical outcome of patients (235-242). The oncology research network, established by the University of Texas Health Science Center at San Antonio and Nichols Institute reference laboratory, which was established in order to evaluate the clinical utility of new prognostic factors for patients with primary breast cancer, reported in 1993 on 127,000 breast cancer patients. In their report they found that steroid receptor negative tumours were more often aneuploid and had a higher median S-phase fraction than receptor positive tumours (243). The strong inverse relationship between steroid receptor status, ploidy and S-phase fraction has been reported by other investigators (237-251) while a few earlier studies had failed to observe this finding (252-254).

9. Receptor heterogeneity

Breast cancer patients undergoing hormone treatment with anti-oestrogens invariably become resistant (208,255). As it has already been stated on the basis of conventional biochemical receptor assays

oestrogen, and in particular, progestin receptor status is a significant prognostic variable with regard to recurrence free and overall survival (121-123). At the cellular level most breast cancers are heterogeneous and composed of varying proportions of individual receptor positive and receptor negative cancer cells. It has been found that in a heterogeneous tumour primarily the receptor negative cells synthesize DNA and proliferate more rapidly than receptor positive cells (248,256-258).

A variety of mechanisms have been proposed for development of the acquired resistance to tamoxifen that arises in animal model systems (168,259) and in virtually all patients (208) undergoing hormonal therapy. Genetic mechanisms include the variant and mutant forms of oestrogen receptor, which may exert dominant positive or dominant negative controls over oestrogen and anti-ostrogen regulated growth (141,143,260,261). Epigenetic mechanisms centres on pharmacokinetic issues related to drug absorption, distribution and metabolism. Recent data indicates that the metabolite, trans-4-hydroxytamoxifen, may be selectively excluded from tamoxifen-resistant breast cancers or be further metabolized to relatively inactive forms (262).

It is possible that tumour progression to the resistant state includes a mechanism involving selection and expansion of cell subpopulations, some of which remain strongly influenced by tamoxifen. That hormone treatment may itself provide the selective remodelling pressure has been

suggested (168). While normal tissue may respond uniformly to the regulatory signals of hormones, the genetic instability and heterogeneity that characterize malignant cells may render them incapable of uniform response. Evaluation of heterogeneity is important for analysis of tumour behaviour in response to therapy and in attempting to explain the lack of correlation between response and marker levels that so often arises.

Hormone responsive or resistant breast cancer cell lines with various oestrogen and progesterone receptor levels have been useful in studies of steroid hormone action and growth regulation (263). However, these breast cancer cell lines have been shown to have unstable biological properties. In addition to changes in steroid receptor levels and hormone sensitivity, some breast cancer cell lines have also been shown to be genetically unstable, with spontaneous DNA duplication occuring. (264-271). Studies into these unstable breast cancer cell lines in culture may lend valuable clues to the aggressive nature of breast cancer tumours, particularly their involvement in the development of hormone resistance, which may be due to a collection of reasons. These include defects in the oestrogen and progesterone receptor, progression from diploid to aneuploid and tetraploid to hypertetraploid states, and the relationship that exists with the S-phase fraction.

This thesis is an attempt to explore further the relationship of oestrogens and anti-oestrogens on the progestin receptor in *in vitro* cell culture models.

MATERIALS AND METHODS

1. Reagents and chemicals

Oestradiol-[17-ß[2,4,6,7-^{3}H] specific activity 80-110 Ci/mmol and 16-*A*-ethyl-21-hydroxy-19-nor-[6,7,-^{3}H]-pregn-4-ene-3,20-dione (Organon 2058) specific activity 42 to 59 Ci/mmol, unlabelled Organon-2058, Z-4-OH-(N-methyl)-tamoxifen, tamoxifen and Serotec GC serum free media, were purchased from Amersham International, UK. Oestradiol 17-*beta,* diethylstilbestrol, glycerol, dithiothreitol, and sodium molybdate were obtained from Merck (Darmstadt, Germany), while dextran T-70 was procured from Pharmacia (Bromma, Sweden). RPMI 1640, foetal calf serum, antibiotic-antimycitic solution, 0.1% EDTA, phosphate buffered saline and trypsin were obtained from Highveld Biological (Johannesburg, South Africa). Trypan blue, ribonuclease A, propidium iodide and all other chemicals including EDTA were of reagent grade and were products marketed by Sigma Corporation U.S.A.

2. Cell culture procedures

MCF-7 and T47-D human breast cancer cell lines were obtained through the American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A. and routinely grown at 37°C with 5% CO_2 in RPMI 1640 with 5% foetal calf serum containing 2mM glutamine and the antibiotics Penicillin (100ug/ml). Streptomycin (100ug/ml) and Fungizone (0.25ug/ml). Aliquots of 10^7 cells in 2ml media containing dimethylsulfoxide as

preservative were stored in liquid nitrogen. Cells were revived by rapid thawing and stock cultures were maintained in continuous exponential growth by weekly passage of the appropriate number of cells following trypsinization of the monolayer with (1:1) 0.25% trypsin in 0.1% EDTA phosphate buffered saline, pH 7.0. MCF-7 and T47-D cells were then conditioned to serum free cell culture media, consisting of Iscove modified Dulbeccos medium supplemented with bovine serum albumin, transferrin, lipids, 15mg/L phenol red and antibiotics. The serum concentration was gradually reduced from passage to passage until the cells were proliferating in the serum free media. When optimum growth was reached the cells were inoculated with 17ß-oestradiol 10^{-6M}, diethylstilbestrol 10^{-6M}, 1*um* tamoxifen and 1*um* 4-hydroxytamoxifen were added for 24, 48, 72, hours and 1 week respectively.

Stock solutions of the hormones were prepared in ethanol and kept frozen. Ethanol 0.1% was added to a control culture of each cell line. Cells were dislodged using two washes of 0.1% EDTA phosphate buffered saline, pH 7.4 and pelleted at 800x g for 5 minutes in a Hettich tabletop centrifuge and placed on ice. All subsequent procedures were performed at 4°C. Mean doubling times were calculated from the initial and final cell numbers for each hormone and results were expressed as:

Proliferation rate =

Doubling time (control)/Doubling time

in order to account for the variation in number of control population

doubling among the two cell lines. Cells were ruptured in 10mM PEDG (10mM potassium phosphate, 1.5 mM EDTA, 1mM DTT, 10% glycerol) buffer using a 3ml teflon glass homogenizer on ice to obtain a final cytosol protein concentration of 2-5 mg/ml. Cytosols were prepared by centrifugation at 100,000x g for 30 mins at 4°C in a Beckman L8-80M ultracentrifuge fitted with a Type 65 fixed angle head rotor.

3. Ligand binding reactions

All ligand binding reactions were carried out at 0-5°C. Cytosols were incubated with a 5nM concentration of ^{3}H Organon-2058, either in the presence or absence of a 200 fold excess of unlabelled Organon-2058 to estimate non specific binding in the case of progestin receptor and a 5nM concentration of 17-*beta*-[2,4,6,7-^{3}H] oestradiol, either in the presence or absence of a 200 fold excess of unlabelled diethylstilbestrol to estimate non specific binding in the case of oestrogen receptor. The reactions were terminated by the addition of the reaction mixture to pellets of dextran coated charcoal for thirty seconds for progestin receptor and 5 minutes for oestrogen receptor and centrifuged at 12000 rpm for 4 mins. Proteins were estimated by the colorimetric dye binding method of Bradford 1976 (272) using bovine serum albumin (BSA) as a standard.

4. Sucrose density gradient centrifugation

Linear gradients of 10 to 20% (w/w) sucrose were formed by layering

equal volumes of 10%, 15%, and 20% sucrose prepared in P10EDGM buffer into 13x57*mm* Beckman Quick-seal centrifuge tubes. Gradients were allowed to diffuse overnight at 4°C. Cleared cytosol (100 *ul*) was carefully layered on top of each gradient , sealed and centrifuged in a VTi-80 vertical tube rotor for 2½hrs at 405 000x g and 5°C using the slowest acceleration and deceleration program. Gradients were fractionated from the top in 4 drop (100*ul*) aliquots using a peristaltic pump and a Beckman gradient fractionation system.

5. Calculation of specific binding capacity

Gradient fractions were counted in a Packard Tri-Carb Model 2000 CA scintillation counter giving an average counting efficiency of 55%. Specific binding capacity was expressed as femtomole steroid bound per milligram cytosol protein using the difference between the total binding and the binding in the presence of excess unlabelled ligand (non-specific binding). Non-specific binding is the result of the ligand binding to non-receptor sites which are usually of low affinity and high capacity relative to the receptor. The total amount of steroid bound in such a system is the sum of that bound to receptor sites plus that bound to non-specific sites. The quantities of receptor steroid complex are calculated by subtracting non-specific binding from the total steroid bound to give specific binding capacity.

6. High performance ion-exchange chromatography

All chromatography was performed in a cold box at 5°C utilizing a Beckman Altex (6mmID x 210 mm) glassed column packed with a DEAE Spherodex-M obtained from Bio technics (USA). The column was equilibrated with P10EDG buffer, which had been passed through a 0.45 micron HAWP filter. The column was linked to a Packard flow-through *B*eta scintillation counter equipped with an in line LKB (Sweden) conductivity meter. Labelled cytosol was applied in 200 ul volumes. A programmed gradient elution was carried out as follows: 0 to 60% P500EDG buffer from 0 to 40 minutes, 60% P500EDG buffer from 40 - 45 minutes and 100% P10EDG buffer from 45 to 60 minutes.

7. Flow cytometry

Prior to harvesting, cells were checked for viability. This was done by taking confluent cells and using 0.1% EDTA, the cells are centrifuged and aspirated to a volume of 20 ml cells and medium that does not contain foetal calf serum. Serum is known to take up trypan-blue dye and the background would appear excessively dark. Cells and media (0.5ml) were added to 1.0ml of 0.4% trypan blue in phosphate buffered saline. The dye mixture was then agitated and counted using an improved Neubauer counting chamber taking care not to overflow the chamber. Care was taken so as not to allow the dye mixture to stand for longer than 15 minutes as the viable cells would start to take up the dye. The remaining confluent cells were harvested and washed in media

containing 10% glycerol and frozen until assayed. On day of assay cells were washed in phosphate buffered saline and lightly centrifuged. The cells were then exposed (at a concentration of 1 million cells per ml of propidium iodide) to modified Krishan's reagent, containing propidium iodide, the detergent nonident-P-40 and sodium citrate prepared in phosphate buffered saline and then incubated for 20 minutes at 4°C. The cells were then exposed to 15,000 units/ml of ribonuclease A for 15 minutes at 37°C. To stop the reaction, cold phosphate buffered saline was added and the cells pelleted lightly and filtered through a nylon mesh to obtain single cell suspension. A Coulter Epics Profile II linked to the multicycle software modelling computer programme was used. Diploid DNA female red cells were used as controls. A 488nm argon laser light run at 200mw was used for fluoresence exitation. CV of flourescent beads was set < 2.0% and at low rates of 100-500 cells per second to maximise resolution. Simultaneous gating on both forward and 90° light scatter was used to exclude signals from cleaved nuclei and other debris.

RESULTS AND DISCUSSION

1. Growth of human breast cancer cell lines grown in serum-free media

The MCF-7 and T47-D human breast cancer cell lines were conditioned over a period of a few weeks to grow in a serum-free environment. This was done by reducing the starting foetal calf serum concentration of 5% during each passage by 50% of the previous serum concentration. The proliferation rates of the MCF-7 and T47-D cell lines grown in serum free-media were compared with the cell lines grown in their original media which was RPMI-1640 containing 5% foetal calf serum. Figure 5(a) and 5(b) compares the proliferation rates of MCF-7 and T47-D human breast cancer cells grown in RPMI-1640 and serum-free media. There appears to be little effect on the proliferation rates of either cell lines. A higher cell density than usually employed was used during adaptation ($1x10^4$).

Application of the principles of defined cell culture environments to the isolation, selection and culture of cells from various tissues have revealed that the culture environment, nutrients and extracellular matrix is the key determinant of the relative kinetics of growth expression in cells. It seems unlikely that a single media will ever be devised suitable for all cell types. It is important for cells to survive and proliferate under culture conditions. Therefore, the culture media must carry out the functions previously performed by the complicated *in vivo* environment. The serum-

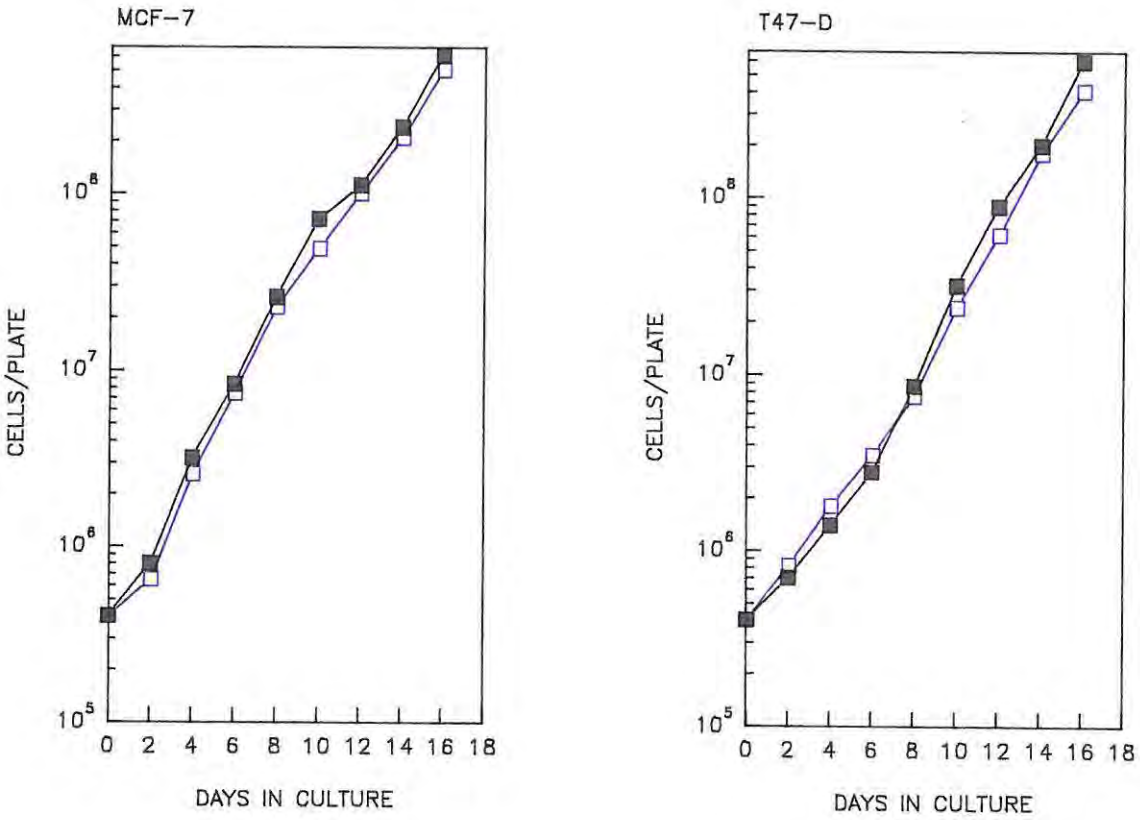


FIGURE 5: Proliferation rates of:
a) MCF-7 human breast cancer cells grown in ■ RPMI-1640 culture media + 5% foetal calf serum and adapted to grow in □ serum-free media containing bovine serum albumin, lipids, antibiotics and 0.015% phenol red.
b) T47-D human breast cancer cells grown in ■ RPMI-1640 + 5% foetal calf and adapted to grow in □ serum-free madia containing bovine serum albumin, lipids, antibiotics and 0.015% phenol red.
No statistical differences in their proliferation rates were noted.

free media used in these experiments was developed for activation of mammalian cells and had been found to be sufficient for the stimulation and propagation of most cells (273-280). The media was supplemented with albumin, transferrin, and lipids. Table 11 lists the complete constituents of the media used in these experiments. MCF-7 and T47-D human breast cancer cell lines appear very sensitive to their growth conditions, hence the importance that the culture media utilized contain adequate lipids and proteins. Clonal culture methods have revealed the extreme dependence of cell behaviour on how populations of cells modify their micro-environment (281,282). Many cells also excrete novel-like polypeptides that can act directly on cells regulating their metabolism and proliferation (283-285). Various combinations of nutrients and purified proteins may be used with little perceivable lag time.

2. The effects of oestrogen and anti-oestrogens on cell proliferation

To ascertain the effects of oestrogen and anti-oestrogens on proliferation rate of cells grown in a serum-free environment, oestradiol *17B* 10^{-6M}, diethylstilbestrol 10^{-6M}, tamoxifen *1um* and 4-hydroxytamoxifen *1um* were added to the cell cultures using 0.1% ethanol as carrier. Ethanol 0.1% was added to a control flask and cell proliferation rates were monitored over a period of one week. As illustrated in figures 6(a) and 6(b) the proliferation rates of the oestrogen and synthetic oestrogen-exposed MCF-7 and T47-D cells increased above the levels of the control groups. This result confirmed the proliferative properties of oestrogens

TABLE II COMPOSITION OF SERUM FREE MEDIA

AMINO ACIDS	mg/mL	OTHER COMPONENTS	mg/L
L-Alanine	19,87	Bovine serum	
L-Arginine HCL	84,00	albumin	1000,00
L-Asparagine	27,22	Cholesterol	57,50
L-Aspartic acid	23,48	D-Glucose	4500.00
L-Cystine	28,00	Hepes	3574,50
L-Cystine 2HCL	62,57	Human Transferrin	32,00
L-Glutamic acid	59,60	Phenol red	15,00
L-Glutamine	584,00	Sodium pyruvate	87,42
Glycine	30,00	Soybean lipids	100,00
L-Histidine HCL H20	42,00	2-Mercaptoethanol	50uM
L-Isoleucine	105,00		
L-Leucine	105,00	**VITAMINS**	
L-Lysine HCL	146,00	D-Biotin	0,013
L-Methionine	30,00	D-Ca-Pantothenic	
L-Phenylalanine	66,00	acid, Ca salt	4,00
L-Proline	31,79	Choline chloride	4,00
L-Serine	42,00	Folic acid	4,00
L-Threonine	95,00	I-Inositol	7,20
L-Tryptophan	16,00	Nicotinic acid-	
L-Tyrosine	103,79	amide	4,00
L-Valine	94,00	Pyridoxal HCL	4,00
		Riboflavin	0,40
SALTS, INORGANIC	mg/L	Thiamine HCL	4,00
CaCl2	200,00	Vitamin B12	0,013
FeCl3 6H20	0,075		
Fe(NO3)3 9H20	0,10		
KCL	400,00		
MgSO4	96,67		
NaCl	6400,00		
NaHCO3	2500,00		
Na2O3Se	0,022		
NaH2PO4H20	125,00		

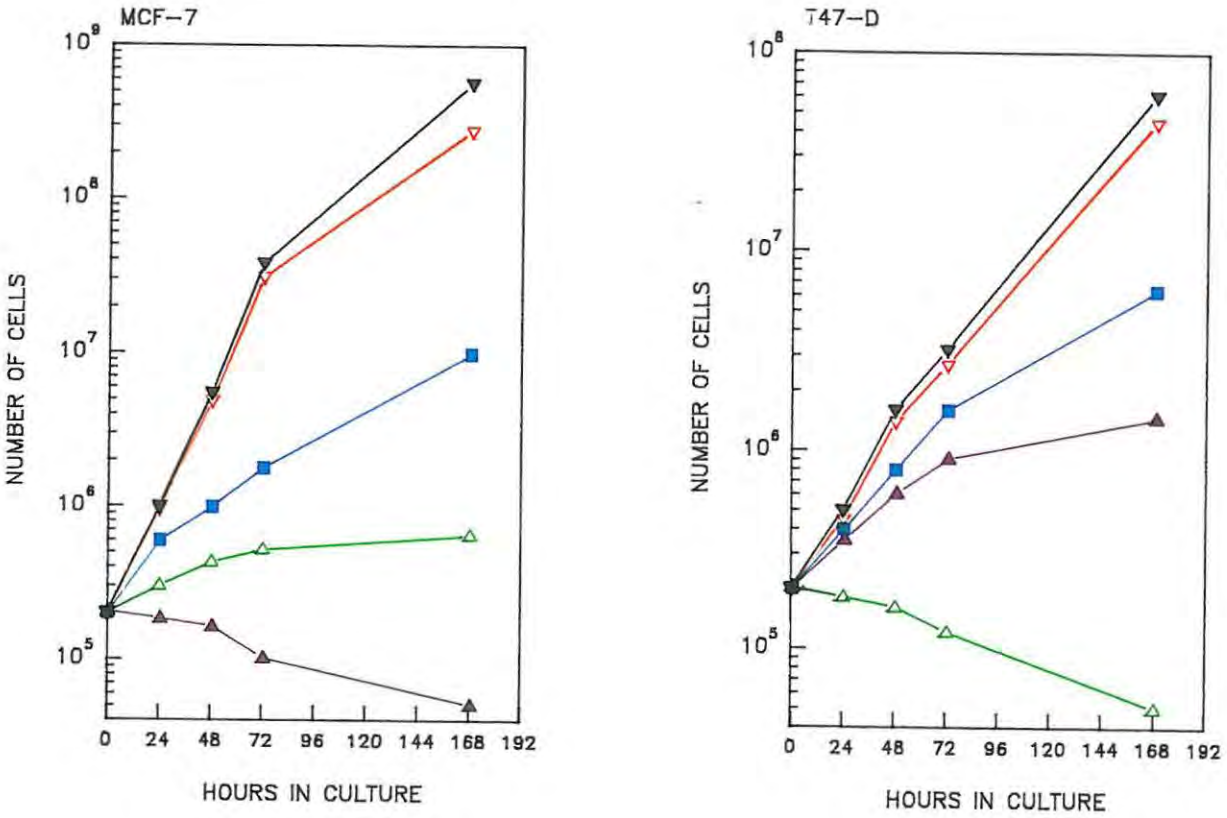


FIGURE 6: Proliferation rates of:
a) MCF-7 human breast cancer cells adapted to grow in serum free media under the influence of ▼ *17B* oestradiol 10^{-6m}, ▽ diethylstilbestrol 10^{-6m}, △ tamoxifen 1*u*m, ▲ 4-OH-tamoxifen 1*u*m and ■ control with 0.1% ethanol.
b) T47-D human breast cancer cells adapted to grow in serum free media under the influence of ▼ *17B* oestradiol 10^{-6m}, ▽ diethylstilbestrol 10^{-6m}, △ tamoxifen 1*u*m, ▲ 4-OH-tamoxifen 1*u*m ■ and control with 0.1% ethanol.
Under the influence of oestrogens proliferation rates increased above those of the control.
Under the influence of tamoxifen and its' metabolites a decline in proliferation rates was noted.

on human breast cancer cells even in a serum-free environment. The anti-oestrogen groups, in particular the tamoxifen groups of both cell lines, showed a negative growth pattern over a period of a week. The hydroxylated tamoxifen group did not, however, approach the negative growth pattern of the tamoxifen group. This happened despite the fact that 4-OH-tamoxifen is regarded as an active metabolite of tamoxifen. These results did lend credence to the supposition that the MCF-7 and T47-D human breast cancer cells used in these experiments are oestrogen growth-responsive and growth-inhibited in the presence of the anti-oestrogens tamoxifen and its' metabolite, the hydroxylated tamoxifen, under serum-free growth conditions.

3. Oestrogen receptor content of MCF-7 and T47-D cell lines

To test the productivity of MCF-7 and T47-D human breast cancer cell lines grown in serum-free culture media, oestrogen receptor levels were assayed using sucrose density gradient ultracentrifugation (SDG). Figure 7 illustrates the oestrogen receptor sedimentation profile of a) MCF-7 cells grown in RPMI-1640 culture media containing 5% foetal calf serum and b) MCF-7 cells grown in serum-free media.

The sucrose density gradient technique involves firstly the reversible formation of a complex between receptor proteins and a labelled hormone. Once formed, the protein hormone complex dissociates slowly (half life of dissociation complex is greater than 20 hours at 0°C. The

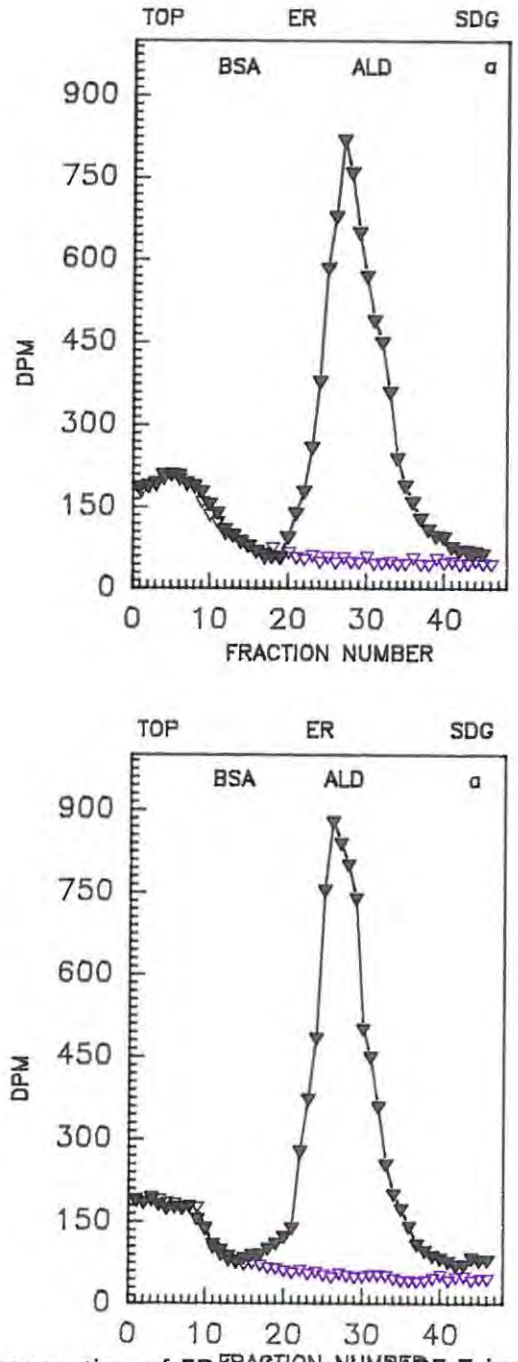


FIGURE 7: SDG separation of ER isoforms in MCF-7 human breast cancer cell line grown in: a) RPMI-1640 plus 5% foetal calf serum and b) serum-free media. Cytosols were incubated overnight with 5nM ^{3}H 2,4,6,7 oestradiol *17B* in the presence ▼ or absence ▽ of a 200 fold excess of unlabelled DES. ALiquots of each reaction were cleared of unbound ligand with DCC and layered onto preformed sucrose gradients. Molecular markers albumin (4-5S) and aldolase (8-9S) were included. Total protein = 2.0mg/ml. The 4S isoform was undetectable. The 8S isoform = 35 fmol/mcp and 33 fmol/mcp respectively.

sedimentation coefficient of the receptor-hormone complex can be estimated from the migration of protein-bound radioactivity relative to the migration of internal markers of known sedimentation coefficient. A clear cytosolic extract labelled with tritiated steroid is layered onto a preformed sucrose gradient in a cylindrical tube and centrifuged in a vertical tube rotor in a preparative ultracentrifuge. The gradient run is terminated before the molecules of interest have reached the bottom of the tube. The gradient is then fractionated and analyzed to obtain a distribution of radioactivity as a function of fraction number. From this limited data, the sedimentation coefficient for the steroid components can be evaluated in these experiments by comparison with macromolecules such as albumin (4-5S) and aldolase sedimenting as 8-9S. (286-288).

With this method it has been determined that the sedimentation profiles of ER and PR in human breast cancer cells fall into 4 catergories. Breast tumours contain specific steroid-binding components migrating at either 4S,8S or both 4S and 8S and those in which receptors are undetectable. A quantitative assessment of the various molecular forms obtained by SDG can be calculated by subtracting the sum of the non-specific binding fractions (in distintergrations per minute DPM) from the sum of the total binding fractions (DPM) present in the isoforms to give specific binding. With the protein content of the homogenized cells, the receptor isoforms are calculated as being present in fmol per cytosol protein concentration.

In the case of ER present in the MCF-7 cells, whether grown in RPMI-1640 or serum free media only the 8S receptor is present and in almost equal amounts. Figure 8(a) and 8(b) illustrates that in the case of T47-D cells there are no measurable isoforms of ER in either culture media used.

4. Progestin receptor content of MCF-7 and T47-D human breast cancer cell line

Figure 9(a) and 9(b) illustrates the sedimentation profile of PR in the MCF-7 cell line. There is virtually no difference between the MCF-7 cell line grown in RPMI-1640 or serum-free media. The PR is present solely in the 8S isoform and quantitatively the calculated receptor amounts are similar.

It is interesting at this point to observe the role that "non-specific binding" plays in the determination of receptor binding parameters. Non-specific binding sites are defined to be of low affinity and high capacity relative to the receptor system. However, non- specific actually means non-displaceable by a competitive steroid in the range of tritiated steroid used in the assay. The non-labelled steroid is, as a rule, generally used as the competitive inhibitor. It is essential that the binding of tritiated steroid and steroid competitor have reached equilibrium at the time of measurement.

Gold standard biochemical methods are mainly based on the

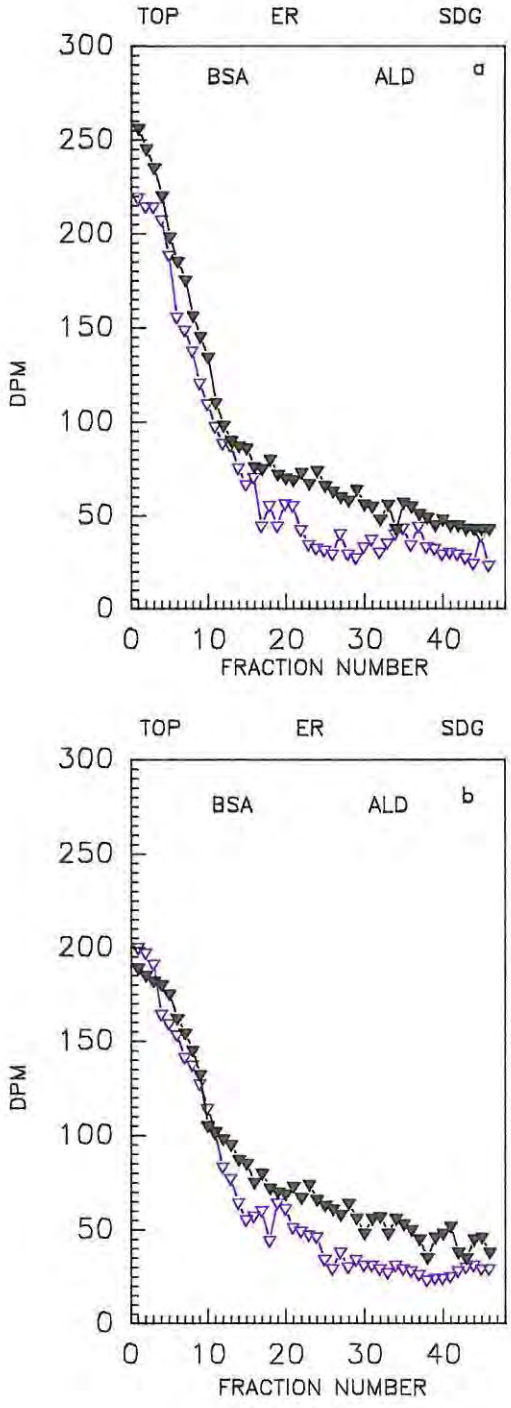


FIGURE 8: SDG separation of ER isoforms in T47-D human breast cancer cell line grown in: a) RPMI-1640 plus 5% foetal calf serum and b) serum free media. Cytosols were incubated overnight with 5nM ^{3}H 2,4,6,7 oestradiol 17*B* in the presence ▽ or ▼ absence of a 200 fold excess of unlabelled DES. Aliquots of each reaction were cleared of unbound ligand with DCC and layered onto preformed sucrose gradients. Albumin (4-5S) and aldolase (8-9S) were used as markers. Total protein = 2.4 mg/ml. There were no measurable ER isoforms.

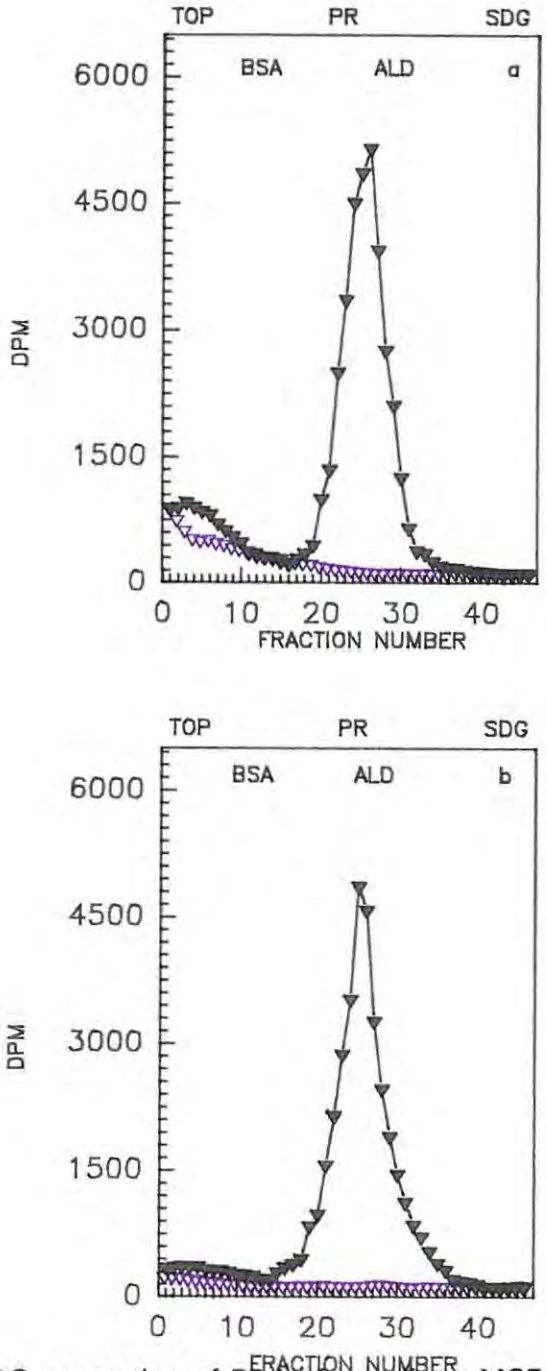


FIGURE 9: SDG separation of PR isoforms from MCF-7 human breast cancer cells grown in a) RPMI-1640 media plus 5% foetal calf serum and b) serum-free media containing 0.015% phenol red. Cytosols were incubated overnight with 5nM ^{3}H organon 2058 in the presence ▽ or absence ▼ of a 200 fold excess of unlabelled organon. 100ul aliquots of each reaction were cleared of unbound ligand with DCC and layered onto preformed gradients. Albumin (4-5S) and aldolase (8-9S) were used as markers.Total protein = 2.0mg/ml. The 4S isoform was undetectable. The PR 8S isoform = 380 fmol/mcp and 330 fmol/mcp respectively.

measurement of a radiolabelled steroid, usually to soluble proteins contained in the supernantant (cytosol) of a tissue homogenate. These methods differ primarily in the procedure employed to separate hormone bound to the receptor protein from that that remains unbound. The separation of bound from unbound hormone is complicated by the fact that steroids may bind to a number of proteins in addition to the receptor, including albumin, *al*-acidic glycoprotein, corticosteroid-binding globulin (CBG), and steroid-binding globulin (SHBG) (289). These are plasma proteins, but since human tumour specimens, including breast tumour cell lines, nearly always contain substantial amounts of plasma protein contamination, the ability of a method to distinguish these from true receptors is critical. The greater the excess of labelled hormone the greater the competition for binding to low affinity sites, but no more than 100-200 fold excess need be used. In addition, unlabelled hormone should be added either before or with labelled hormone but never after, since it will replace label only very slowly if added afterwards (48,73,128).

The specificity of the SDG assay can be further enhanced by the appopriate choice of radioactive ligand and competitors. Labelled progesterone, for example, binds PR with a very high affinity for SHBG. In order to reduce this high degree of interference, a synthetic progestin is used which has high affinity for the receptor and a greater specificity.

The unbound receptor is not very stable, and the presence of

ligand tends to stabilize the receptor by formation of a receptor-ligand complex. It is well known that many binding proteins are more stable with bound ligand than without (128,290). The earliest possible addition of ligand produces the most stabilty to the experiment.

Progestin receptor isoforms of T47-D cells by SDG again figure 10 (a) and (b) shows that the PR levels are almost identical in either RPMI-1640 or serum-free media used for cell culture. In both cell lines and in both culture media used, the PR isoforms are present almost exclusively as the large oligomeric untransformed 8-10S. It is this 8-10S assembly that has been shown to be a predictive indicator of an endocrine responsive breast tumour (120,121). This 8S binds to non-hormone binding proteins which includes two molecules of the 90kDa heat shock protein (hsp) and one molecule of hsp 70 (111-113,116). In this oligomeric state, the receptor proteins are basally phosphorylated and unable to bind DNA, but they are maintained in a stable conformation that allows them to bind to progestins.

Hormone binding to the 8S receptor form activates a rapid series of changes, the exact sequential order of which is still unknown. After hormone binding *in vivo,* the receptor becomes hyperphosphorylated (116-118) and several of the associated proteins (but not hsp70) dissociate, leading to a generation of the faster-sedimenting 4S receptor form (111-113,116-118). What we can assume from these results is

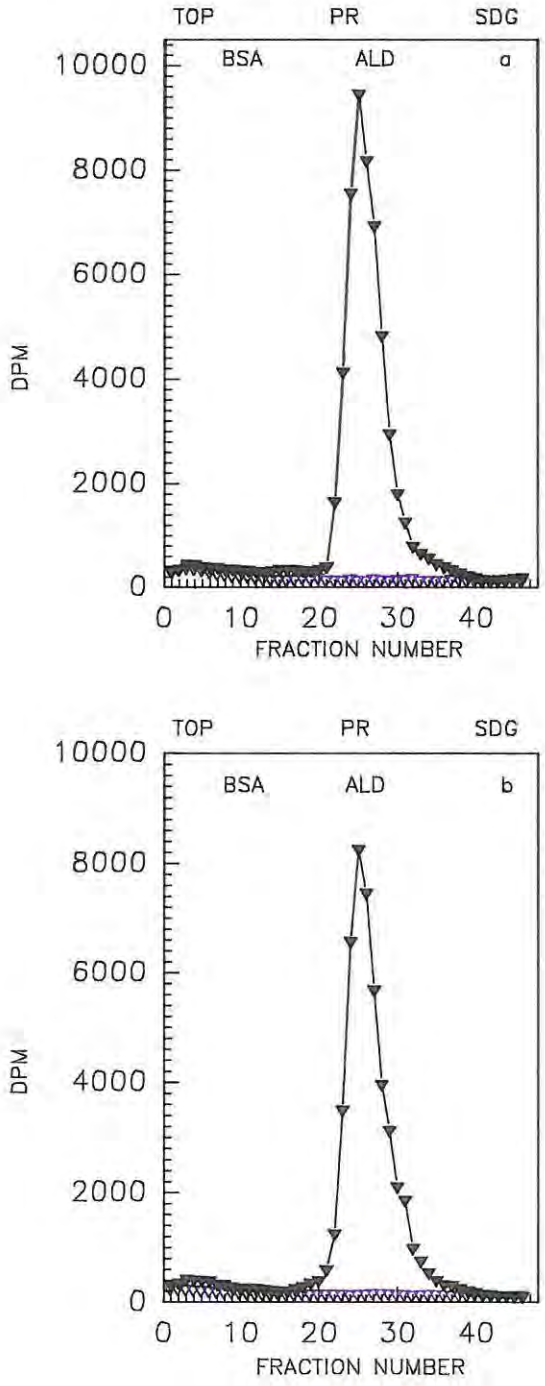


FIGURE 10: SDG separation of PR isoforms from T47-D human breast cancer cells grown in: a) RPMI-1640 media plus 5% foetal calf serum and b) serum free media containing 0.015% phenol red. Cytosols were incubated oc\vernight with 5nM ^{3}H organon 2058 in the presence ▽ or absence ▼ of a 200 fold excess of unlabeeled organon. 100 *u*l aliqouts of each reaction were cleared of unbound ligand with DCC and layered onto preformed gradients. Albumin (4-5S) and Aldolase (8-9S) were used as markers. Total protein = 2.4mg/ml. The 4S isoform was undetectable. The PR 8S isoform = 1360 fmol/mcp and 1280 fmol/mcp respectively.

that the PR assayed from the MCF-7 and T47-D cells grown in serum-free media is indeed the intact untransformed receptor and that the lack of serum in the culture media used has not affected the conformation or binding characteristics of the progestin receptor.

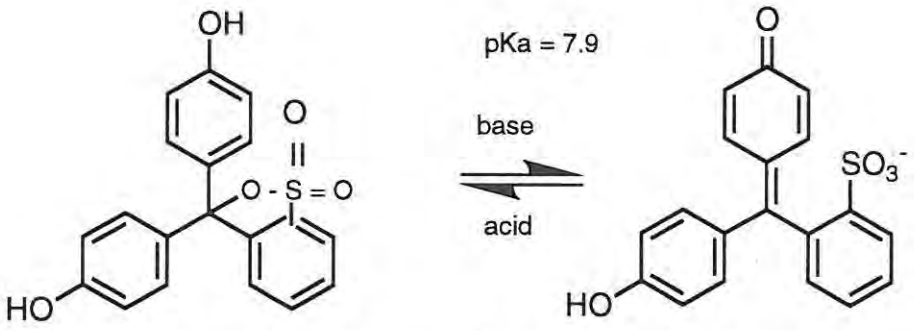
Careful consideration must be given to the reported findings that while MCF-7 human breast cancer cells from various cell banks have similar morphology, they nevertheless differ in their receptor content and that despite having similar karotypes, the different sourced cells have unique biological properties. Cell selection, due to different culture conditions, have also been known to take place (291). The inherent genetic instability of cancer cells in long term culture cannot be ignored (292). Biological properties of the T47-D cell line have not always been consistent between laboratories While the original cell line was reported ER-positive, some workers found that the T47-D cells actualy lacked measurable ER (293). This suggests that variants of the cell line may have arisen, or even that the wild-type ER has given rise to mutant cDNA's. This genetic-damage may even be largely random. It is also conceivable that unrestrained growth and imperfect intracellular relations are conditions that may arise in so many ways that no common denominator exists at the molecular level but only at a crude descriptive biological level (263).

Phenol red, which bears a structural resemblance to some steroidal

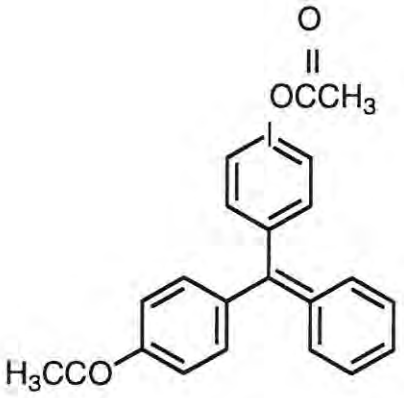
oestrogens, is used as a pH indicator in tissue culture media (Figure 11). Phenol red has been found to bind to the oestrogen receptor of MCF-7 human breast cancer cells with an affinity of 0.0001% that of oestradiol ($k^d=2x10^{-5}m$). It stimulates the proliferation of oestrogen receptor positive breast cancer cells in a dose-dependent manner (294,295). Levels of progestin receptor are also reportedly higher in media containing phenol red (294,295). In these sets of experiments, however, the phenol red was retained in the serum-free media so as to allow a small amount of low affinity oestrogen-like compounds to mimic an *in vivo* situation. Other growth factors such as insulin, epidermal growth factor, insulin growth factor and hormones such as prolactin, androgens, glucocorticoid steroids and thyroid hormone have also been implicated in the induction of cell proliferation (296-303).

5. High performance ion-exchange chromatography of progestin receptor in MCF-7 and T47-D breast cancer cell lines

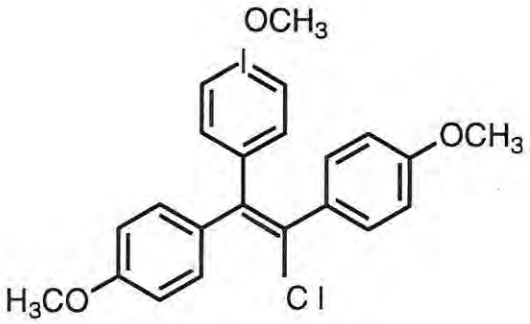
High performance ion-exchange liquid chromatography (HPIEC) has been found to be not only an ideal research tool for the measurement of steroid receptors but also an ideal medium for receptor isoform resolution (304-307). Figure 12 illustrates the assay of PR by HPIEC on MCF-7 breast cancer cell lines grown in a) RPMI-1640 plus 5% foetal calf serum and b) serum-free media. As may be discerned from the chromatograms, they appeared almost identical. The chromatogram gave three distinct isoforms of the PR, although the first peak fell in the void volume (10mM



(yellow - acidic) **PHENOL RED** (red - basic)



Cyclofenil



Chorotrianisene

FIGURE 11: Structure of Phenol red (phenolsulfonphthalein) and some structurally related nonsteroidal oestrogens.

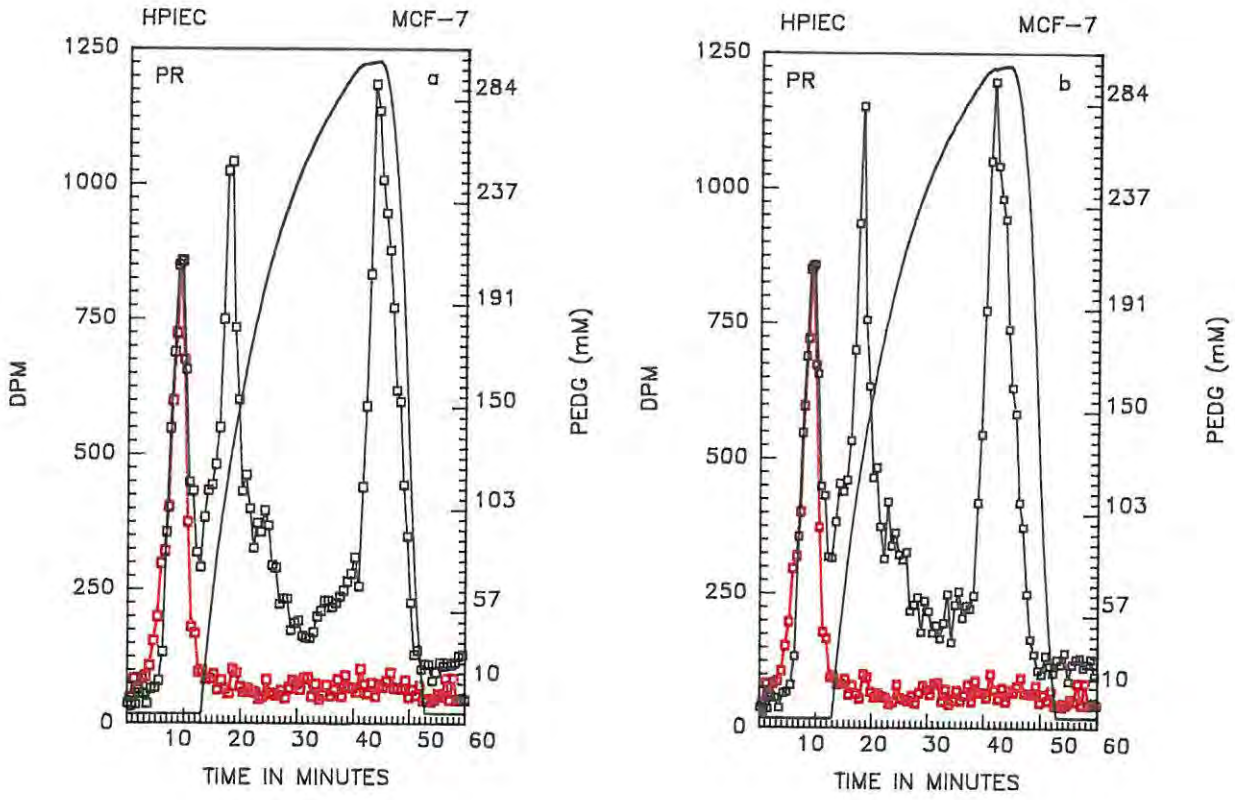


FIGURE 12: HPIEC of PR from MCF-7 human breast cancer cells.
a) MCF-7 cells grown in RPMI-1640 media.
b) MCF-7 cells grown in serum free media.
Cytosols were incubated overnight with 5nM ^{3}H organon 2058 in the presence ▣ or absence ▢ or a 200 fold excess of unlabelled organon 2058. 200*ul* aliquots of each reaction were cleared of unbound ligand with DCC and injected onto a glass column packed with DEAE Spherodex-M. The column was linked to a flow-through *beta* counter equipped with an in line conductivity meter. A linear gradient elution of P10EDG to 60% P500EDG was used. Three isoforms were eluted, at the void volume, P120EDG, P300EDG, and a shoulder peak at P180EDG respectively.

phosphate buffer), and as may be observed, most of the "non-specific" binding occured in this area. The second peak was at 120mM phosphate buffer and the third occurred at 300mM. There did appear to be a discernible shoulder peak at 180mM phosphate buffer which was extremely difficult to fully isolate. Column recoveries were consistently in the order of 95%.

Figure 13 illustrates the isoforms of PR by HPIEC on T47-D cells grown in (a) RPMI-1640 plus 5% foetal calf serum and (b) serum free media. The chromatograms appeared almost identical. Three distinct isoforms of the PR were noted. The first peak fell in the void volume and also contained most of the "non-specific" binding. The second peak was at 120mM phosphate buffer and the third was at 300mM phosphate buffer.

HPIEC is a very effective technique with high powers of resolution provided the isoforms to be separated are capable of existing as positively or negatively charged ions. The stationary phase of this method of chromatography consists of a solid substance that participates in the separation process by interacting directly with components of a mixture. The majority of ion-exchangers are synthetic resins that are fabricated in the form of very small, uniform beads.

Chemically, each solid bead is composed of large polymeric chains. The ability of the resin material to function as an ion-exchanger is due to the

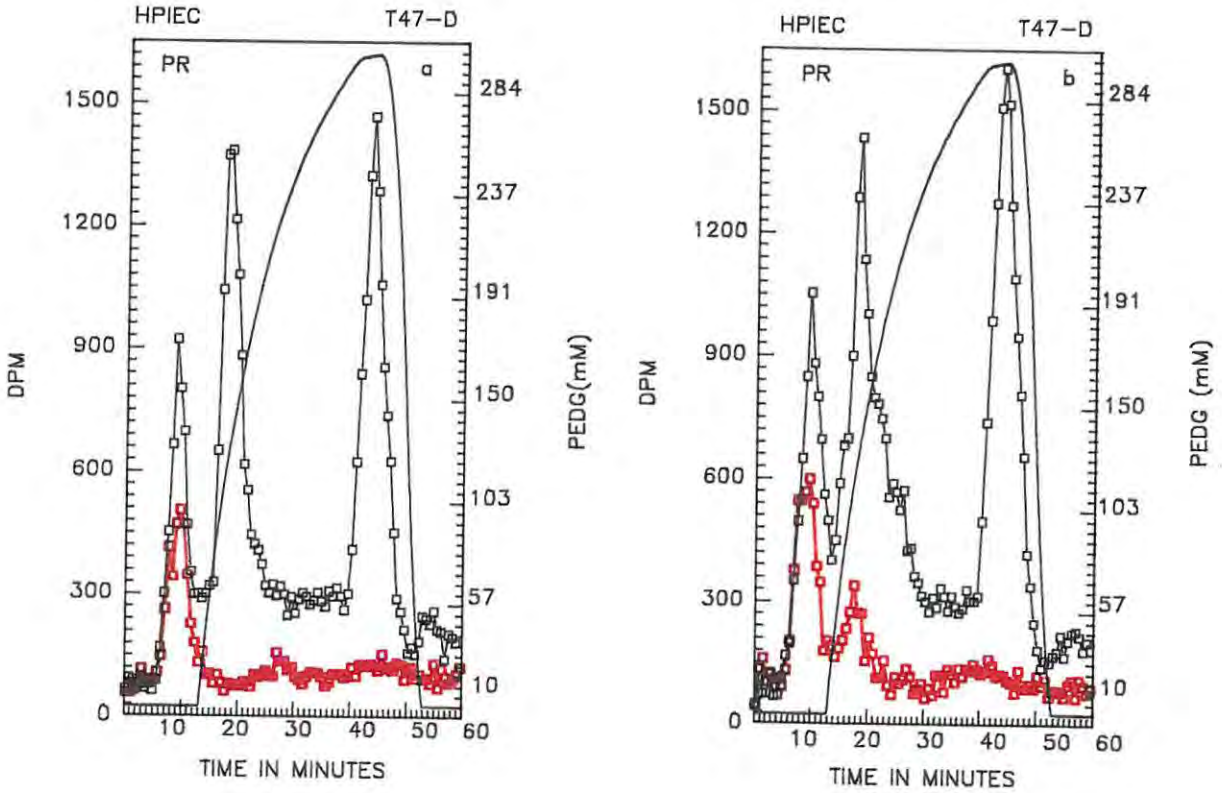


FIGURE 13: HPIEC of PR from T47-D human breast cancer cells.
a) T47-D cells grown in RPMI-1640 media.
b) T47-D cells grown in serum free media.
Cytosols were incubated overnight with 5nM ^{3}H organon in the ◘ presence or□ absence of a 200 fold excess of unlabelled organon 2058. 200*ul* aliquots of each reation were cleared of unbound ligand with DCC and injected onto a glass column packed with DEAE Spherodex-M. The column was linked to a flow-through *beta* counter equiped with an in line conductivity meter. A linear gradient elution of P10EDG to 60% P500EDG buffer was used. Three isoforms were eluted, at the void volume, P120EDG, P300EDG and a shoulder peak at P180EDG respectively.

presence of several ionisable chemical groupings that are attached along the length of the polymeric chain. The stationary phase thus has fixed charges, each having an associated counter-ion of an opposite charge to the starting buffer. The ionic solute distributes itself between the mobile liquid and the stationary phases by exchanging ions with a counter ion of the solid electrolyte as it passes through the column. Since various ionic solutes have different affinities for the exchange site, the solutes pass through the column at different rates (Figure 14). The analysis of steroid hormone receptors on the basis of their surface charge properties has been generally successfully performed on polyionic columns such as ATP-sepharose (308), carboxymethyl-sephadex (309), phosphocellulose (310), DNA-cellulose (311) or alternatively on anion-exchangers such as DEAE-Sephadex (312).

The extensive literature on both conventional gel-type ion-exchange resins for proteins and HPLC packings indicate that the ideal packing for HPIEC should be mechanically stable to mobile phase, of high ion-exchange capacity, completely hydrophilic and chemically stable over a broad pH range, available in 5 to 10 micron size as well as spherical, easy to pack and inexpensive (313). Unfortunately, no packing material is available at the present time that appears to possess all these necessary properties. Retention on IEC is controlled by two independent phenomena, i.e. the inherent size-exclusion contribution from differential penetration of solutes of macroporous matrices and electrostatic

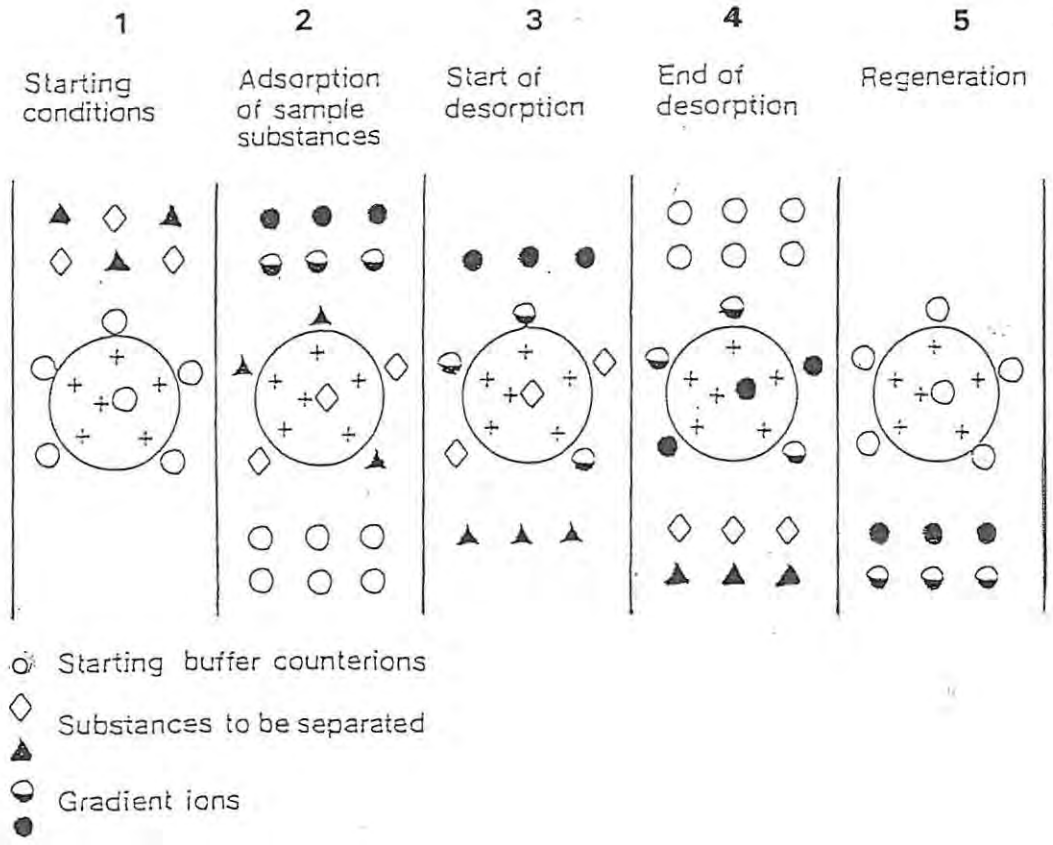


FIGURE 14: Explanatory diagram of IEC.

partitioning at the surface of the ion-exchanger (314).

This inherent size-exclusion properties of HPIEC packings causes band spreading to occur as solute size approaches pore diameter. Because IEC is a surface-directed process, it would seem that ion-exchange capacity should be proportional to the surface area of a packing. Greater than 95% of the surface of a porous support is, however, inside the pore network, and a molecule must be able to penetrate the pore matrix to reach the surface. It has also been determined that column length plays a minimal role in the resolution of proteins. Resolution on 5cm columns is almost equivalent to that of 25cm columns (315), although with short columns low and dead volume connecting tubing and flow cells are essential.

Several laboratories have utilized HPIEC to resolve ionic isoforms of steroid hormone receptors (266,305,306). The speed and reproducibility of this method make it a desirable research tool and the recovery of applied receptor as a function of radioactivity may, as in our case, be 90% or greater. This methodology is suited to rapid, multidimensional analysis, especially coupled to attributes such as dead volume, and the relative ease of the application of on line, flow-through methods of conductivity and radiation detection which are available in our laboratory (307).

6. HPIEC of PR in oestrogen treated MCF-7 and T47-D breast cancer cell lines

Over the past few years there has been great interest in understanding the mechanisms by which hormones affect cell proliferation and protein synthesis (13-15,17,26,34,37,50). Culture systems have been used in many analyses, since they enable responses to be monitored under carefully controlled conditions of hormone exposure. Oestrogens are well known to stimulate a variety of biosynthetic processes in hormone responsive target cells (87). They appear to influence progestin receptor synthesis (98,110).

Concurrently it must be borne in mind that the *in vitro* cell culture conditions may give a very simplistic view of the biology of tumourgenesis and that *in vivo,* the regulation of growth of breast tumour cells is the product of complex interactions among stromal, vascular and epithelial tumour elements. At present these interactions are still poorly understood. No model system currently available can hope to reproduce *in vivo* conditions accurately. Experimental result differences obtained from a single model system should be viewed with caution, since substantial differences in the characteristics and behaviour of the same breast tumour cell line from different laboratories have been noted(291).

Oestradiol-*17B* and DES were added to the MCF-7 and T47-D cells

grown in serum-free media. PR levels and HPIEC analysis was carried out on these cell cultures at set time intervals of 24, 48, 72 and 168 hours to ascertain the effects these oestrogens may elicit on PR levels and PR isoform distribution. Figure 15 indicates the changes that occurred to the PR in the MCF-7 breast cell lines over a period of a week. There was a marked decrease in the PR levels, particularly with oestradiol-*17B* administration; this despite the fact that there was over the same time period and on the same cells an increase in the proliferation rates over the control group of cells. HPIEC of the PR was carried out on the same cells taken at the same time as the PR levels were assayed.

Figure 16 illustrates the results of the MCF-7 group of cells treated with oestradiol-*17B* . The most striking event of these chromatograms is that there is confirmation of the decreasing levels over time of PR and the dissapearance of the isoform at 120mM phosphate buffer coinciding with a dramatic fall in PR levels.

Figure 17 illustrates the DES-treated group of MCF-7 cells. Once again the same phenomena has occurred. Accompaning the fall of PR levels there is the corresponding dissappearance of the isoform eluting at 120mM phosphate buffer after 72 hours of the MCF-7 cells being under the influence of DES in culture.

In another group of experiments, the T47-D breast cancer cells were

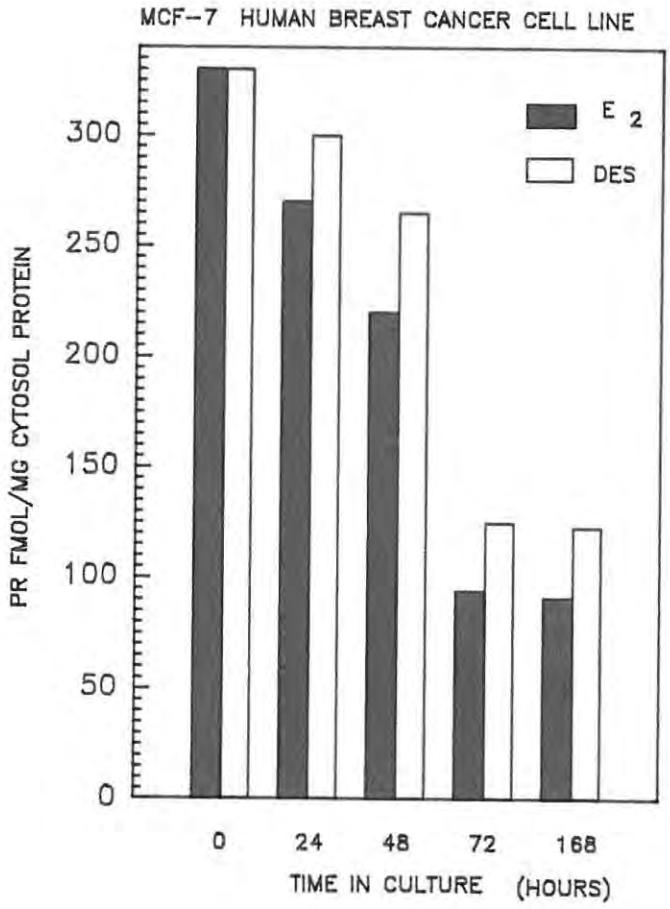


FIGURE 15: Histogram of PR levels from MCF-7 human breast cancer cells grown in serum-free media under the influence of *17B* oestradiol and DES. PR levels were measured before inoculation of oestrogens and again at 24,48,72 and 168 hours respectively.
Cytosols were incubated overnight with ^{3}H organon 2058 in the presence or absence of a 200 fold excess off unlabelled organon 2058. Aliquots of each reaction were cleared of unbound ligand with DCC and added to scintillation fluid and counted in a *beta* scintillation counter with a counting efficiency of 60%. Specific binding capacity was expressed in fmol/mcp using the difference between the total binding and the non-specific binding.

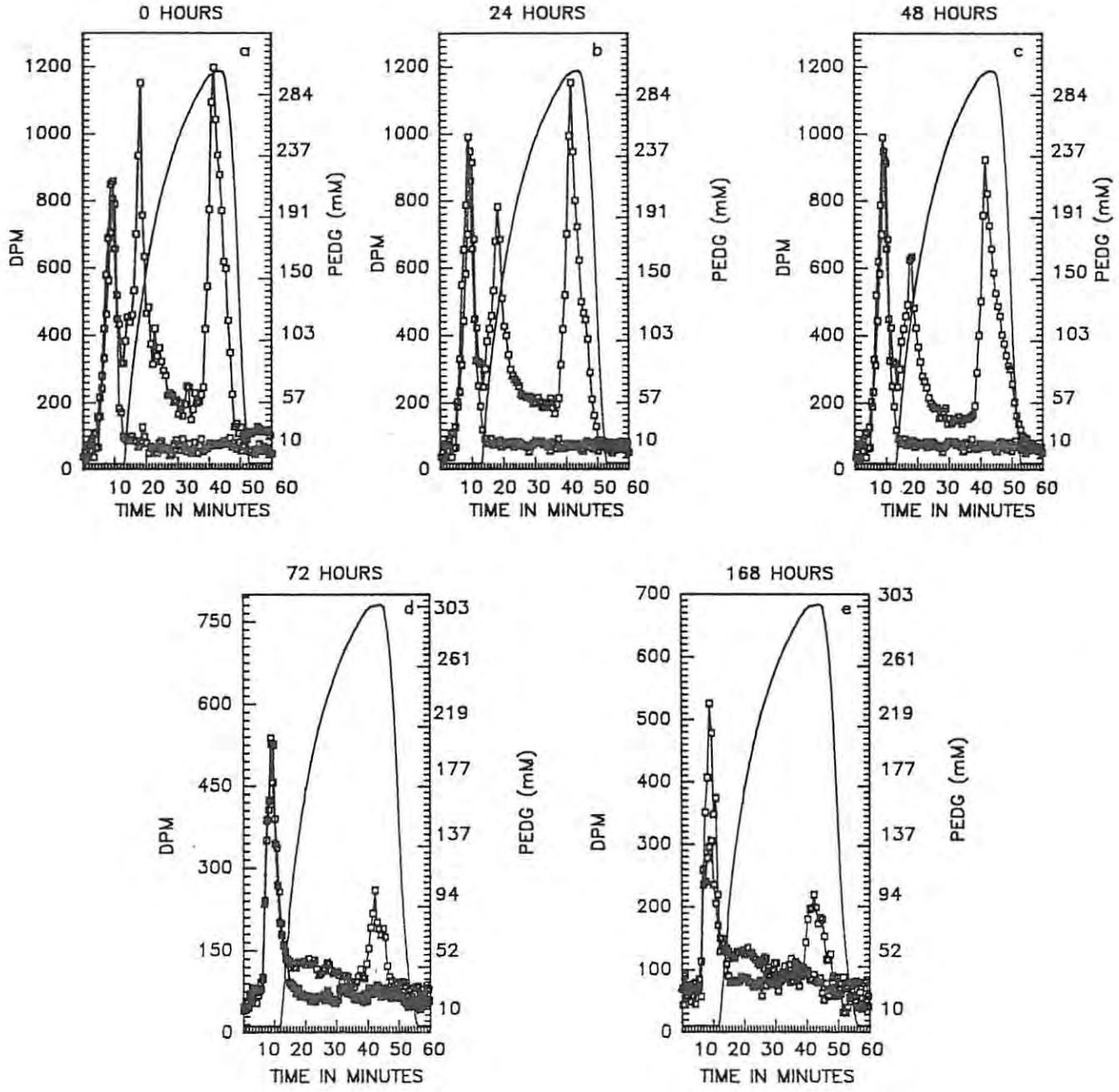


FIGURE 16: HPIEC of PR from MCF-7 cells grown in serum-free media under the influence of *17B*-oestradiol for varying periods of time.

a) PR from control MCF-7 cells not under oestrogen influence.
b) PR from MCF-7 cells 24 hours after *17B*-oestradiol inoculation.
c) PR from MCF-7 cells 48 hours after *17B*-oestradiol inoculation.
d) PR from MCF-7 cells 72 hours after *17B*-oestradiol inoculation.
e) PR from MCF-7 cells 168 hours after *17B*-oestradiol inoculation.

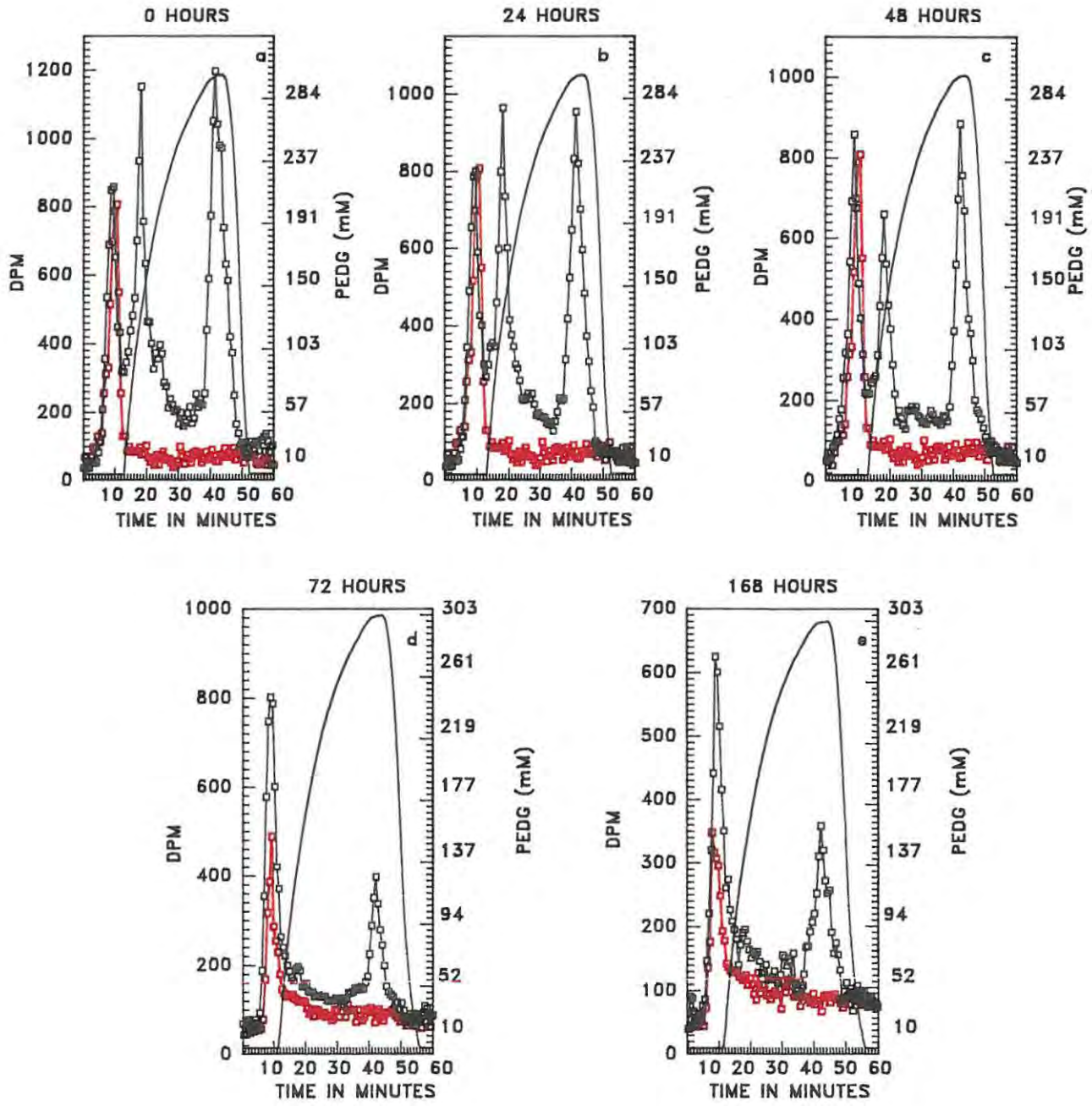


FIGURE 17: HPIEC of PR from MCF-7 cells grown in serum-free media under the influence of DES for varying periods of time.
a) PR from control MCF-7 cells not under DES influence.
b) PR from MCF-7 cells 24 hours after DES inoculation.
c) PR from MCF-7 cells 48 hours after DES inoculation.
d) PR from MCF-7 cells 72 hours after DES inoculation.
e) PR from MCF-7 cells 168 hours after DES inoculation.

treated in an identical fashion to the MCF-7 cells. The T47-D cells were inoculated with oestradiol-*17B* and DES and grown under the influence of these oestrogens for a week respectively. Determination of PR levels, were carried out at 24,48,72 and 168 hours. The PR levels of the control was 1280 fmol/mg cytosol protein and may be discerned from figure 18, decreased over the period of 1 week to 340 fmol/mcp for the oestradiol-*17B* group and 590 fmol/mcp in the DES group. As was the case with MCF-7 cells, the decrease of PR levels with the synthetic oestrogens was not as great as with the Oestradiol-*17B* group.

Figure 19 illustrates that,although a decrease in PR levels (over the time period of a week) was evident in the oestrogen group, the HPIEC isoform at 120mM phosphate buffer and as the isoform at 300mM phosphate buffer remained largely intact over the inoculation period of one week. Figure 20 shows that the same occurred in the synthetic DES-treated T47-D group of cells.

The behaviour of the T47-D cells while under the influence of oestrogens appeared to have similar proliferation rates, loss PR levels. On HPIEC, it was evident that, although loss of PR level occurred, the chromatogram remained largely similar to the control chromatogram viz.no loss of PR isoform occurred. However, with the MCF-7 cells under the influence of oestrogens, there was on HPIEC a distinct loss of isoform at 120mM phosphate buffer.

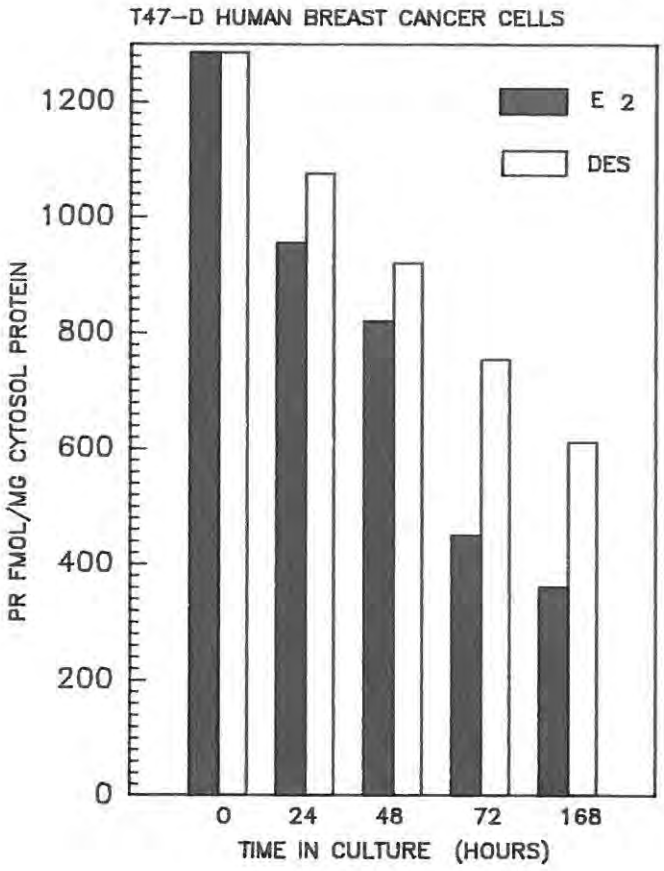


FIGURE 18: Histogram of T47-D human breast cancer cells grown in serum-free media under the influence of *17B*-oestradiol and DES. PR levels were measured before inoculation with oestrogen and again at 24,48,72 and 168 hours respectively. Declines in PR were noted.Cytosols were incubated with 5nM ^{3}H organon 2058 in the presence or absence of unlabelled organon 2058. Aliqouts of each reaction were cleared of unbound ligand with DCC and added to scintillation fluid and counted in a *beta* scintillation counter with 60% efficiency. Specific binding capacity was expressed in fmol/mcp using the difference between the total binding and the non-specific binding.

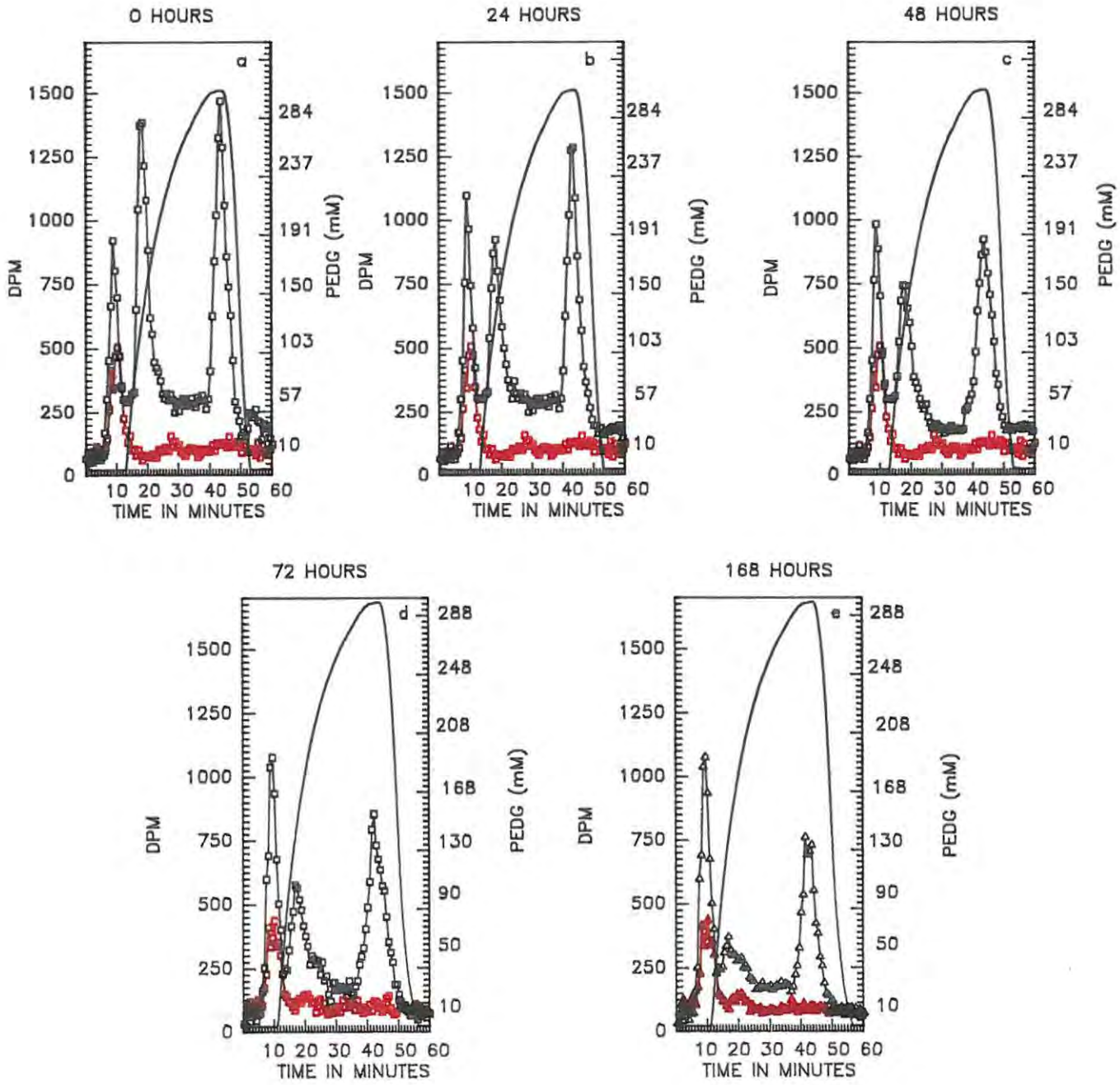


FIGURE 19: HPIEC of PR from T47-D cells grown in serum-free media under the control of *17B*-oestradiol for varying periods of time.
a) PR from control T47-D cells not under oestrogen control.
b) PR from T47-D cells 24 hours after *17B*-oestradiol inoculation.
c) PR from T47-D cells 48 hours after *17B*-oestradiol inoculation.
d) PR from T47-D cells 72 hours after *17B*-oestradiol inoculation.
e) PR from T47-D cells 168 hours after *17B*-oestradiol inoculation.

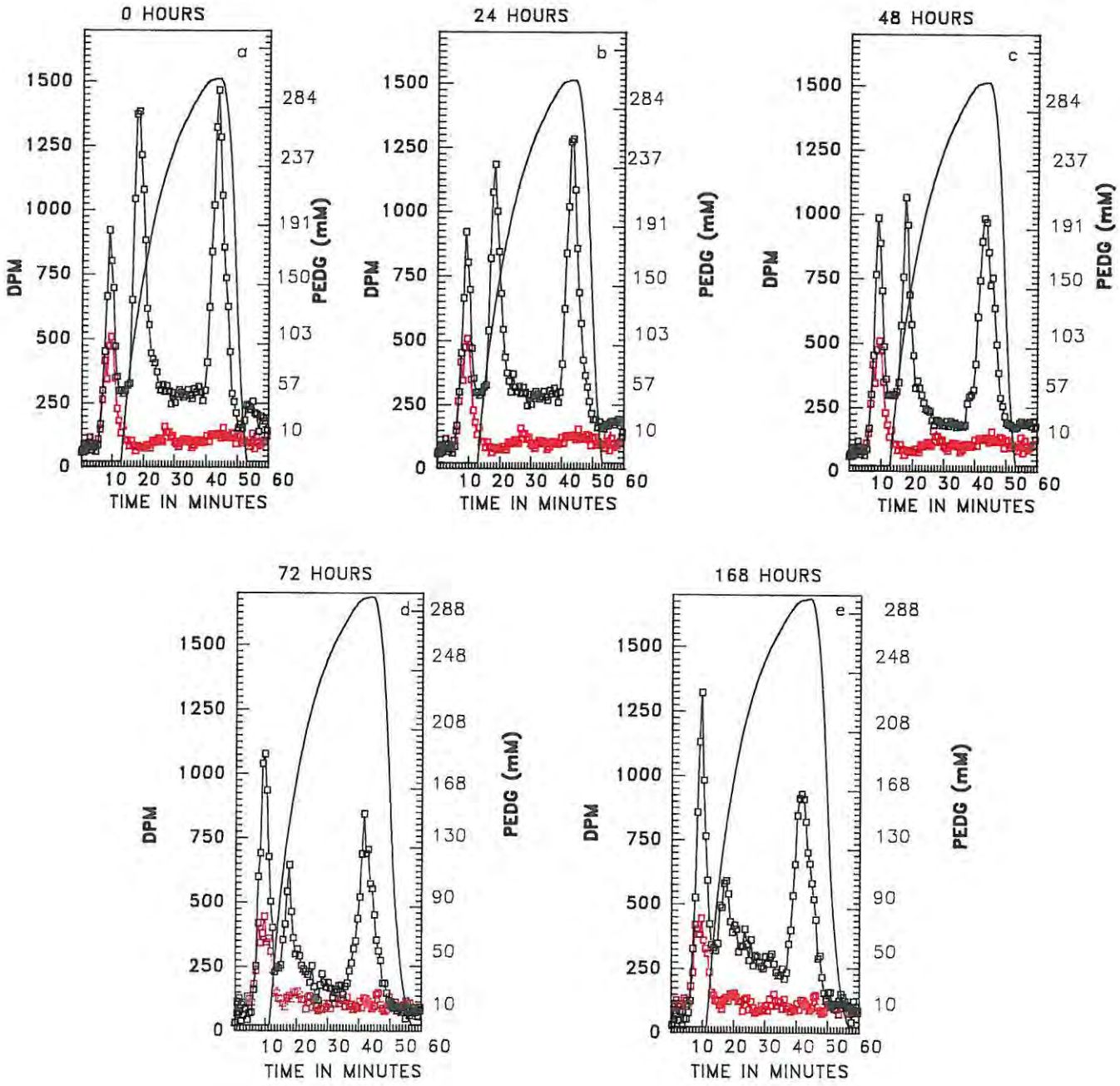


FIGURE 20: HPIEC of PR from T47-D cells grown in serum-free media under the influence of DES for varying periods of time.

a) PR from T47-D cells not under oestrogen control.
b) PR from T47-D cells 24 hours after DES inoculation.
c) PR from T47-D cells 48 hours after DES inoculation.
d) PR from T47-D cells 72 hours after DES inoculation.
e) PR from T47-D cells 168 hours after DES inoculation.

7. Progestin receptor levels and HPIEC of anti-oestrogen treated MCF-7 and T47-D cells.

Tamoxifen and 4-hydroxytamoxifen were added to the MCF-7 cell line grown in serum-free media. The PR levels were measured at timed intervals for 24, 48, 72 and 168 hours respectively. Figure 21 illustrates the downward trend of the PR levels over a period of one week. The tamoxifen group of cells showed a greater fall in PR levels than the hydroxylated group of cells. This decrease was accompanied by a decline in the proliferation rates of treated MCF-7 cells.

Figure 22 gives a pictorial indication of the PR chromatograms on HPIEC from MCF-7 cells cultured in a serum-free environment under the influence of tamoxifen carried out at timed intervals after treatment commenced, viz. 24,48,72 and 168 hours. It was noted that the loss of the isoform at 120mM phosphate buffer occurred at 72 hours after tamoxifen inoculation and that the isoform at 300mM phosphate buffer was barely discernible after one week. This may have been a function of the extremely low levels of PR reached at that time.

Figure 23 with the hydroxylated tamoxifen inoculated MCF-7 group, the loss of the isoform at 120mM phosphate buffer was noted at one week but a small discernible peak at 300mM phosphate buffer was still observed after one week. At the end of a week's incubation with tamoxifen and 4-hydroxytamoxifen, the anti-oestrogens were withdrawn

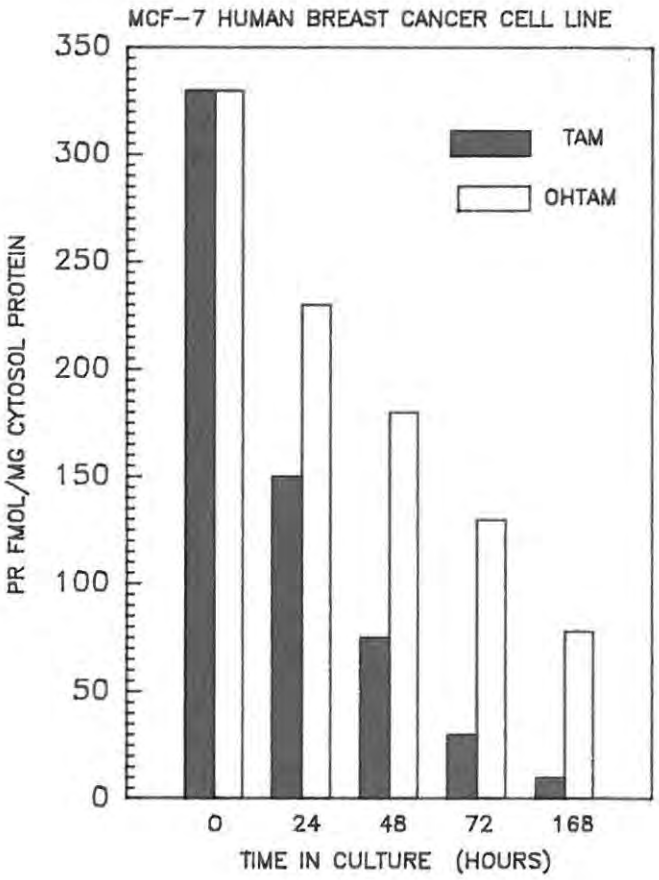


FIGURE 21: Histogram of PR levels from MCF-7 human breast cancer cells grown in serum-free media under the influence of tamoxifen and 4-hydroxytamoxifen. PR levels were measured before inoculation of anti-oestrogens and again at 24,48,72 and 168 hours respectively.
Cytosols were incubated overnight with 5nM ^{3}H organon 2058 in the presence or absence of a 200 fold excess of unlabelled organon 2058. Aliquots of each reaction were cleared of unbound ligand with DCC and added to scintillation fluid and counted in a *beta* scintillation counter. Counter efficiencies were in the order of 60%. Specific binding capacity was expressed in fmol/mcp using the difference between the total binding and the non-specific binding.

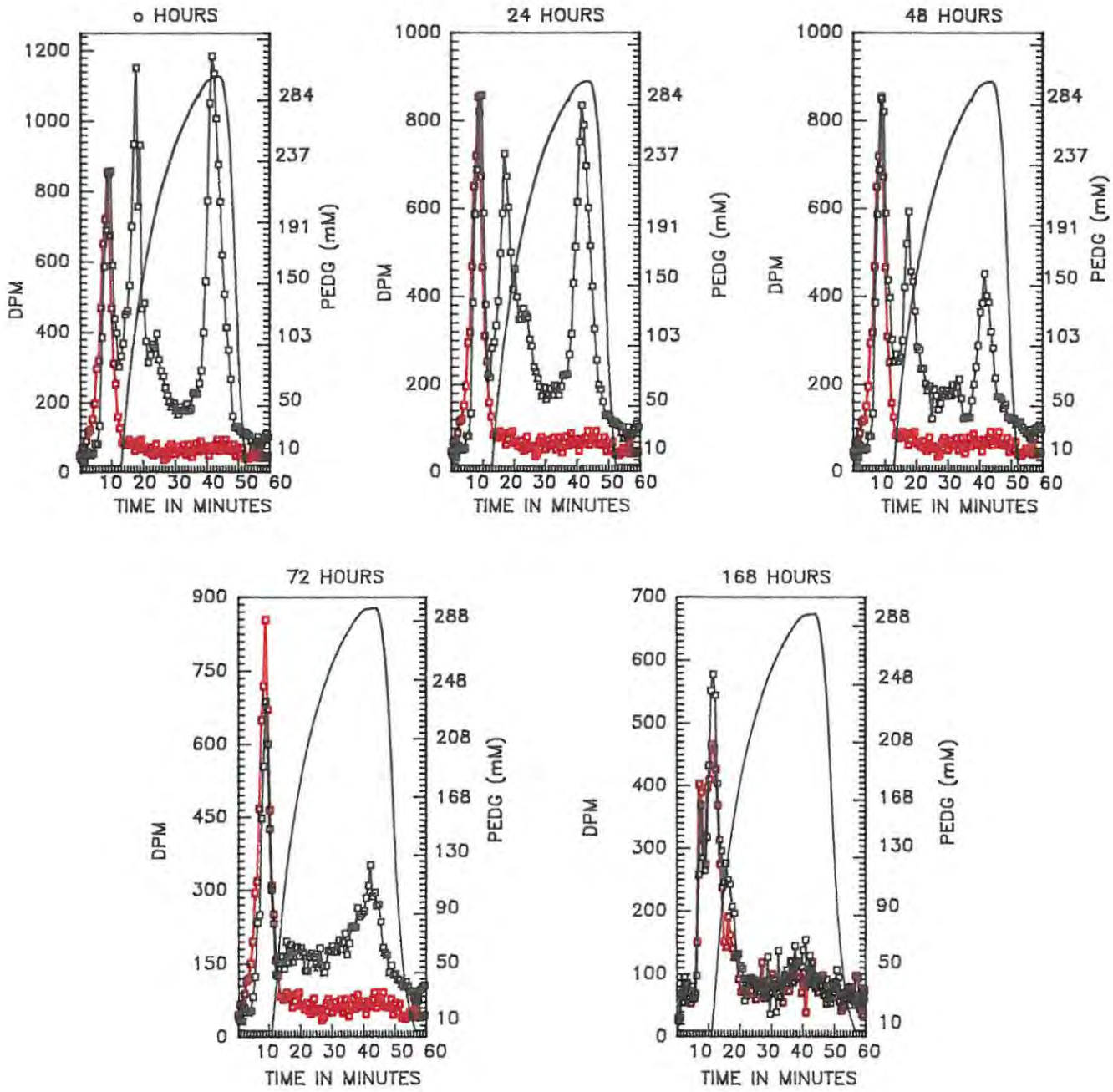


FIGURE 22: HPIEC of PR from MCF-7 cells grown in serum-free media under the influence of tamoxifen for varying periods of time.
a) PR from MCF-7 cells no under tamoxifen control.
b) PR from MCF-7 cells 24 hours after tamoxifen inoculation.
c) PR from MCF-7 cells 48 hours after tamoxifen inoculation.
d) PR from MCF-7 cells 72 hours after tamoxifen inoculation.
e) PR from MCF-7 cells 168 hours after tamoxifen inoculation.

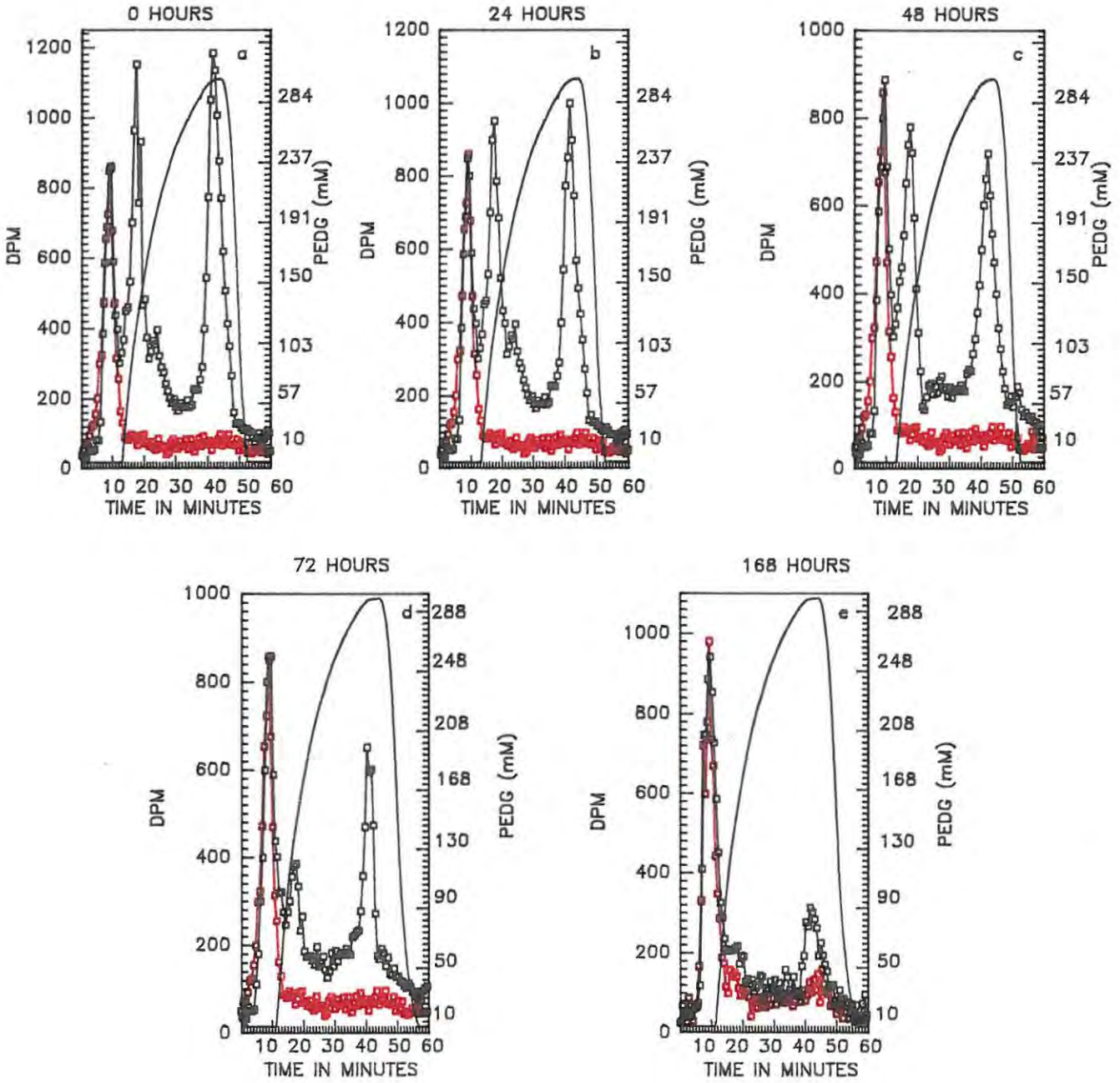


FIGURE 23: HPIEC of PR from MCF-7 cells grown in serum-free media under the influence of 4-hydroxytamoxifen for varying periods of time.
a) PR from MCF-7 cells no under tamoxifen control.
b) PR from MCF-7 cells 24 hours after 4-hydroxytamoxifen inoculation.
c) PR from MCF-7 cells 48 hours after 4-hydroxytamoxifen inoculation.
d) PR from MCF-7 cells 72 hours after 4-hydroxytamoxifen inoculation.
e) PR from MCF-7 cells 168 hours after 4-hydroxytamoxifen inoculation.

from the culture media and an attempt was made to grow these cells in either RPMI-1640 plus 5% foetal calf serum or in serum free-media, but to no avail. Proliferation of cells could not be achieved.

With T47-D cells under the influence of anti-oestrogens in a serum-free environment, it was noted that there was also a sharp decline in PR levels over the same period of a week as was the case with the MCF-7 cells. Figure 24 illustrates this. Figure 25 shows the chromatograms of the PR of the tamoxifen-treated group of T47-D cells. Unlike the chromatograms of the MCF-7 anti-oestrogen groups, the PR isoforms at 120mM and 300mM phosphate buffer respectively have largely remained intact. In Figure 26 the same applies to the hydroxylated tamoxifen-treated T47-D cells. Once again the HPIEC chromatograms have remained largely intact during the inoculation period of one week.

8. DNA Analysis by flow cytometry of oestrogen and anti-oestrogen treated MCF-7 and T47-D cells.

Flow cytometry allows the assessment of the DNA content and estimation of cell cycle characteristics of tumours. This may provide information on many common cancers (316). Optical resolution is a prerequisite to prevent inaccurate calculations of the percentage cells in the cell cycle phases. In the context of DNA analysis, resolution reflects the capability of the instrument to distinguish similar but not identical DNA quantities.

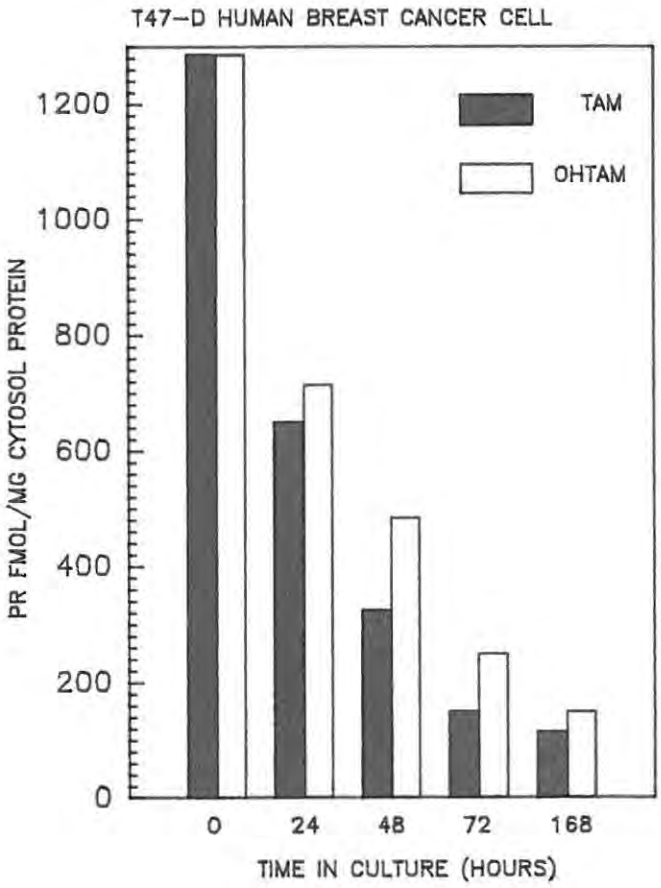


FIGURE 24: Histogram of PR levels from T47-D human breast cancer cells. grown in serum-free media under the influence of tamoxifen and 4-hydroxytamoxifen. PRlevels were measured before inoculation of anti-oestrogens and again at 24,48,72 and 168 hours respectively. Decline in PR levels were noted. Cytosols were incubated overnight with ^{3}H organon 2058 in the presence or absence of a 200 fold excess of unlabelled organon 2058. Aliquots of each reaction were cleared of unbound ligand with DCC and added to scintillation fluid and counted in a *beta* sctillation counter with counting efficiency of 60%. Specific binding capacity was expressed in fmol/mcp using the difference between total binding and non-specific binding.

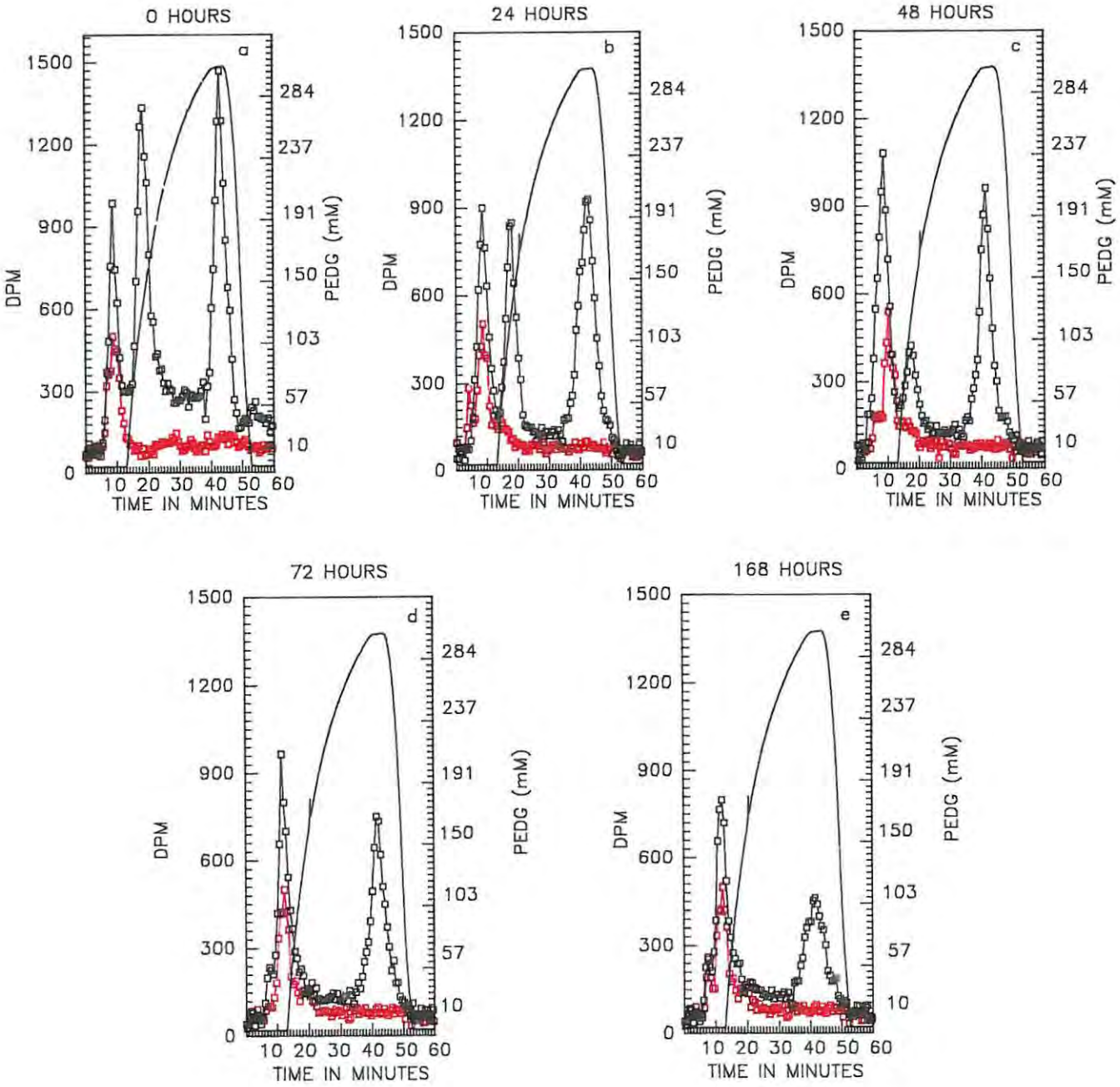


FIGURE 25: HPIEC of PR from T47-D cells grown in serum free media under the influence of tamoxifen for varying periods of time.
a) PR from T47-D cells not under tamoxifen control.
b) PR from T47-D cells 24 hours after tamoxifeninoculation.
c) PR from T47-D cells 48 hours after tamoxifen inoculation.
d) PR from T47-D cells 72 hours after tamoxifen inoculation.
e) PR from T47-D cells 168 hours after tamoxifen inoculation.

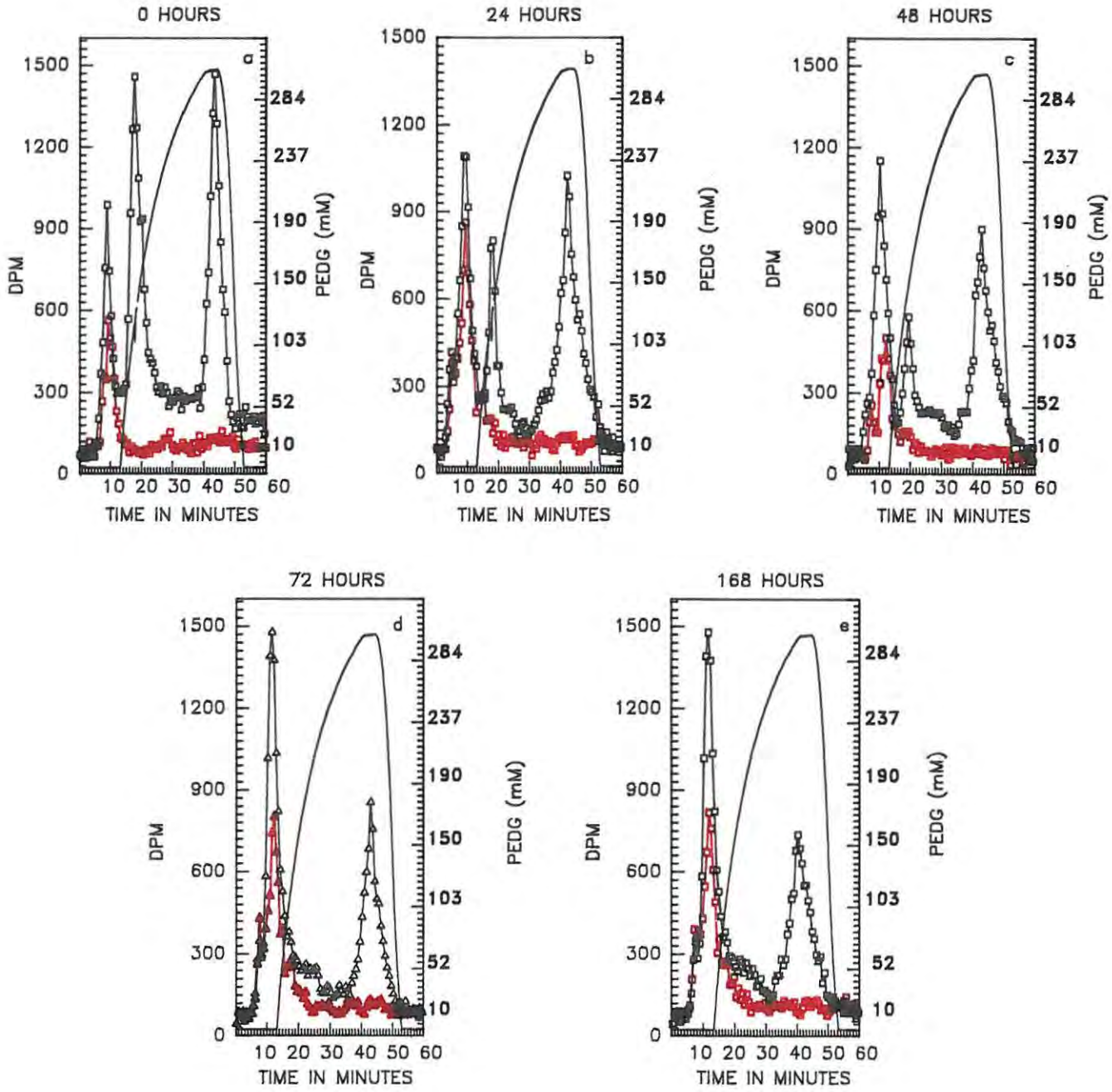


FIGURE 26: HPIEC of PR from T47-D cells grown in serum-free media under the influence of 4-hydroxytamoxifen for varying periods of time.
a) PR from T47-D cells no under 4-hydroxytamoxifen control.
b) PR from T47-D cells 24 hours after 4-hydroxytamoxifen inoculation.
c) PR from T47-D cells 48 hours after 4-hydroxytamoxifen inoculation.
d) PR from T47-D cells 72 hours after 4-hydroxytamoxifen inoculation.
e) PR from T47-D cells 168 hours after 4-hydroxytamoxifen inoculation.

In order for the analysis of the treated MCF-7 (and for that matter the T47-D cells) to be understood. Figure 27 represents an example of a histogram that is obtained from the Coulter Epics^{R} flow cytometer. When cells are labelled with fluorescent molecules, extremely sensitive, specific and rapid measurements can be made using flow cytometry. Large numbers of cells prepared in free suspension flow within a fluid stream in a single file past a laser beam where they are individually evaluated. Light is scattered as the cells pass through the laser light and generates fluorescent light. Scattered light is at the same wavelength as that of the laser beam. If the cells have been bound by a reporter fluorescent molecule, the flourochrome will transiently absorb the laser light and then emit it at another light frequency.

The flow cytometer is equipped with photomultiplier tubes for this emitted light which not only detects the flourescent signal, but quantitates its intensity. The intensity of the emitted light is directly proportional to the amount of the fluorescent compound bound to the cells or particle (figure 28). Evaluating large numbers of cells quantitatively and reproducibly by flow cytometry increases the statistical confidence and precision of the data. In DNA content analysis the relative DNA content of the nucleus of the cell is evaluated. The fluorochrome most commonly used for this analysis is propidium iodide (figure 29). One molecule propidium iodide (PI) binds stoichiometrically to five DNA nucleotides. Propidium iodide binds to DNA in cells at all

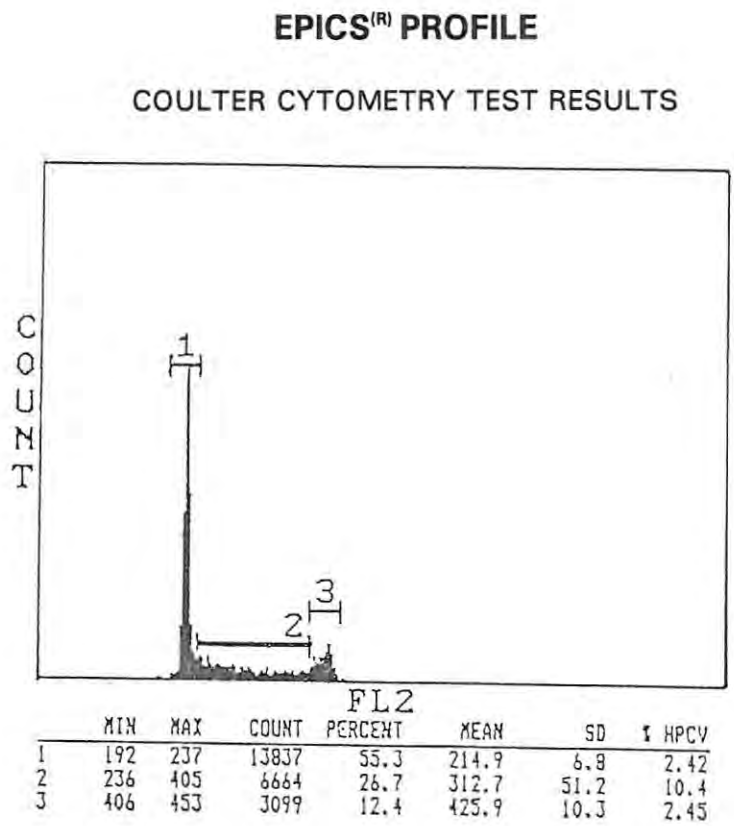


FIGURE 27: Flow cytometer histogram.
Representative histogram of cell number (y-axis) versus fluorescence/FL2 (x-axis). The percentage cells in the G_0G_1, S-phase and G_2M- phases were designated by cursors 1 (55.3%), 2 (26.7%) and 3 (12.4%) respectively. The sum of the percentages included in cursors 1,2, and 3 was 94.4% - the remaining 5.6% reflected cellular debris.
The mean channel number of G_2M (426 representing 4n) is approximately twice that of the mean channel number of G_0G_1 (214 representing 2n). The percentage HPCV values for cursor 1 (2.42) and 3 (2.45) were less than 3%. The large % hpcv value for cursor 2 (10.4) reflects the width of the S-phase "peak" - the amount of DNA in the S-phase varied predictably from 2n right up to 4n. The SD values showed a similar trend for the same reason (6.8 for cursor 1; 10.3 for cursor 3 and 51.2 for cursor 2).
The DNA index for the histogram was 1.07. This was calculated by dividing the mean channel number of the G_0G_1-phase peak by the mean channel number (always calibrated to 200) of the control female DNA derived from peripheral blood lymphocytes.

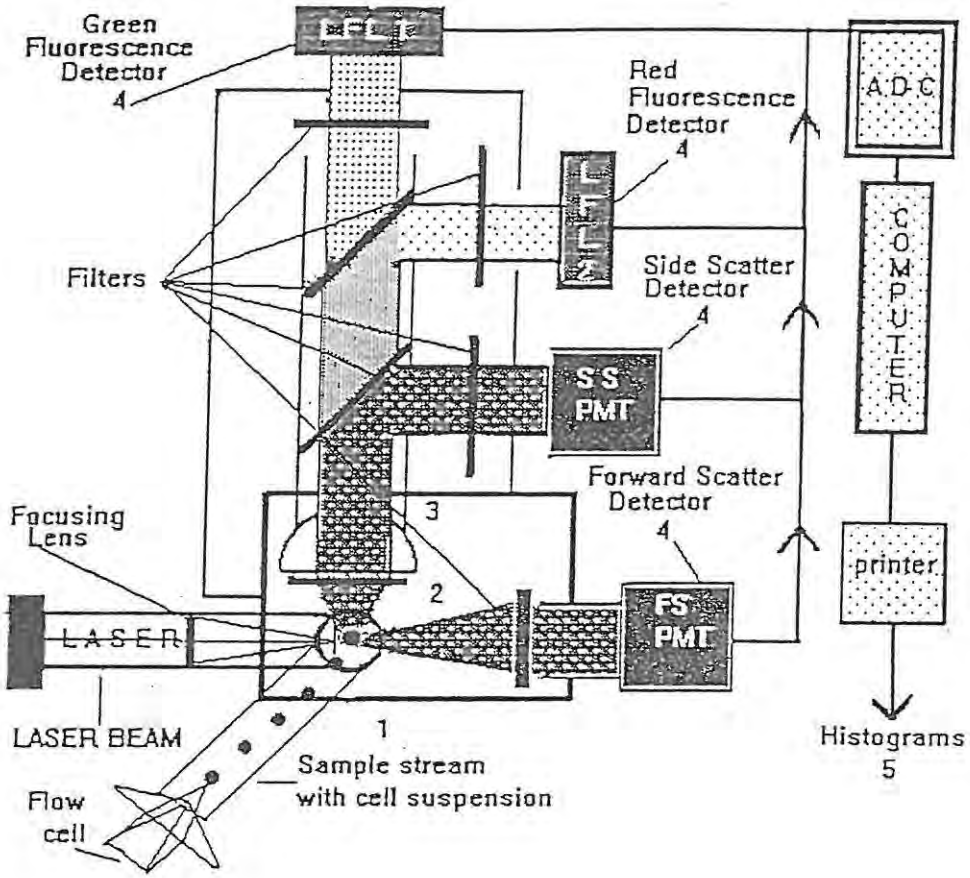


FIGURE 28: Schematic representation of a flow cytometer.

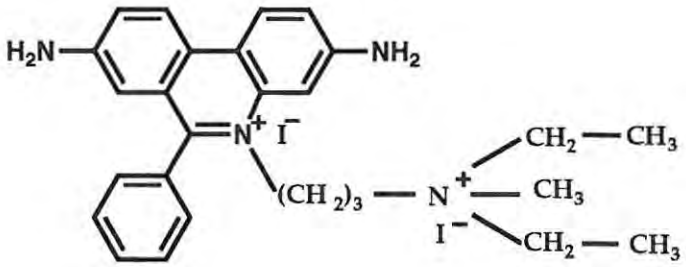


FIGURE 29: Chemical structure of propidium iodide.

stages of the cell division cycle and the intensity with which a cell's nucleus emits light is directly proportional to it's DNA content (317).

Accessibilty to the cell nucleus was acccomplished by permeabilizing the cell membrane or by stripping away the cytoplasm with detergents such as Triton-100 and Nonident-40. Polyethylene glycol was used to stabilize the DNA. Optimal staining with propidium iodide was obtained at a cell concentration of 2-3 x 10^6cells/50*ug*/ml of propidium iodide. PI staining usually occurs in the dark. Since many nucleotide binding dyes bind to both DNA and double stranded RNA, the latter was removed by digestion with ribonuclease. The highest quality ribonuclease should be used to minimise the presence of contaminating proteolytic enzymes and DNase (318,319).

One of the characteristics of many malignancies is the presence of an abnormal chromosome number, one that is not a multiple of 23. These particular cells are called DNA "aneuploid". Some of the DNA abnormalities in cancer can be very subtle and others may involve gross chromosomal aberrations. Most normal cells in the body at any one time are in a quiescent stage, termed G_0. As cells respond to signals to divide they move into the G_1 stage where RNA and the proteins necessary for DNA synthesis are produced. The replication of total cellular DNA occurs in the synthetic stage termed S-phase. At the end of the S-phase the cell contains twice as much its original DNA content but is still bound by one

external membrane. In preparation for cell division further proteins are elaborated in a phase termed G_2. The stage at which the cell actually divides into two daughter cells is termed *M* for "mitosis". The length of each of the phases of the cell cycle is a characteristic of the individual cell population.

The representative DNA histogram in figure 30 highlights the effects of one week exposure of oestradiol-*17B,* DES, tamoxifen, 4-hydroxy-tamoxifen had on DNA synthesis, and consequently on the distribution of MCF-7 cells grown in serum-free media in the various cell cycle phases. The control MCF-7 cells grown only in serum-free media without any hormone supplementation showed that 56% of the cells were in the G_0G_1 phase and were possibly diploid in DNA content. Fifty six percent of the cells appeared to be in the synthetic S-phase. Unfortunately, exact ploidy definitions could only be possible by the use of Bromodeoxyuridase (BrDU), which is a halogenated thymidine analogue and is only incorporated into the S-phase DNA cells (320).

Following a week in an oestrogen environment cell cycle perturbations seem to have taken place in the MCF-7 cells and the S-phase transit times have been accelerated. The G_0G_1 phase was now 13%, while the S-phase was 66% and the M-phase had moved to 12%. The MCF-7 cells that were exposed to anti-oestrogens also showed a decrease in the G_0G_1 phase with a flux of cells into the S-phase. However, repeatedly great

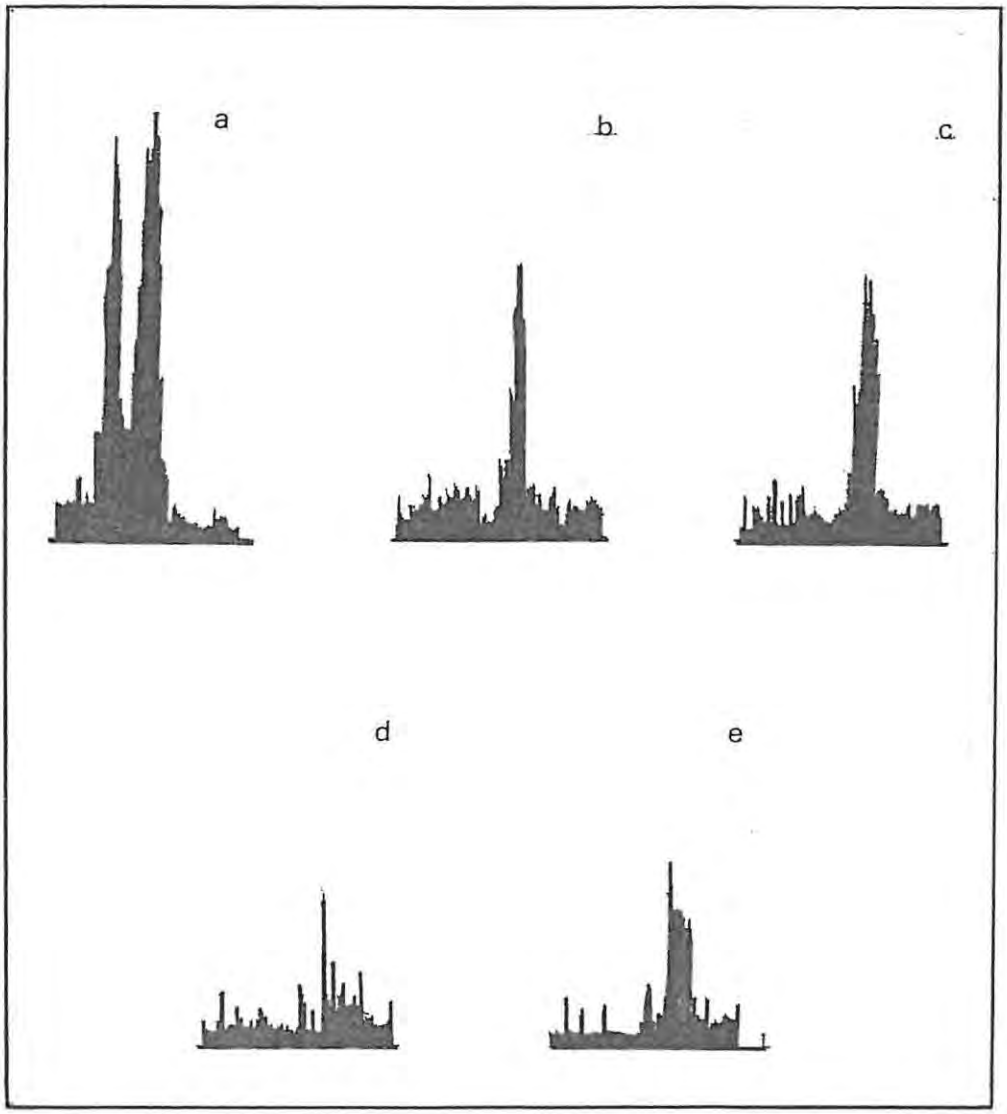


FIGURE 30: Representative cytokinetic data from MCF-7 human breast cancer cells under the influence of oestrogens and anti-oestrogens.

Representative histograms of cell number (y-axis) versus DNA content (X-axis).

a) Control MCF-7 cells grown in serum-free media without hormone or anti-hormone. G_0G_1 phase = 36%, S-phase = 56%.

b) MCF-7 cells exposed to *17B*-oestradiol for one week. G_0G_1 phase = 13%, S-phase = 66%.

c) MCF-7 cells exposed to DES for one week. G_0G_1 phase = 14%, S-phase = 66%.

d) MCF-7 cells exposed to tamoxifen for one week. G_0G_1 phase = 11%, S-phase = 71% and cellular debris comprised 14%.

e) MCF-7 cells exposed to 4 hydroxytamoxifen for one week. G_0G_1 phase = 11%, S-phase = 71% and cellular debris comprised 14%.

difficulty was experienced in finding nuclear particles. This is an indication of either cell death or apoptosis.

The representative DNA histogram in figure 31 highlights the effects that a weeks exposure of oestradiol-*17B*, DES, tamoxifen and 4-hydroxytamoxifen had on DNA synthesis and consequently on the distribution of T47-D cells grown in serum free-media in the various cell cycle phases. The control T47-D cells had 40% of the cells were in G_0G_1 phase and 43% were in S-phase. After an oestrogen-rich environment, there appeared to be a marked shift to the left with 81% of the cells in the G_0G_1 phase and only 9% in the synthetic S-phase. The T47-D cell line under the influence of anti-oestrogens showed a marked shift to the left with cellular debris comprising 64% of the total cells.

Remodelling of both the MCF-7 and T47-D cell lines occurred within an oestrogen-rich environment. With the observation that DNA remodelling with a shift to the right occurred in the MCF-7 cell line. These results may be coupled with those obtained by HPIEC where the PR level decline was accompanied by a shift in receptor isoforms. This leads to the speculation that these MCF-7 cells may indeed be a heterogenous population. The MCF-7 cells under the influence of anti-oestrogens appeared to be hormone responsive although there were cell cycle perturbations with a shift to the S-phase which is highly suggestive of a heterogenous population within the cell line.

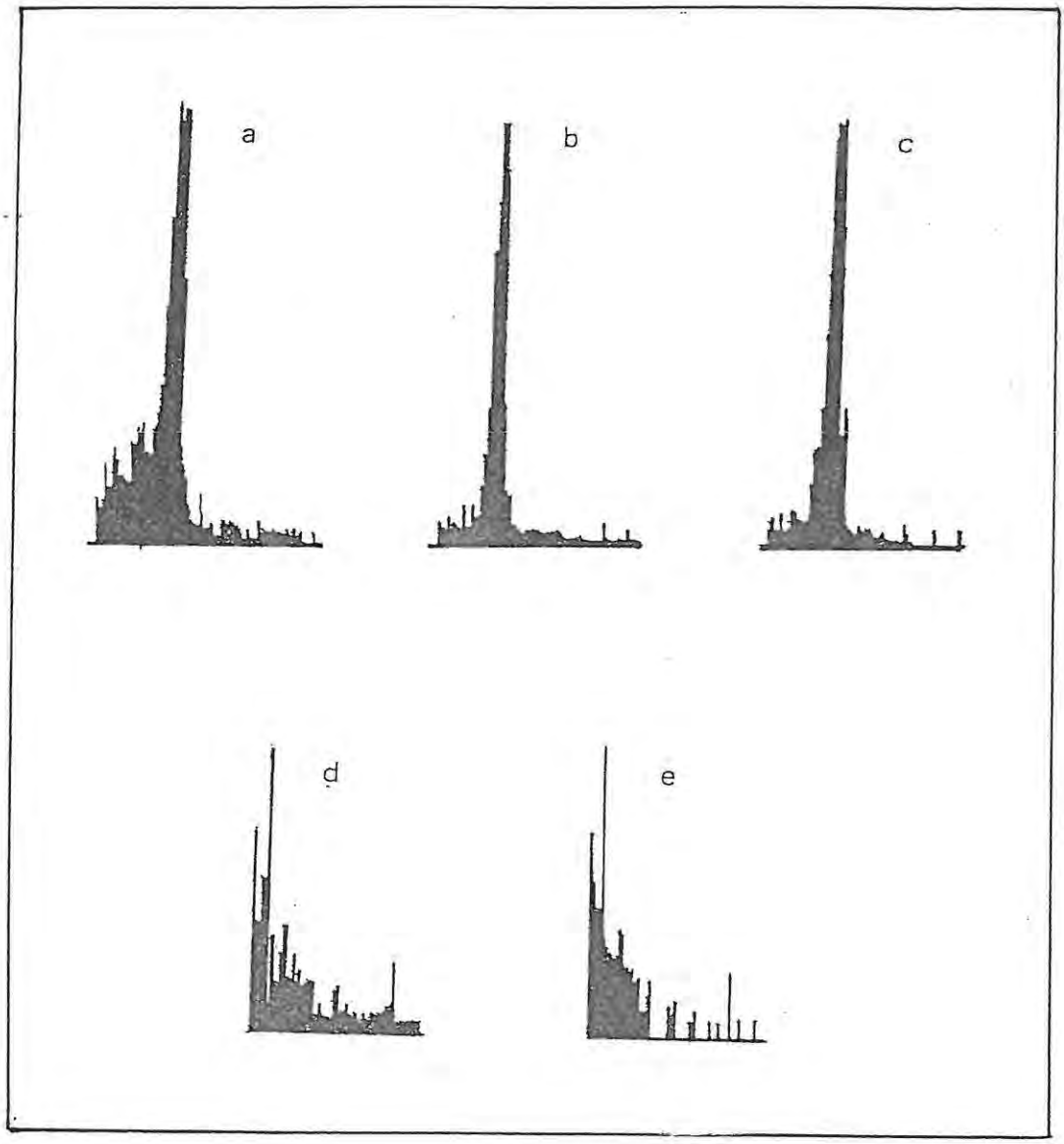


FIGURE 31: Representative cytokinetic data from T47-D human breast cancer cells under the influence of oestrogens and anti-oestrogens.

Representativenhistograms of cell number (y-axis) versus DNA content (x-axis).

a) Control T47-D cells grown only in serum-free media without hormone or anti-
b) hormone. G_0G_1 phase =40% and the S-phase =40%.
T47-D cells exposed to *17B* oestradiol for 1 week. G_0G_1 phase =81% and the S-phase =9.0%.

c) T47-D cells exposed to DES for 1 week. G_0G_1 PHASE =81% and the S-phase 11%.

d) T47-D cells exposed to tamoxifen for 1 week. A marked shift to the left with cellular debris comprising 64% of the total cell population.

e) T47-D cells exposed to tamoxifen for 1 week. A marked shift to the left with cellular debris comprising 66% of the total population.

With the T47-D cells under the influence of oestrogens, the shift away from the synthetic phase is indicative of remodelling of the cells. However, with the T47-D cells under the influence of anti-oestrogens and the marked increase in cellular debris may be indicative of hormone responsiveness.

The MCF-7 and T47-D human breast cancer cell lines have been successfully grown long-term in our laboratory in RPMI-1640 plus 5% foetal calf serum. The conditioning of these cells to grow in serum free media was relatively problem free. The baseline PR levels, SDG profiles and HPIEC resolutions were repeatedly almost identical as was the cell lines proliferation rates. After a week in an oestrogen-rich environment, a declination in PR levels occured. On HPIEC, altered chromatograms were observed. Decreases were also observed when the cells were placed in anti-oestrogen rich culture media. There were also subtle differences on flow cytometry between the two cell lines with the T47-D cell line appearing to be more hormone-responsive in an anti-oestrogen rich environment than the MCF-7 cells.

Flow cytometric analysis allows an assessment of the extent of heterogeneity within a population of cells. An assessmnent was made of the two cell lines used before oestrogen loading and at the end of a week of loading. In the MCF-7 line it appeared that a "remodelling" of the cell had occurred i.e. one population of cells had a distinct growth advantage

over the other. With the decrease in PR levels and isoform alterations on HPIEC, this may imply that the oestrogen-regulatory mechanism rendered defective beyond the initial interaction of the steroid-receptor complex.

While a normal tissue may respond uniformly to the regulatory signals of hormones, the genetic instability and heterogeneity that characterize malignant cells may render them incapable of a uniform response. Instead subpopulations, having unique programs, respond differently to the hormone signal in different culture conditions. The result could possibly be a waxing and waning of the selected cell subsets, ultimately leading to remodelling of the tumour to one with different biological properties. If oestrogen not only leads classically to the synthesis of PR but also has the ability to decrease PR levels and remodel PR biochemically under altered culture conditions, this makes for great concern. This underpins our simplistic view of *in vitro* models of MCF-7 and T47-D human breast cancer cells and reminds us of the complexities that exist in unraveling tumour cell growth and differentiation.

Tumour heterogeneity, while becoming one of the major prognostic determinants of treatment selection in the late 20th century remains one of the major hurdles in the cure of a large number of patients, even when optimal drug dose intensity is delivered. Our understanding of the interactions between clinical and biological factors and how to construct probabilities for prognosis from these interactions, remains largely

incomplete. In the final analysis survival, depends primarily on the presence or absence of micro-metastasis and on the ability of systemic treatment to control growth and the inherent instability of the tumour cell.

The precise mechanism/s by which tamoxifen achieves it's anti-tumour effects are still unknown. The drug appears to act primarily by competing with oestradiol for receptor sites in the cell nucleus, causing oestrogen blockade (151-157). This, however, does not explain the reason why the T47-D cells, being ostensibly ER poor, should in fact be tamoxifen responsive. Several alternative mechanisms of action have been postulated. Tamoxifen may inhibit cell proliferation by modulating the production of transforming growth factors *a* and *b* that help to regulate cell proliferation (161,162). It may bind to cytoplasmic anti-oestrogenic binding sites, increasing intracellular drug levels. In turn this may lead to an increase in SHBG, reducing the availabilty of free oestrogen for diffusion into tumour cells and decreasing the circulating insulin like growth factor 1 (163). This would modify the endocrinological regulation of cell kinetics. It has also been reported that tamoxifen may act as an inhibitor of tumour angiogenesis (155,156,166). Tamoxifen has also been shown to impair tumour initiation for as long as the administration of the drug is maintained (1).

Many parts of the jigsaw puzzle still need to be identified and clarified.

Breast cancer is indeed a multifactorial disease with steroid hormones and their receptors representing only a small component of that jigsaw. Questions that need to be addressed include the loss of functional ER. This in turn encompasses the genomic deletion of the gene itself, mutations or rearrangements of the gene, down regulation of transcription at the promotor level, methylation within the coding domain or the promotor region and altered message which occurs with alternative splicing.

CONCLUSION

In vitro cell culture models designed to assess the role of oestrogen or progestin agonists in tumour cell proliferation have generated contradictory results. Experiments can literally be cited in support of any argument that these agonists inhibit, stimulate or have no effect on growth. Explanations for the lack of a consensus are thus as varied as the results.

Responses of cells in culture are critically dependent on the conditions in which they are grown. In a rich medium, where the growth is optimized, further growth may be difficult to demonstrate experimentally while inhibitory stimuli may be exaggerated. In a deprived medium the reverse may be true, although here key cofactors may be lacking. There is no simple solution to these inherent problems. Couple this generic uncertainty with other variables including: the use of different cell lines, heterogeneity and genetic instability (even within the the same cell line), factors such as insulin, insulin-like growth factors, serum, epidermal growth factors, transforming growth factors, retinoic acid (other than oestradiol) and its antagonists, then these may directly or indirectly modulate sensitivity through regulation of PR levels. It is possible that no physiological consensus is likely to be forthcoming using these *in vitro* models. Nevertheless, these models remain invaluable for the analysis of molecular mechanisms of oestrogen and anti-oestrogen actions and to demonstrate the complexities inherent in tumour biology.

Biochemical assays remain the best available quantitative assessment of steroid receptors at present. The use of highly efficient synthetic progestins as ligands for the biochemical assays have given credence to reliable quantitative PR levles. HPLC, particularlarly in ion-exchange mode, has proven to be a very useful tool in the isolation of steroid receptor isoforms. The use of flow cytometry, however, allows one the opportunity to confirm hetrogeneity.

There is little doubt that the tumour cell is largely heterogeneous and that the PR present in MCF-7 and T47-D exhibit a degree of heterogeneity. Receptors are also generally shown to be capable of remodelling under the influence of the given environment. Nevertheless, the questions as to why and how this remodelling takes place remain. It is not really surprising that due to the inherent genetic instability of the tumour cells, mutations that occur are likely to be selective. Since steroid hormones have important growth regulatory functions, and PR heterogeneity could also be a reflection of underlying ER heterogeneity. Mutant and variant ER'S have also recently been described.

The progesterone receptor exists in a A and B isoform, and since both homo-and heterodimers can form between the A and B isoforms, three possible classes of receptor dimers can bind to a PRE, each having a potentially different transcription regulatory capacity. This diversity in the repertoire of responses to one hormone sets progesterone apart from the

regulatory information carried by other sex steroid hormones. However, no proof exists that both isoforms are in the same cell. The tissue-specific effects of the two PR isoforms still remain to be studied. Do mutant/variant PR's exist? What is the role these variants are likely to play in the development of tumour resistance to hormonal therapy? How may they be modified by drugs? These questions are but a few where future focus may concentrate in our endeavour to understand the complexities of cell regulation.

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