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The Interaction Between the 90 kiloDalton Heat Shock Protein and Steroid Hormone Receptors.

Part 1.

The Association of hsp 90 and ER in the MCF-7 Human Breast Cancer Cell Line.

Part 2.

The Effect of Temperature on the Androgen Responsiveness of two Human Prostate Cancer Cell Lines.

A thesis submitted for the degree of Doctor of Philosophy
in
the Faculty of Sciences.

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This Thesis is Dedicated to my Father

Whose Interest, Help and Encouragement
I will Always Be Thankful For.

Table of Contents.

Part 1: *The Association of hsp 90 and ER in the MCF-7 Human Breast Cancer Cell Line.*

Experimental.

Discussion.

Part 2: *The Effect of Temperature on the Androgen - Responsiveness of two Human Prostate Cancer Cell Lines.*

Introduction.

Experimental.

Discussion.

Citations.

Abbreviations Used.

A	amperes
AEV	avian erythroblastosis virus
AR	androgen receptor
Arg	arginine
ATP	adenosine triphosphate
BSA	bovine serum albumin
°C	degrees centigrade
cDNA	complementary DNA
CEF's	chicken embryo fibroblasts
Ci	curie
cpm	counts per minute
Cys	cysteine
DCC	dextran coated charcoal
DES	diethylstilboestrol
5αDHT	5α-dihydrotestosterone
DMEM	Dulbecco's modification of Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
EIA	enzyme immunoassay
eIF-2α	eukaryotic initiation factor 2 alpha
ER	oestrogen receptor
ERE	oestrogen response element
ETN	EDTA-Tris-NaCl buffer
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
OH-Flut.	4-hydroxy flutamide
g	gravitational force
GR	glucocorticoid receptor
GRE	glucocorticoid response element
^3H	tritium label
HED	HEPES-EDTA-dithiothreitol
HEPES	4-(2-hydroxyethyl-1-piperazine)-ethanesulphonic acid
HIDCCFCS	heat-inactivated dextran coated charcoal stripped FCS
His	histidine

HPLC	high performance liquid chromatography
HRE	hormone response elements
hrp	horse raddish peroxidase
HRT	hormone replacement therapy
HSE	heat shock regulatory element
hsp	heat shock protein
HST	heat shock treatment
HSTF	heat shock transcription factor
IgG	immunoglobulin G
Kd	dissociation constant
kDa.	kiloDaltons
LBA	ligand binding assay
Lys	lysine
M	molar
Mr.	relative molecular mass
mRNA	messenger ribonucleic acid
MMTV	mouse mammary tumour virus
NGS	normal goat serum
NP-40	Nonidet P-40
NSS	normal sheep serum
PAGE	polacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBS-A	Dulbecco's buffered saline
$pp60^{src}$	Rous sarcoma virus transforming gene product
PR	progesterone receptor
PRE	progesterone response element
RA	retinoic acid
RAR	retinoic acid receptor
rpm	revolutions per minute
RNA	ribonucleic acid
SAPU	Scottish Antibody Production Unit
Std. Devn.	standard deviation
SDS	sodium dodecyl sulphate
T_3	triiodothyronine (thyroid hormone)
T_3R	triiodothyronine receptor
TAM	tamoxifen
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethylene-diamine
TGF-α	transforming growth factor alpha

TGF-β	transforming growth factor beta
TRIS	N-tris (hydroxymethyl) methyl-2-amino- -ethanesulphonic acid.
tRNA	transfer ribonucleic acid
V	volts

Figures & Tables.

Materials.

REAGENT	SOURCE
Acrylamide	FSA Laboratories Ltd.
Antisera*	(*See METHODOLOGY section 6.1.)
Bisacrylamide	FSA Laboratories Ltd.
Bradford Reagent	BioRad Ltd.
BSA Standard	Pierce Inc.
5-α Dihydrotestosterone	Sigma
Dithiothreitol	Boehringer Mannheim, GmbH.
DNA (calf thymus)	Sigma
DPX-mountant	BDH Ltd.
Ecoscint A	National Diagnostics. Ltd.
Foetal Calf Serum	Flow Labs. Inc./Gibco BRL. Ltd.
Fluorescein Isothiocyanate	Sigma
Flutamide	Schering Plough, Inc.
Gentamicin	Gibco BRL. Ltd.
Hoechst 33258	Hoechst GmbH
Mibolerone	Sigma
[^{3}H]-Mibolerone	Amersham
Nonidet P-40	BDH Ltd.
Oestradiol-17β	Sigma
[^{3}H]-oestradiol-17β	Amersham
Penicillin/Streptomycin	Gibco BRL. Ltd.
Progesterone	Sigma
RPMI-1640 medium	Gibco BRL Ltd.
TEMED	BDH Ltd.
Trypsin	Gibco BRL Ltd.

Suppliers of any other specific materials are specified in the text.

All other basic chemicals were purchased from BDH and Sigma.

All plasticware for use in cell culture was supplied by Bibby Ltd.

Summary.

Breast cancer is a major killler of adult women, yet effective therapies are few. Prostate disease (both benign prostate hypertophy-BPH and prostate cancer) afflicts a large proportion of adult males.

Both breast cancer, and prostatic disease, represent abnormal growth of reproductive cells. Epithelial cells of the male and female reproductive systems respond to a variety of stimuli, notably including the sex steroid hormones. These hormones exert their effect by binding to an intracellular receptor, or *steroid receptor.* Hormone sensitivity - the basis of successful endocrine therapy - is best defined, for breast cancer, as the presence of specific receptors for oestrogen and progesterone. Presence of functional steroid receptors may also reflect better prognoses.

Steroid receptors are proteins that represent one category in the superfamily of intracellular receptors. They are composed of two types of protein subunits: steroid-/DNA-binding subunits and seemingly inert nonsteroid-/nonDNA-binding subunits. It is proposed that the two sets of subunits form multimeric complexes which appear as *"non-activated"* steroid receptors. This large complex is unable to bind DNA. Binding of the correct steroid to the steroid-binding site of the steroid-/DNA-binding subunit(s) is thought to induce the dissociation of the inert subunit(s) to reveal the hitherto "masked" DNA-binding sites. This process has been termed *activation*, and it is fundamental to productive hormone action.

In recent years the "inert" subunit has been the subject of much interest. This protein has been identified as the highly conserved 90 kiloDalton

heat shock protein (hsp 90); a heat inducible protein that has also been found to associate with various components of the cytoskeleton. Since this protein was both a vital component of steroid receptors, and a member of the thermally-inducable heat shock protein family, the work reported in this thesis investigated whether the endocrine functions of hsp 90 could be enhanced or compromised after heat shock.

1. *HSP 90 and MCF-7 Breast Cancer Cell Oestrogen Receptor.*

The effects of heat shock on steroid receptor action were studied in the MCF-7 human breast cancer cell line. This epithelioid cell contains receptors for oestrogen (ER), progesterone (PR), and androgen (AR) in amounts convenient for laboratory assay.

In order to investigate whether the expression of hsp 90 could modulate steroid receptor activity, initial experiments tested the effect of heat on hsp 90 in MCF-7 cells. Growing MCF-7 cells were exposed to a range of temperatures in order to gauge the point at which temperature became lethal. A fixed exposure time of two hours was always used so as to allow direct comparison between different experiments. These titration experiments found that the lethal temperature for MCF-7 cells fell between 42°C and 43°C.

Later experiments showed that a prior 2 hour exposure of the cells to 41°C enabled the cells to survive 2 hours exposure at 44°C. This conditioning process of double heat shock was recognised as the acquisition of cellular thermotolerance.

When an anti-hsp 90 monoclonal antibody (AC 88) was used to immuno-precipitate hsp 90 from MCF-7 cells, the greatsest amount of hsp 90 was precipitated from the cells subjected to double heat shock. Since this treatment also promoted thermotolerance, hsp 90 may represent an integral part of the thermotolerance machinery.

To assess whether this increased expression of hsp 90 had any effect on the activity of steroid receptors, oestrogen receptor assays were carried out on control and thermotolerance-induced (double heat shocked) MCF-7 cells. Conventional ligand-binding assay showed that ER-binding capacity was almost totally abolished as a result of heat shock. When time-course studies were carried out, this abolition of "available" ER was shown to be a transient phenomenon which seemed to mirror the known kinetics of hsp 90 induction.

Interestingly when enzyme immunoassay (a method independent of the ligand-binding ability of the receptor) was used to measure the levels of ER the effect of heat shock was drastically different from the results of the ligand-binding assay. MCF-7 cells subjected to heat shock maintained 67% of the levels of ER in cells maintained at 37°C. This result suggested that heat shock results in an abolition of ER-binding ability through a mechanism other than by merely a down regulation of ER protein.

Since ER is located within the nucleus of target cells, the subcellular distribution of hsp 90 under "normal" (37°C) and heat shocked conditions was investigated. Fluorescent immunocytochemical staining using the AC 88 anti-hsp 90 antibody was used to visualise the effects of heat shock on

hsp 90 expression. The absolute amount of hsp 90 present was only marginally higher in the heat shocked cells, the result predicted by the immunoprecipitation experiments. However, the effect of heat shock on the distribution of hsp 90 was very obvious: a very pronounced nuclear accumulation was seen in these cells. This phenomenon has never before been witnessed for hsp 90, although heat shock-induced nuclear accumulation of hsp 70 is well documented. Thus, the action of heat shock is to induce the over expression of nuclear hsp 90 and so create a population of ER unable to bind oestrogen.

These results indirectly substantiate the stabilisation role of hsp 90 in the mechanism of steroid receptor action. Furthermore, they illustrate the need for assays of receptor-functionality as well as receptor-positivity in tumour biopsies. In addition, these data suggest one possible explanation that may account for the discrepancies between tumour receptor status and disease responsiveness in some patients of breast cancer.

2. *Hsp 90 and Prostate Cancer Cell Androgen Receptor.*

With these results in mind, it was decided to investigate a clinical scenario where thermal and endocrine manipulations are used in conjunction. The treatment of BPH has recently involved microwave probes to supply local irradiations of heat to promote regression of prostate disease. Treatment is often used in conjunction with antiandrogen therapy to the combined benefit of the patient.

To investigate any possible interactions between thermal and endocrine

manipulations of prostate cancer, it was decided to use prostate cancer cell lines as an *in vitro* model. The LNCaP and DU-145 cell lines were chosen as although they are both thought to contain AR, only LNCaP cells are known to respond to exogenous androgen.

In order to confirm the AR-status of these two cell lines immuno-cytochemical staining was carried out using an anti-AR antibody. This illustrated the presence of AR within the nuclei of both cell lines, although the LNCaP cells showed significantly more intense staining of nuclear AR. This methodology was also used in conjunction with the AC 88 antibody to visualise hsp 90 staining within these cells; interestingly, this staining was predominantly cytoplasmic.

When the prostate cell lines were tested for thermal tolerance and the induction of thermotolerance, it was found that the sub-lethal temperature (41°C) for the MCF-7 breast cancer cell line was in fact a lethal dose for both the LNCaP and DU-145 cell lines. However, when a priming exposure to 39°C was administered, both cell lines could withstand exposure to 42°C. These results showed that both prostate cell lines exhibited the thermotolerance phenomenon, and revealed a means of hsp 90 induction open to exploitation.

Immunocytochemistry showed that double heat shock (thermotolerance-induction) increased the overall levels of intracellular hsp 90. This staining was more prominent in the LNCaP cells, with some nuclear staining being obvious; though not the dramatic nuclear accumulation of hsp 90 that was displayed by the MCF-7 cell line.

DU-145 cells failed to respond to either androgen or antiandrogen in terms of growth stimulation, but LNCaP cells were stimulated by both ligands. Positive growth stimulation by 5α-dihydrotestosterone (5αDHT) was predictable, and occurred at "physiological" concentrations of 10^{-10} to 10^{-12}M. Positive stimulation by 4-OH flutamide (OH-Flut.) was not expected, although there have been a few reports of this *in vitro* phenomenon.

The level of LNCaP cell AR was also shown to be under the influence of both androgen and antiandrogen, being down- and up-regulated by 5αDHT and OH-flut. respectively. DU-145 cell AR on the other hand failed to regulate the levels of intracellular AR in response to either ligand, again suggesting the nonfunctional nature of this receptor.

Heat shock of LNCaP cells did not result in loss of androgen-binding in stark contrast to the activity of the heat shocked MCF-7 cell ER. In order to assess whether this difference was due to the nature of the cells or of the receptors, AR assay was carried out on heat shocked MCF-7 cells. Once again, heat shock did result in a significant inhibition of steroid receptor binding ability, although this was only a partial diminution (40% reduction of AR as assayed by ligand-binding assay).

When antiandrogen (OH-Flut.) was administered in conjunction with heat shock no significant differences were seen in the DU-145 cells treated with antiandrogen (either prior or subsequent to heat shock) when compared to cells grown in the absences of any exogenous ligands. However, when LNCaP cells were exposed to both heat and anti-

androgen an additive suppression of growth was observed. This result, in contrast to the observed stimulatory effect of antiandrogen alone, was demonstrated irrespectively of the sequence of the two manipulations; although heat shock preceded by antiandrogen exposure resulted in the most profound inhibition of growth.

These results reflect the observations recorded *in vivo* when benign prostate hypertrophy patients undergoing antiandrogen therapy are subjected to thermal therapy. It is possible that either the heat shock-induced excess hsp 90 is interfering with the androgen/ antiandrogen stimulatory pathway; or alternatively, the presence of antiandrogen may inhibit efficient function of hsp 90 in the intracellular machinery of cellular resistance to heat. In either case, these studies suggest that the combination of endocrine and thermal therapies are most appropriate when used to treat patients possessing androgen-responsive tumours.

INTRODUCTION.

1. Breast Cancer.

1. Breast Cancer.

1.1. BREAST CANCER: A GLOBAL ISSUE.

Between 1948 and 1985 the most frequent cause of cancer death among American women was breast cancer. In 1990, only carcinoma of the lung and of the colorectum are bigger killers. A woman's lifetime risk of developing breast cancer was recently estimated at 1 in 10 (SPRATT *et al*, 1988). Fear of the disease itself, and its treatment in particular, have an immense psychological effect.

A study comparing the annual breast cancer mortality rate among age-standardised women in the 42 most industrialised countries gives us some indication of the disease occurrence (SEIDMAN & MUSHINSKI, 1983). The annual death rate was highest in Denmark, closely followed by England & Wales with 33.8 and 33.6 annual deaths per 100,000 females respectively. Scotland lay eighth in the table with a death rate of 32.8, the United States lay fourteenth with 28.3, Greece lay twenty-eighth with 15.6, Japan lay thirty-ninth with 5.8 and finally Nicaragua with 0.5 annual deaths per 100,000 females. Obviously the magnitude of these numbers illustrate that breast cancer is a disease of enormous international impact; however, the reasons for the disease epidemiology still remain unclear.

1.2. A BRIEF HISTORY OF THE DISEASE.

The human impact of breast cancer as a disease is made obvious by the amount of records left to us from many older cultures. Breast tumours were first described by the Egyptians *circa* 3000 B.C. Later, Greek and Roman physicians preserved their records, but these were not advanced further by the barbarian societies of the Dark Ages. The surgical treatments endorsed by Roman medicine gave way in medieval times to various extremes of folk and quasi-religeous therapies: these varied from mass prayer and the laying on of hands, to topical application of various natural agents such as milk, goat dung, frogs and bisected chickens. The sixteenth century saw a resurgence of surgery; the practice and technique of mastectomy being refined from the original Græco-Roman protocols. However, any advances in terms of survival and patient morbidity awaited the arrival of anesthesia and antiseptic.

In 1757, a French surgeon Henry Françoise LeDran significantly advanced the hope of a surgical cure by proposing the theory that the cancer began as a local disease, spreading first to the lymphatics and then on to the general circulation. Over the next century, many surgical procedures involving different degrees of breast, muscle and lymphoid tissue were employed with varying results, culminating in the operations described by William Halsted. The Halsted radical mastectomy was used almost exclusively for around fifty years until in 1933 Emile Grubbe demonstrated the use of X-rays in the treatment of breast cancer; and so radiotherapy was born. The last half century has seen modification of

surgical procedures with a view to breast preservation. The development of investigative biopsy techniques now allows more selective decisions on possible beneficial adjuvant therapy; chemotherapy or endocrine therapy are now almost always used in conjunction with surgery for patients with node positive disease. However, more recently the body's own immune system has been identified as a possible weapon in the fight against the disease, and a positive relationship seems to exist between a patient's psychological state and the activity of her immune system. This is interesting in the light of the observation of the Greek physician Galen, who comments on the occurrence of breast cancer among Roman women as being more frequent among the "melancholic" than the "sanguine".

1.3. CAUSATION OF THE DISEASE.

1.3.1. EPIDEMIOLOGY

There are many epidemiological studies available to those studying the aetiological basis of human breast cancer. Drawing useful conclusions from these studies, however, is complicated by the interplay of various factors.

A known factor is the woman's age at menarche and the time elapsing between this event and her age at first parturition. Recently, the freedom of women to control their own fertility means that the variation of this time differential among cultures has increased; women of industrial and more affluent societies have fewer children and wait longer before having them. At first sight it seems possible to correlate earlier and more frequent motherhood with a lowered risk of breast cancer; a conclusion that is reflected geographically. However there are other possible risk factors which vary between societies: environmental, cultural and dietary, and these may all act to cloud our objective conclusions.

For example, a third-world low protein diet may mean a delay in the menarche; however the woman may become pregnant on her first ovulation, and may also become pregnant after her subsequent infrequent ovulations. A Northern European woman eating a diet rich in animal proteins and fats may reach the menarche in her early teens, but the dictates of her lifestyle may mean that her first pregnancy, if any at all, could be 20 or more years later. Is the correct conclusion, therefore, that it is infrequency of parity (excess of menstrual periods), contrasting diets or

some other environmental factor that causes Northern European women to have an 8-10 fold greater chance of developing breast cancer than her third-world counterpart? Against such a background of confounding factors certain conclusions have been drawn.

1.3.2. GENETIC & FAMILIAL FACTORS.

There is certainly a genetic predisposition to breast cancer inherent in certain populations and individuals. Daughters and sisters of breast cancer patients have a significantly increased risk of the disease, even after other common familial factors are discounted (ANDERSON, 1974). In the most extreme case, that of monozygous twins, the number of breast cancers codeveloping in twins after the first diagnosis was found to increase by a factor of six (HOLM *et al*, 1980).

1.3.3. REPRODUCTIVITY.

Lactation and childbearing are also important factors, although it seems to be childbearing rather than breast feeding that is important (KALACHE *et al*, 1980). Contrastingly, a preponderance of right-breast cancer is manifest among certain nomadic tribes who traditionally suckle from the left breast. High levels of breast cancer among nuns has been noted for almost 300 years. What seems critical is the length of time between the menarche and the first pregnancy; it has even been suggested that the number of menstrual cycles before the first pregnancy may be the ultimate determinant. If we compare the rat model for breast cancer which

assumes an early carcinogenic event; pregnancy in the rat protects against a mammary carcinogen if it precedes exposure but promotes cancer formation if it follows exposure (SPRATT *et al,* 1988). This model correlates well with human epidemiological data.

1.3.4. ORAL CONTRACEPTION & HORMONE REPLACEMENT THERAPY.

Genetic factors and frequency of parity are probably modulating etiology of the disease through the endocrine-paracrine environment. This begs the question as to whether exogenous hormones; either in the form of contraception or hormone replacement therapy (HRT), can increase the risk of breast cancer. Studies from the Royal College of General Practitioners (1981) suggest that no increased disease risk, or alternatively no protective effect, are associated with oral contraception. HRT may slightly increase the disease risk (HUNT *et al*, 1987; HOOVER *et al*, 1976) but studies are so far unsatisfactory.

1.3.5. VIRAL, RADIATION & DIETARY CARCINOGENESIS.

Viruses are known effecters of carcinogenesis in animals. Of particular interest is the mouse mammary tumour virus (MMTV), the oncogenic potential of the virus being influenced by hormones. It seems possible (although as yet unproven) that MMTV, or a similar virus, is instrumental in the genesis of human breast cancer.

Doses of ionising radiation during repetitive fluorographic examination of the chest for tuberculosis is associated with breast cancer (LOWELL *et al*, 1968). More importantly, repetitive, poorly measured mammography may increase a woman's lifetime risk of disease (UPTON, 1976). Conclusions on cumulative low dose environmental exposure are uncertain; however data from Hiroshima suggest that there is no threshold dose for the breast, but that risk is much increased in the actively growing breast of puberty.

Dietary carcinogenesis can be split into three areas: 1) dietary contaminants, i.e. xenobiotic carcinogenesis; 2) dietary excess, i.e. obesity; and 3) the proportions of constituents within the diet.

Polycyclic hydrocarbons have been shown to be potent tumourogenic agents in rats, but the extent of their responsibility for carcinogenesis in the human breast is unknown. Obesity is associated with an increase in the synthesis of oestrone from the liver and the fatty tissues (DEWAARD, 1975). This hormone is the major circulatory oestrogenic agent in postmenopausal woman after the cessation of ovarian oestrogen; and it has been proposed that the increased oestrone levels result in an increased breast cancer risk (WYNDER *et al,* 1976). Most interesting, however, is the constituents of the diet; in particular, the amounts of dietary fat, and to a lesser extent sugar and also animal protein. One notable study found a significant positive association between breast cancer and fat and animal protein (KOLONEL *et al,* 1981). These conclusions confirm numerous other investigations (for a review see GRAY

et al, 1979).

Nicotine and alcohol fail to demonstrate strong correlations with an increased risk of breast disease; as any increased risk is comparable with that of other cancers (MEARA *et al*, 1989).

1.4. BREAST CANCER AND OESTROGENS.

1.4.1. HISTORICAL OVERVIEW.

The earliest report suggesting that normal human female breast development is under the influence of the ovary was made by Pott in 1775. He removed the normal ovaries of a twenty-three year old woman while repairing a bilateral groin hernia; subsequently, the woman ceased to menstruate and experienced significant loss in breast size, but remained otherwise healthy. A most important observation that a similar relationship existed between malignant growth of the breast and the ovary was made in Glasgow by Beatson at the end of the nineteenth century (BEATSON 1896). He described the healing of locally recurrent cancer of the breast of a thirty-three year old woman after undergoing ovariectomy.

Thirty years later oestrone and oestradiol-17β were characterised and proposed as oestrogenic agents, but it was not until after the Second World War that oestrogens were proven to have a stimulatory effect on human breast cancer, reversing the benefits of ovariectomy (PEARSON *et al*. 1954). The next decade saw the utilisation of new biochemical techniques to identify and estimate steroids in blood and urine samples; but the synthesis and target sites of oestradiol remained unknown.

The adrenal cortex was known to secrete oestrogens, or their precursors, because women with advanced breast cancer who initially responded to castration, would later further respond to adrenalectomy (WEST *et al*, 1952). However, the adrenal cortex was eliminated as a primary source of oestrogen synthesis by Baird and colleagues (BAIRD *et al*, 1969); and the human ovary, known to secrete oestrogen (DOISY, 1941) was shown by Mikhail (1970) unable to secrete oestrogen after the menopause. Then in 1975 it was demonstrated that human breast fat could synthesise oestradiol from testosterone (NIMROD & RYAN, 1975). The enzyme responsible for the catalysis, aromatase, has since been shown to be of greater activity in the breasts of patients with the disease; and the activity was most enhanced in the quadrant of the breast harbouring the tumour (O'NEILL *et al*, 1988). Thus, in post-menopausal women, oestrogens are known to be synthesised in the breast (and other peripheral tissues) from 19-carbon precursors from the adrenals.

A critical step forward, so far undiscussed, was the discovery of an intracellular receptor for oestrogen by the groups of Gorski and Jensen (GORSKI *et al*, 1968; JENSEN *et al*, 1968). They proposed the classical two step model where steroid enters the cell, binds to a "cytosolic" receptor, which then enters the nucleus and activates an oestrogen responsive gene. This subject will be discussed in much more detail elsewhere, but the importance of receptors in the history of breast cancer should be noted. Due largely to the results of McGuire and coworkers, it was found

that receptors for oestrogen were present in about two thirds of human breast cancers; furthermore, their concentration was relative to the age and menstrual status of the patient, and to the degree of differentiation of the tumour (McGUIRE *et al*, 1975). Oestrogen receptors have since been used in the diagnosis, prognosis and treatment of breast and gynaecological cancers. (For a review see SOUTTER & LEAKE, 1987).

1.4.2. GROWTH AND DEVELOPMENT OF THE NORMAL BREAST.

The development of the breast (thelarche) begins about two years prior to the menarche, which for 95% of women in the UK, is reached by the end of the 15th year. The pubertal release of luteinizing hormone causes the secretion of ovarian oestrogens, but it is not until pregnancy that any further development occurs. Early in pregnancy, breast alveoli form ducts under the influence of ovarian oestrogens. Later, pituitary prolactin and placental lactogen induce synthesis of the enzymes involved in milk production - lactogenesis. Milk secretion is prevented until parturition by high levels of progesterone; when upon delivery, the suckling child stimulates milk ejection. The milk ejection reflex comes about as a result of pituitary secreted oxytocin acting directly on duct vessels to release the milk. (For a thorough review see NEVILLE, 1983).

1.4.3. OESTROGEN AND DEVELOPMENT OF THE CANCEROUS BREAST.

When discussing breast cancer it is useful to consider the two-stage,

initiation-promotion model of carcinogenesis (BERENBLUM, 1954). This model assumes that the neoplastic event is triggered by some chemical, biological or physical initiator which acts by irreversibly altering the DNA. Promoters, then, are agents which result in the expression of initiated cells by either permitting or stimulating proliferation to detectable tumours. There is little evidence to suggest that oestradiol could itself be considered an initiator, even though covalent binding of oestrogen to DNA has been shown. However, it is possible that oestrogen may indirectly enhance initiation due to its proliferative effects resulting in an increase in the number of carcinogen target sites. This is reflected in the number women who were exposed to irradiation after the atomic bombing of Hiroshima. Women who were in their teens at the time of explosion show higher levels of invasive breast cancers when compared to older women who received comparable exposure (HRUBEC *et al*, 1989).

Rat models suggest that oestrogen is a very efficient promoter of breast cancer (HOWELL, 1989). Prospective studies carried out by Bullbrook and colleagues (1986) involved measuring the blood oestradiol levels in 5000 women and then examining clinically for signs of breast cancer. The results obtained suggest that women having a higher amount of bioavailable oestrogen in their blood were at greater risk from breast disease. Curiously, these findings are reflected in earlier reports of breast cancer in male to female transsexuals who were found taking high doses of oestrogen illicitly over a number of years (SYMMERS, 1968). Unlikely to act as an initiator of breast cancer therefore, oestrogen seems to exert its

neoplastic effect as a potentiator or promoter of transformed cells.

Oestrogenic induction of epithelial cell mitogens, or growth factors, is well documented (LEAKE, 1988), and will be discussed in due course. However, a more direct mitogenic action of oestrogen has been shown (LOOSE-MITCHELL *et al*, 1988) and is demonstrated by the induction of the c-*fos* and c-*myc* proto-oncogenes in rat uterus. It has been suggested (WEISZ & BRESCIANI, 1988) that the oestrogenic mechanism of mitogenisis arises as a result of direct activation by the oestrogen receptor complex of c-*fos* gene expression. These oncogenes are associated with proliferation and differentiation (THOMPSON *et al*, 1986) possibly by encoding a trans-acting promoter (RAUSCHER *et al*, 1988) and so may be directly involved in transformation.

1.5. STEROID RECEPTOR RELEVANCE IN BREAST CANCER.

1.5.1. A BREAK-THROUGH.

As previously mentioned, the identification of an intracellular receptor for oestrogen by the groups of Gorski and Jensen (GORSKI *et al*, 1968; JENSEN *et al*, 1968) was an important step forward in understanding the underlying endocrine mechanisms of steroid hormone action. Prior to then, endocrine ablative surgery was found to be successful in about one third of patients, but no preoperative estimation of the outcome could be offered to the individual. In the early 1970's it was hoped that a tumour could be "typed" according to its receptor status, and so the success of

subsequent endocrine therapy predicted. It is now known that 50-60% of patients of advanced disease presenting ER-positive tumours will respond to endocrine therapy, correspondingly less than 10% of patients presenting ER-negative tumours respond (CLARK & McGUIRE, 1989).

1.5.2. STEROID RECEPTOR ASSESSMENT.

Analysis of fresh breast biopsies for the presence of steroid hormone receptors can be carried out using a variety of methods, although some of which are more suited to research purposes (WITTLIFF, 1988). These include; firstly, and most frequently, titration using ligand binding assay, density gradient centrifugation, various classes of HPLC, isoelectric focussing, immunohistochemical staining and finally, enzyme-immuno assay (EIA).

Ligand binding assay is the method most frequently used to quantify steroid receptors on a regular clinical basis. This method satisfies the major criteria describing a receptor assay; being able to accurately quantify a specific receptor, and to determine a single binding affinity while eliminating non-biological binding (LEAKE, 1981). These assays are basically titrations of radioactively labelled hormone with a suitably prepared tissue homogenate (LEAKE & HABIB, 1987), the specificity of receptor detected is discriminated by the labelled ligand and the unlabelled competitors selected. (See METHODOLOGY section 3.1.).

1.5.3. STEROID RECEPTOR STATUS.

The initial poor correlation between the levels of cytosolic ER and the patient response rate to endocrine therapy meant that nuclear ER were also assayed in the hope that this would prove a better diagnostic test (LAING *et al*, 1977). This tactic resulted in an increased correlation between ER positivity and responsiveness to endocrine manipulations; and was later justified by the discovery that the nucleus was the intracellular location of these receptors *in vivo* (KING & GREEN, 1984). Since one of the early effects of oestrogen receptor is the induction of progesterone receptor synthesis (FREIFELD *et al*, 1974), Horwitz and McGuire proposed that the additional assay of progesterone receptor would confirm the presence of *active* oestrogen receptor and hence oestrogen dependence and anti-oestrogen sensitivity (HORWITZ *et al*, 1975a). The combination of assaying both oestrogen and progesterone receptors in both the cytoplasm and nuclear fractions of tumour homogenates led to an improved correlation of from 71% to 91% between receptor status and patient response to endocrine therapy (DEGENSHEIN *et al*, 1980). Prognostic decisions on breast and gynaecological cancer patients could now be made with greater confidence (For a review see SOUTTER & LEAKE , 1987).

1.5.4. STEROID RECEPTORS AS PROGNOSTIC INDICATORS.

The most important determinant of prognosis is the degree of invasiveness demonstrated by the tumour. The number of histologically-

involved axillary nodes is the critical parameter; the likelihood of treatment failure rising directly with the number of such deposits. Steroid receptor status is independent of axillary node status and can aid discrimination among patients within either the positive or negative nodal groups (CLARK & MCGUIRE, 1989).

Initial findings from McGuire and colleagues suggested that patients presenting with ER-positive primary breast tumours had a significantly increased disease free survival when compared to their ER-negative counterparts (KNIGHT *et al*, 1977). This group (OSBORNE *et al,* 1980) and others (LEAKE *et al*, 1981) later confirmed that the quantitative amount of ER was not only indicative of the response to endocrine therapy; but also the length of disease free survival.

There remains a significant number of patients whose tumours are ER-positive yet fail to respond to endocrine manipulations (MAASS *et al*, 1980). As cytoplasmic ER assays only indicate the levels of ER and not its functionality, it has been proposed that these patients may display non-functional ER. This underscores the requirements for either nucler ER assay or PR assay; found to be the critical determinant in the prognosis of human breast cancer (CLARK *et al*, 1983; LEAKE *et al*, 1981).

1.6. OESTROGEN INTERACTION WITH OTHER GROWTH FACTORS AND THE REGULATION OF BREAST GROWTH.

1.6.1. EPIDERMAL GROWTH FACTOR (EGF) & TRANSFORMING GROWTH FACTOR α (TGF-α).

Although oestrogenic hormones remain the most important endocrine influence for the development and mitogenic control of both the normal and neoplastic breast, much evidence supports the concept that they act by triggering production of locally acting hormones or growth factors (LEAKE, 1988). EGF and TGF-α are small related polypeptides which are both known to bind to the EGF receptor (CARPENTER *et al.*, 1983). Most breast cancer cell lines derived from epithelial tumours produce TGF-α (LIPPMAN & DICKSON, 1989). Oestrogen has been shown to regulate growth factor expression by inducing production of TGF-α messenger RNA (BATES *et al*, 1988). Expression of TGF-α can be induced by up to 14-fold in the ER positive cell lines MCF-7 and ZR-75-1 after the addition of physiological levels of oestrogens (DICKSON *et al*, 1986). Work from our own laboratory (RINALDI & LEAKE, in press) and from others has shown EGF to be a potent mitogen of cultured breast cancer epithelial cells. Consequently, a hypothesis has evolved suggesting that malignant proliferation may arise as a result of the excessive local actions of growth factors; this paracrine mechanism is presumed to be oestrogen-induced, although EGF or TGF-α may then continue to act as growth promoting agents in an autocrine manner (SPORN & ROBERTS, 1985).

It has also been shown that a blockade of the EGF-receptor using monoclonal antibodies results in only a transient suppression of growth stimulation (ARTEAGA *et al*, 1988), and oestrogen is known to be mitogenic independent of paracrine mechanisms (WEISZ & BRESCIANI, 1988). These data suggest that autocrine/paracrine systems are not solely responsible for oestrogenic proliferation of breast cancer cells.

As for the transformation of breast epithelia to neoplasia it has been suggested that some initiating lesion may result in the novel expression of a growth factor or its receptor (NISHIMURA & SEKIYA, 1987). There exists an oncogenic gene product, v-*erb* B, which shows significant structural and functional homology to the EGF receptor (CARPENTER & ZENDEGUI, 1986). EGF is known to undergo tyrosine autophosphorylation as a result of EGF binding and activation (DOWNWARD *et al*, 1985) and it has been suggested that the truncated v-*erb* B, which is devoid of the EGF/TGF-α binding domain, may achieve transformation by acting as a constitutively activated EGF receptor (SCHLESSINGER, 1988).

1.6.2. TRANSFORMING GROWTH FACTOR β (TGF-β).

After the discovery of transforming growth factor activity which permitted anchorage-independent growth of normal cells in soft agar (DELARCO & TODARO, 1978) it was discovered that there was in fact two peptides present, both being required for transformation (ROBERTS *et al*, 1982). Structurally similar TGF-α or EGF are required together with the unrelated

polypeptide TGF-β in order to induce phenotypic transformation. There has since been found multiple forms of both TGF-β and its receptor. (For a review see HSUAN, 1989). There is much confusion as to the function of TGF-β as it has been shown to be both mitogenic and inhibitory in the same cell line. Growth of subconfluent normal rat kidney (NRK) cells is inhibited by TGF-β (ROBERTS *et al*, 1985) while growth arrested NRK cells can be stimulated to proliferate in response to rat TGF-β (MASSAGUE, 1984). It is thought then that TGF-β is a modulator of some other growth factor activity rather than being considered as a direct mitogen itself.

Recently, experiments from our group (KERR *et al,* 1989) have shown that interferon-α can inhibit the growth of ZR-75-1 breast cancer cells by increasing transcription and expression of the TGF-β gene. This is interesting as low concentrations of interferon-α have been shown to increase oestrogen receptor expression in this cell line (van den BERG *et al*, 1986) sensitising the cells to subsequent inhibition of proliferation using the antioestrogen tamoxifen, although this mechanism may not work at the current clinical levels of interferon-α (KERR *et al*, 1989). Tamoxifen has been shown to upregulate TGF-β expression in MCF-7 breast cancer cells by Lippman and colleagues (KNABBE *et al*, 1987) and so it has been suggested that there could be a dual action of this antioestrogen: a

receptor-mediated anti-proliferative effect which can be enhanced using interferon-α; and possibly a receptor-independent mechanism acting through TGF-β (LEAKE, 1988).

1.6.3. PROLACTIN.

The precise role of prolactin in the continuous proliferation of breast disease is unclear. It is known that women categorised as having a high risk of breast cancer present elevated serum prolactin and that this may result in a stimulation of breast epithelial proliferation (WANG *et al*, 1987). In addition, birth of first child (known to reduce the relative risk of breast cancer in women under 30) and of subsequent children causes a decrease in circulating prolactin. There are many different physiological effecters of prolactin secretion and it may be the case that the higher levels observed in these patients may be symptomatic rather than aetiological. However, work from our laboratory, (RINALDI & LEAKE, *in press*) has shown that prolactin may act in synergy with oestrogens to increase EGF receptor expression in cultured ER positive breast cancer cells. Work is continuing to assess the implications of prolactin in breast cancer although this will depend on a fuller understanding of the neoplastic significance of the EGF receptor.

1.7. ENDOCRINE MANAGEMENT OF BREAST CANCER.

Treatment of primary breast cancer usually involves surgery of varying degrees followed up with an appropriate adjuvant therapy such as radiotherapy or chemotherapy. A comprehensive description of these treatments is too involved to be included in this thesis, however one line of adjuvant therapy is directly concerned with this research: endocrine therapy.

The concept underlying endocrine therapy is that certain tumour cells retain their sensitivities to the hormones which regulate their normal progenitor cells. Oestrogen is a highly potent mitogenic agent in the normal mammary gland and is, directly or indirectly, the major stimulus for the growth of hormone-dependent breast cancer (SANTEN, 1982). Endocrine therapy must then be able to negate or even reverse the proliferative effects of oestrogens. Two lines of treatment exist which achieve this goal in a significant number of patients: ablative hormonal therapy and additive hormonal therapy.

1.7.1. ABLATIVE HORMONE THERAPY.

Ablative hormonal therapy is principally castration; as adrenalectomy or hypophysectomy are today seldom practised. Oophorectomy, by surgical or radiation methods, results in a response rate of 31% in non selected, pre-menopausal advanced breast cancer patients (KARDINAL, 1988). This response rate can be increased to 55% by selecting ER positive patients and to 78% by selecting ER and PR positive patients. Ablative hormone

therapy is therefore still a common method of treatment among pre-menopausal women, with ER positive tumours (DAO & NEMOTO, 1980).

1.7.2. ADDITIVE HORMONE THERAPY.

Post-menopausal patients presenting with ER positive advanced breast cancers are now routinely treated using antioestrogen therapies with success rates similar to oophorectomy, although again depending on the receptor status of the tumour (BROOKS *et al*, 1980). Additive endocrine therapy probably inhibits neoplasmic growth principally via a receptor specific mechanism rather than a general cytotoxic effect. The original practices of using androgens as oestrogen antagonists have now been almost wholly superseded by the use of antioestrogenic agents; exemplified by the drug tamoxifen and progestins.

Tamoxifen (Nolvadex®) is a non-steroidal antioestrogen that binds to the oestrogen receptor forming an abortive complex (BAULIEU, 1987a & b.; the significance of this binding will be discussed in INTRODUCTION section 2.5.5), but note that the tamoxifen-receptor complex can induce PR synthesis. In oestrogen receptor positive cell lines tamoxifen acts in a cytostatic manner (OSBORNE *et al*, 1983). Response to tamoxifen among post-menopausal women has been shown restricted to the oestrogen receptor positive patients (ROSE *et al*, 1985); together with 5-10% of oestrogen receptor negative tumours.

Synthetic progestins including Megestrol acetate (Megace®) act in an

antioestrogenic manner by decreasing the amount of the oestrogen receptor (ROCHEFORT, 1984). Patient response is predictably governed by the levels of progesterone receptor, however antioestrogen therapy remains preferable because of reduced side effects. (MUSS *et al*, 1988).

Adjuvant therapy is now very much geared to the meta-analysis study of breast cancer trials (PETO *et al*, 1988). Node positive, pre-menopausal patients receive chemotherapy, while node positive post-menopausal patients receive tamoxifen. Node negative patients do not normally receive additive, adjuvant therapy until relapse.

2. Steroid Receptors.

2. Steroid Receptors.

2.1. THE STEROID RECEPTOR SUPERFAMILY.

2.1.1 STEROID RECEPTORS.

The steroid receptor superfamily includes receptors for what are generally thought of as steroids in the traditional sense: the male and female sex steroids, the glucocorticoids and mineralocorticoids, and vitamin D_3. More recently added are the receptors for thyroid hormone, retinoic acid and various aryl hydrocarbons as well as the receptor-like product of the oncogene *erb* A. These receptors are grouped together as a superfamily for a variety of structural and functional reasons:

- All (identified) ligands for these receptors are hydrophobic in nature and as such are permeable in the cell membrane.
- All these receptors are intracellular; none are membrane bound.
- These receptors show a marked homology of general primary structure; there is broad continuity in the order of the various functional domains.
- There is a very high degree of sequence homology shown among the different DNA binding domains; and a lesser, though still significant homology among the ligand binding domains.
- There is some inherent cross-reactivity of agonists and antagonists among receptors. In addition, it is possible to "transplant" DNA and hormone binding domains to form hybrid "chimæric" receptors

which maintain some degree of functionality.

- Finally and most importantly, all these receptors represent ligand-responsive transcription activation factors. There exists a common mechanism of gene activation exploiting similar hormone responsive elements.

These phenomena will be discussed in the following pages of this thesis; extensive reviews of this area have been published by O'MALLEY (1990), BEATO (1989) and EVANS (1988).

2.1.2. STEROID RECEPTOR COMPARATIVE ANATOMY.

In recent years the primary sequences of the steroid receptor superfamily have been elucidated using complementary deoxyribonucleic acid (cDNA) cloning technology. Receptors for oestrogen (ER; GREEN *et al*, 1986; GREENE *et al*, 1986), progesterone (PR; CONNEELY *et al*, 1986), glucocorticoid (GR; HOLLENBERG *et al*, 1985), androgen (AR; LUBAHN *et al*, 1988), mineralocorticoid (MR) and vitamin D_3 (VD_3R; MCDONNELL *et al*, 1987) have been cloned and the primary sequences obtained. FIGURE 1. is a schematic summary of these receptors showing their comparative lengths and the regions of most significant homology. It is obvious that these receptors have evolved from one primitive receptoroid and it has been suggested that the ancestral receptor may resemble the *erb*-A protooncogene product (WEINBERGER *et al*, 1985).

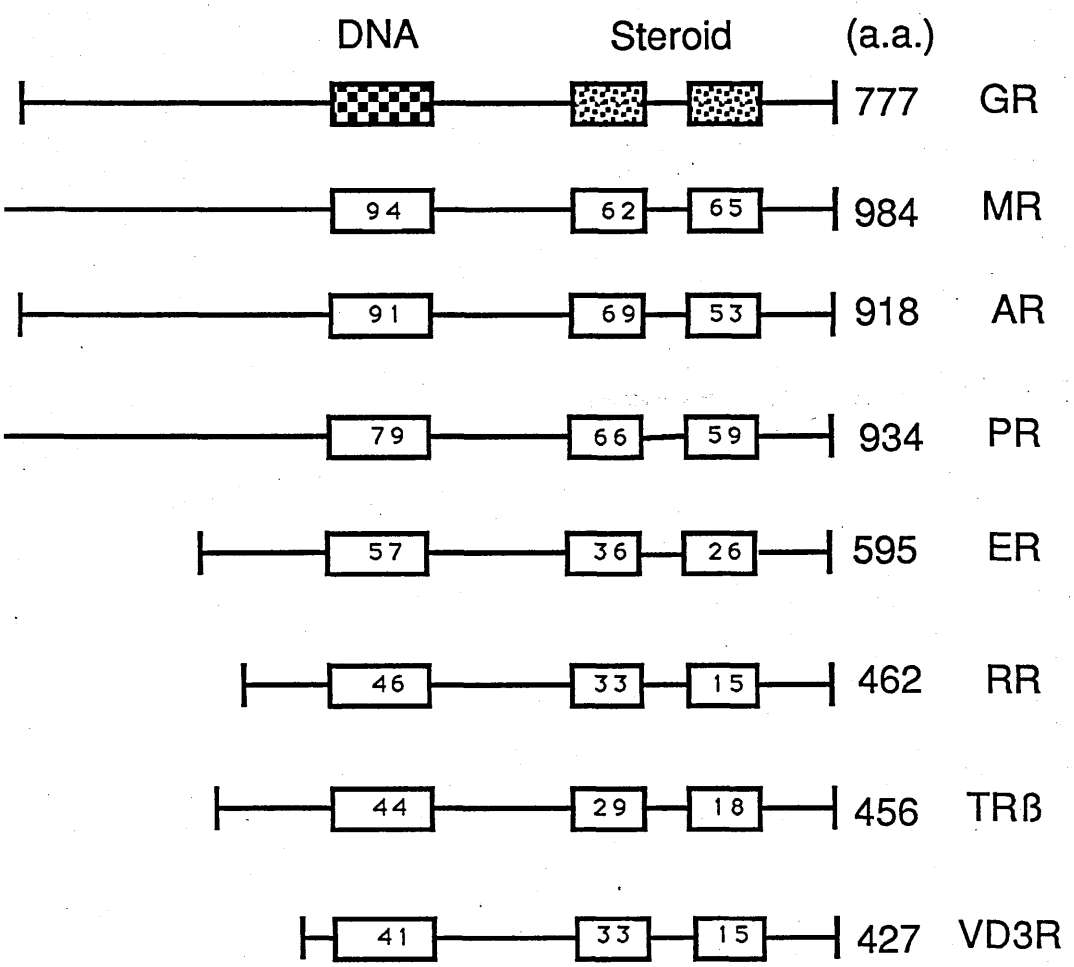
FIGURE 1. **The Steroid Receptor Superfamily.**

The human steroid receptors are shown in terms of primary amino acid structure to compare the significant sequence homologies. The figures within the boxes show the similarity of both the DNA- and steroid-binding domains, defined as % homology to human GR.

(Adapted from O'MALLEY, 1990)

The Steroid Receptor Superfamily.

Relative Sequence Homologies of the Human Steroid Binding Subunits.



2.1.3. THE THYROID HORMONE RECEPTOR & *erb*-A.

The avian erythroblastosis virus (AEV) is a transforming retrovirus which has a genome encoding two oncogenic sequences, v-*erb*-A and v-*erb*-B (VENNSTRÖM *et al*, 1980). As previously mentioned, v-*erb*-B encodes a membrane protein which represents a truncated, constitutively active EGF/TGF-α receptor (LAX *et al*, 1988). V-*erb*-A however was found to encode a thyroid hormone receptor transduced from the cellular proto-oncogene c-*erb*-A (WEINBERGER *et al*, 1986; SAP *et al*, 1986). Thyroid hormone (triiodothyronine; T_3) is secreted in precursor form (thyroxine; T_4) from the thyroid in response to pituitary stimulation. This hormone modulates a vast array of biological response in different species; although generally speaking, in mammals, T_3 is prinipally responsible for the regulation of metabolic rate and has various "permissive" actions.

When c-*erb*-A was found to encode a T_3 receptor, and the similarity noted between the v-*erb*-A oncogene product and the glucocorticoid receptor (WEINBERGER *et al*, 1985) there was an emergence of the term "superfamily" and the possibility of a universal mechanism of hormone action was entertained.

Originally AEV was known to induce sarcomas *in vivo* and to transform chicken embryo fibroblasts (CEFs) *in vitro*. The transformation was thought to occur as a result of v-*erb*-B; v-*erb*-A enhancing the tumourigenicity of these transformed cells in the same manner it stimulates normal CEFs (GANDRILLON *et al*, 1987). A further publication from this group has since shown that expression of v-*erb*-A alone is

sufficient to transform CEFs *in vitro* (GANDRILLON *et al*, 1989), and it has been proposed that the expression of such functionally altered nuclear receptors may trigger a transforming event that represents an initial step in the development of leukemia.

2.1.4. THE RETINOIC ACID RECEPTOR.

Retinoic acid (RA) is a potent morphogen directing the spatial organisation of cells during the embryonic development and regeneration of vertebrate limbs. These effects, however, are cell-specific; RA inhibits the proliferation of embryonal carcinoma and myeloid leukemia cells but stimulates the growth of keratinocytes (DAVIES *et al*, 1988). In humans, retinol (vitamin A) deficiency is associated with an increased risk of a variety of cancers. Retinoids have also been shown to inhibit tumour progression *in vivo*, and tumour promotion *in vitro*. (MOON *et al*, 1984).

A receptor for RA has been cloned (GIGUERE *et al*, 1987) and its similarity to the steroid receptors and thyroid hormone receptor noted. Recently Chambon and coworkers have shown that there are in fact two human RA receptors; RAR-α and RAR-β; and that these are expressed together within the nuclei of RA-sensitive cells (GAUB *et al*, 1989). Presumably these two receptors are in competition both for the pool of RA ligand and for the RA responsive genes.

What is most interesting is the high level of homology between the RAR and the T_3R; 62% and 33% of amino acids are conserved between the respective DNA and hormone binding domains of these two receptors

(GIGUERE *et al*, 1987). This homology is highly significant, especially when the contrasting two-dimensional ligand structures of T_3 and RA are considered. Furthermore, there has been some recent suggestions that these two receptors may act cooperatively at the DNA-binding level to regulate gene transcription (FORMAN *et al*, 1989). This theory will be further discussed in INTRODUCTION section 2.5.4.

2.1.5. THE ARYL HYDROCARBON RECEPTOR.

Halogenated aromatic compounds are common environmental pollutants which are responsible for many species- and cell-specific toxic effects. These effects include the induction of aryl hydrocarbon hydroxylase activity, a cytochrome P-450-dependent enzyme activity involved in the metabolic activation of environmental carcinogens such as benzo(a)-pyrene (CONNEY, 1982). These effects are thought to be mediated through toxin binding to the aryl hydrocarbon receptor (AhR), a soluble protein capable of binding aryl hydrocarbons such as 2,3,7,8,-tetrachloro-dibenzo-*p*-dioxin, the ligand showing highest affinity for AhR (PERDEW, 1988).

Work carried out by Gustafsson and colleagues has since shown rat AhR to be physicochemically indistinguishable from rat GR (CUTHILL *et al*, 1987). In addition, cDNA analysis has been used to confirm that AhR is in fact a member of the growing steroid receptor superfamily (ISSEMANN & GREEN, 1990).

2.1.6. THE OXYSTEROL RECEPTOR.

Cholesterol and its oxygenated derivatives are vital intermediatory metabolites of many biochemical pathways, not least of which includes the synthesis of steroid hormones. These pathways are, in part, regulated by the expressed levels of 3-hydroxy-3-methylglutaryl CoA reductase (TAYLOR & KANDUTSCH, 1985). These workers identified a soluble protein termed the oxysterol binding protein and have proposed that this protein serves the function of a receptor for oxysterols that are regulating cellular cholesterol levels. Further work from this group (TAYLOR *et al,* 1989) has shown that the oxysterol receptor bears some "general characteristics" to the other members of the steroid receptor superfamily although this remains a very tentative relationship.

2.2. STEROID RECEPTOR STRUCTURE AND FUNCTION.

Steroid receptors have three specific capabilities; ligand-binding, DNA-binding and gene activation. The cumulative knowledge of receptor action has been gleaned from research on individual receptor species. Consequently, some of the seemingly universal properties are part fact and part inference; so bearing this in mind, it is aimed to try and summarise the current understanding of (oestrogen) receptor action, avoiding broad parallelisms.

2.2.1. RECEPTOR SYNTHESIS.

Steroid receptors are synthesised on cytoplasmic microsomes from single mRNA's and it has been suggested that *in vivo* this occurs on the outer nuclear membrane, with the resultant protein transversing into the nucleus (CARSON-JURICA *et al*, 1990). PR is the only receptor which is composed of two hormone-binding subunits, A and B, of Mr. 98 and 86 kDa. in the human (MISRAHI *et al*, 1987); however these isoforms are synthesised from one mRNA by alternate translations initiating from two in-frame AUG codons (CONNEELY *et al*, 1989). Translation of GR mRNA *in vitro* yields a large, nonactivated receptor which can then be activated to a DNA-binding form upon glucocorticoid binding; suggesting that the large heteromeric GR represents the post-translational GR *in vivo* (DENIS & GUSTAFSSON, 1989). Similarly, *in vitro* translation of PR also results in an nonactivated heteromeric receptor, further supporting these conclusions. (CARSON-JURICA *et al*, 1989; See INTRODUCTION section 4.5.).

2.2.2. RECEPTOR ACTIVATION.

Steroid receptors can all be recognised in at least two distinct forms. A large heteromeric complex consisting of steroid-binding and nonsteroid-binding subunits, which have sedimentation coefficients of 8-10S; and smaller homomeric forms consisting only of steroid-binding subunit(s) with sedimentation coefficients of 4-5S (For a review see BAULIEU, 1987a). The 8-10S forms have been termed "nonactivated", as these are unable to bind to DNA, correspondingly the 4-5S DNA-binding forms have been

termed "activated" (GRODY *et al*, 1982). The conversion process is known as receptor "activation" or "transformation" (SAKAI & GORSKI, 1984). There is some bimodal usage of these terms but it should be noted that in the ER at least these represent separate processes (MÜLLER *et al*, 1983). Receptor-activation can be defined as a dissociation of the nonsteroid-binding subunits from the 8-10S complexes producing a DNA-binding receptor (PURI & TOFT, 1984), whereas transformation of ER is seen as a further increase from 4S to 5S producing an ER species demonstrating slower oestrogen dissociation kinetics (MÜLLER *et al*, 1983). Further cross-linking studies (MILLER *et al*, 1985) suggest that this 5S form of ER consists of a dimer of two steroid-binding subunits.

Activation then, is seen as the dissociation of the nonsteroid-binding subunits from the steroid-binding/DNA-binding subunits. It appears that the nonsteroid-binding subunits are necessary for maintainance of the correct steroid-binding conformation of the nonactivated GR prior to ligand binding (BRESNICK *et al*, 1989). Ligand binding is presumed to be the initiator of 10S GR dissociation (MENDEL *et al*, 1990); although this is not the only function of ligand-binding. Glucocorticoid antagonist-binding also results in the dissociation of GR to an activated form, but this dexamethasone-activated form is unable to interact productively with glucocorticoid-responsive genes (RICHARD-FOY *et al*, 1987). Studies from other workers also suggest that both agonist- and antagonist-binding play important roles in gene activation. (see INTRODUCTION section 2.5.5.)

2.3. RECEPTOR PHOSPHORYLATION.

Apart from the binding of the hormone itself, phosphorylation is also thought to be an important mediatory factor regulating steroid receptor activation and action. Steroid receptors are phosphoproteins and consequently both phosphorylation and dephosphorylation have been proposed as regulators of various aspects of steroid receptor action: activation, DNA-binding, processing and recycling (for a comprehensive review see RAO & FOX, 1987). The proposed phosphorylation-effected mechanisms vary between individual receptor species, however the ER system seems the most individual.

2.3.1. OESTROGEN RECEPTOR & PHOSPHORYLATION.

Auricchio, Migliaccio and coworkers have produced a great deal of evidence over the preceding decade to suggest that ER activity is regulated *via* a tyrosine phosphorylation system (AURICCHIO *et al*, 1990). This group initially purified two uterine enzymes, a phosphatase and a kinase (AURICCHIO *et al*, 1984), and showed that *in vitro* ER phosphorylation occurred on tyrosine residues and that it was under the influence of Ca^{2+}-calmodulin (MIGLIACCIO *et al*, 1984). Antibodies directed against phosphotyrosine have been used to demonstrate that tyrosine phosphorylation also occurs *in vivo* (MIGLIACCIO *et al*, 1986). Oestrogen has been shown to promote tyrosine phosphorylation, while anti-oestrogens correspondingly inhibit tyrosine phosphorylation (AURICCHIO *et al*, 1990). This is presumably because they promote ER forms which are

respectively recognised and not recognised by the kinase.

Tyrosine phosphorylation is presumed to be the mechanism whereby newly synthesised ER acquires ligand-binding ability. Incubation of *in vitro* translated ER with a highly purified uterine tyrosine kinase increases oestrogen-binding from 4% to almost 100% (MIGLIACCIO *et al*, 1989), confirming earlier observations of kinase mediated ER "activation" (RAYMOURE *et al*, 1985). This process has been more appropriately termed potentiation (MCNAUGHT *et al*, 1990). These workers implicate both kinase and phosphatase activity as being important in the potentiation of ER (DAYANI *et al*, 1990).

2.3.2. OTHER STEROID RECEPTORS & PHOSPHORYLATION.

There is a great deal of evidence suggesting that phosphorylation/de-phosphorylation reactions are important mediatory mechanisms for other receptor species. A model presented for GR cycling suggests that the net phosphorylation of the steroid-binding and nonsteroid-binding subunits remains constant throughout activation, but that the DNA-binding subunits become de-phosphorylated upon tight DNA-binding (ORTI *et al*, 1989a). Ligand-induced phosphorylation has been associated with receptor cycling. Both agonist-(dexamethasone) and antagonist (RU 486)-binding have been shown to increases GR processing by reducing its half-life, however only agonist binding results in GR phosphorylation, discounting phosphorylation as a processing mechanism. (HOECK *et al*, 1989).

Similar results have been obtained for the PR. Phosphorylation of PR is

hormone dependent (SULLIVAN *et al*, 1988), yet newly synthesised PR is able to bind progesterone and DNA without any potentiating phosphorylation step (SHERIDAN *et al*, 1989). PR activation and subsequent processing are also independent of phosphorylation, although it seems that phosphorylation may affect the interaction of PR with its transcriptional element (SHERIDAN *et al*, 1988).

2.3.3. STEROID RECEPTORS AS KINASES.

There have been periodic reports of kinase activity associated with purified steroid receptor preparations. Baulieu and coworkers first identified an autophosphorylative kinase activity associated with avian progesterone receptor (GARCIA *et al*, 1983). The phosphorylation was confined to different receptor components; specificity being governed by the presence of either magnesium or calcium ions. This group later refuted their initial conclusions (GARCIA *et al*, 1986), confirming that the magnesium-dependent phosphorylative activity was associated with a purifiable protein kinase distinct from known receptor subunits. These conclusions were also supported by others (HAPGOOD *et al*, 1986).

A similar history applies to the glucocorticoid receptor. Initial reports (SINGH & MOUDGIL, 1984) demonstrated protein kinase activity of purified rat GR for a variety of substrates, although no autophosphorylative activity was reported. However, further elegant work (SANCHEZ & PRATT, 1986) found that while immunoprecipitated GR demonstrated ion-dependent protein kinase activity, this was only true if polyclonal antiserum was used.

The lack of any kinase activity with monoclonal antibody immunoprecipitated GR suggested that it was a kinase copurifying with, rather than a specific activity of the receptor.

Evidence for oestrogen receptor kinase activity is very scant, though Witliff and colleagues have made the significant observation that ER immunoprecipitated using a monoclonal antibody exhibited both protein kinase and phosphoinositide kinase activities (BALDI *et al*, 1986). Autophosphorylation seems an unlikely possibility since the ER-associated kinase phosphorylation was on substrate serine residues; whereas Auricchio *et al* have demonstrated exclusively tyrosine phosphorylation of ER (see INTRODUCTION section 2.3.1.). Nonetheless, any relationship between a steroid receptor and a phosphoinositide-mediated response linked through phosphorylation would represent an important link between two distinct intracellular messenger systems.

2.3.4. STEROID RECEPTORS AS KNOWN KINASE SUBSTRATES.

Theoretically, there may exist a battery of protein kinases that incorporate steroid receptors as substrates. One of the most interesting kinase activities that has been identified is that of the growth factor receptors. Fox and coworkers initially demonstrated that affinity purified EGF receptor could tyrosine-phosphorylate purified PR in response to the addition of EGF *in vitro* (GOSH-DASTIDAR *et al*, 1984). PR was found to be a high affinity substrate for EGF-receptor, having a K_m of 100nM for the EGF-induced tyrosine phosphorylation. This group then observed that

insulin receptor (but not platelet derived growth factor receptor) could also phosphorylate PR on tyrosine residues, but while EGF increased the V_{max} of EGFR phosphorylation of PR, insulin decreased the V_{max} of insulin receptor kinase activity (Woo *et al*, 1986). As these receptors phosphorylated both on one common site, and on other differential sites, it was suggested that possible regulatory mechanisms were being observed. These experiments however, remain cell-free observations and fail to be reproducible when EGFR / ER positive MCF-7 breast cancer cells are used (RAO & FOX, 1987).

2.4. STEROID RECEPTORS AND RNA.

There have been numerous reports of the association of RNA with steroid receptors, mainly with GR but also with ER and AR. (For a review see MOUDGIL, 1987) The significance of RNA presence in steroid receptor complexes is still of debate; but it seems likely that RNA could aid in maintainance of structural integrity required for receptor action.

One particular group of workers has isolated and purified the RNA associated with the glucocorticoid receptor (ALI & VEDECKIS, 1987a). Vedeckis has shown the RNA to be specifically transfer RNA and this was largely confined to the tRNA representing the basic amino acids (ALI & VEDECKIS, 1987b). $tRNA^{Arg}$ accounted for 51.8% of the total tRNA, while $tRNA^{Lys}$ and $tRNA^{His}$ accounted for 17.1% and 9.2% respectively. They propose that the tRNA could be involved in a ubiquitin-dependent degradation of the GR, and so aid in GR turnover. The involvement of tRNA in non-lysosomal protein degradation is well documented (FERBER & CIECHANOVER, 1986) and will be discussed elsewhere (see INTRODUCTION section 3.2.5.). It should be noted, however, that although RNA has since been isolated from unactivated GR, only a proportion of this was found to be tRNA (UNGER *et al*, 1988); and so the theories of Vedeckis *et al* require much further investigation.

2.5. STEROID RECEPTORS AND GENETIC ACTIVATION.

2.5.1. THE DNA-BINDING DOMAINS OF STEROID RECEPTORS.

As discussed in INTRODUCTION section 2.1.2., there exists a harmony of structure among the steroid hormone receptors (O'MALLEY, 1990). All receptors analysed contain a 66 amino acid region of highly conserved amino acids. Green and Chambon conclusively demonstrated that this region was the DNA-binding domain by creating a chimæric receptor (GREEN & CHAMBON, 1987). They replaced the 66 amino acid stretch of the ER with that from the GR, and demonstrated oestrogen induction of a glucocorticoid-inducible gene.

Noticeably consistent within this region is a high local density of cysteine residues (EVANS, 1988). Each member of the steroid receptor superfamily contains a single domain of the form: CYS-$X_{(2)}$-CYS-$X_{(13)}$-CYS-$X_{(2)}$-CYS-$X_{(15\text{-}17)}$-CYS-$X_{(5)}$-CYS-$X_{(9)}$-CYS-$X_{(2)}$-CYS-$X_{(4)}$-CYS (after BERG, 1989; where X represents a variable amino acid residue). This type of region has been shown to exist in a number of DNA-binding proteins and is termed a "zinc-finger", as it can be visualised as a finger of amino acids anchored by an atom of zinc and projecting from the surface of the protein (EVANS & HOLLENBERG, 1988). The cysteine-rich region of steroid receptors has been shown to represent two zinc-fingers (FREEDMAN *et al*, 1988); each having an individual function. Chambon has proposed that the first (N-terminal) finger determines the target gene specificity, while the second finger probably stabilises the DNA-receptor interaction (GREEN *et al*, 1988).

Interestingly the superfamily of steroid hormone receptors can be divided

into two subgroups on the basis of the zinc-finger motif (BEATO, 1989). GR, PR, AR, and MR display a common sequence, differing from the common sequence of ER, T3R and RAR, suggesting an early evolutionary divergence. This structural division is reflected in the ability of only the first group to act through the MMTV-hormone response element.

2.5.2. HORMONE RESPONSE ELEMENTS.

Hormone response elements (HRE's) are the DNA loci which participate in receptor-binding. The homology between the various HRE's is reflected by the corresponding similarity among the DNA-binding domains of the receptors. These elements are divisible into three classes with corresponding consensus sequences: oestrogen response elements (ERE's) GGTCAnnnTGACC; glucocorticoid response elements (GRE's) GAACAnnnTGTTC, and the thyroid hormone response elements (TRE's) GGTCATGACC (BERG, 1989). Mutational studies have shown conversion of an ERE to a GRE by changing only two bases (KLOCK *et al*, 1987).

Interestingly these HRE's are found as inverted repeat sequences, i.e. two half-sites having dyad axis of symmetry (O'MALLEY, 1990). This type of motif forms a palindromic DNA-binding site, allowing steroid receptors to bind as dimers. This has in fact been observed (TSAI *et al*, 1988) and it has been suggested that the receptor binds as a dimer to the major grooves of four subsequent turns of the DNA helix, each turn being contacted by a separate zinc finger (HAM & PARKER, 1989).

There is much evidence demonstrating the mixed-matched binding of

receptors and HRE's. Experiments carried out on the T47D human breast cancer cell line, which is biochemically and immunologically devoid of GR, demonstrated dexamethasone-activation of the PR-responsive MMTV after transfection using a GR expression vector (NORDEEN *et al*, 1989). This was a significant observation of dual control of an HRE by two steroid receptors in the same cell line. AR, GR and PR have all been shown to act through the same 15 base pair sequence of MMTV (HAM *et al*, 1988); similarly RAR has been shown to induce transcription by binding to either synthetic or natural T_3 response elements (UMESONO *et al*, 1988). This not only underscores the evolutionary relationship of the HRE's, but also opens up the possibility of synergistic regulation of the HRE's.

2.5.3. DNA-BINDING RECEPTOR DIMERS.

Steroid receptors are thought to exert their effect as dimers bound to the relevant palindromic HRE. Activated GR has been purified as a homodimer whether in the presence or absence of DNA (WRANGE *et al*, 1989), and it seems likely that other steroid receptors bind to DNA in a similar manner. It has also been suggested that the binding of the receptor monomers occurs in a stepwise, cooperative manner (TSAI *et al*, 1988) although this remains unclarified. Chambon generated mixtures of wild type and truncated ER and found that both homodimers and heterodimers were formed on subsequent incubation with an ERE (KUMAR & CHAMBON, 1988), suggesting that dimer formation is necessary for effective DNA-binding.

2.5.4. RECEPTOR COOPERATIVITY & SYNERGY.

Cooperative binding of receptors to HRE's has been demonstrated *in vitro*. O'Malley and coworkers have shown that dimeric PR binding to a tandem GRE/PRE increases in affinity by 100-fold when a second PR dimer binds (TSAI *et al*, 1989). This resulted in a stable complex with a Kd for DNA of approximately 10^{-11}M. Similar synergistic results have been obtained from observations of activation of an ERE and adjacent GRE/PRE (CATO *et al*, 1988) where a progesterone and oestrogen combination was shown to illicit a response greater than the sum of the individual inductions.

Heterodimeric gene activation has also been noted *in vitro*. In T3-responsive rat pituitary cells, transcription can be observed in the absence of T3 (FORMAN *et al*, 1988). This seems to occur though constitutively active c-*erb*-A-α1 forming c-*erb*-A-α1/T3R heterodimers capable of gene activation regardless of ligand. This group later observed that patients presenting thyroid hormone resistance syndrome display dominant-negative c-*erb*-A phenotypes; the transcriptionally inactive form of *erb*-A (FORMAN *et al*, 1989). They suggest that there is major *in vivo* formation of T3R/(neg)*erb*-A heterodimers which are transcriptionally-inactive and so may account for the T3 insensitivity of these patients.

More importantly dimerisation could prove to be a possible interaction mechanism of the various receptors within the superfamily to provide finely tuned hormonal responses.

2.5.5. TRANSCRIPTIONAL ACTIVATION.

Steroid receptors bind to specific DNA sequences inducing transcription. *Trans* -activation is in part due to the activity of their short DNA-binding domain (HOLLENBERG *et al*, 1987), however studies using deletion mutants and chimæric receptors have shown that a more significant proportion of the *trans*-activation activity lies at distant sites of the receptor (HOLLENBERG & EVANS, 1988). Chambon and coworkers have identified at least one *trans*-activation domain within the hormone-binding domain of the ER (KUMAR *et al*, 1987). What is most interesting is that while both oestrogen and antioestrogen (4-OH tamoxifen) seem to cause receptor activation, only oestrogen-binding induces *trans* -activation (WEBSTER *et al*, 1988). These studies confirm the dual role hypothesis of hormone-binding, and demonstrate that the inhibitory effect of antisteroidals arises from the formation of abortive complexes with HRE's (GUICHON-MANTEL *et al*, 1988; RICHARD-FOY *et al*, 1987).

Trans -activation is thought to occur through the *trans* -activation domain of the receptor interacting with other transcription factors; possibly *via* protein-protein contacts with either the TATA box factor or even RNA polymerase II (HAM & PARKER, 1989). It has been suggested that different steroid receptors could compete for these factors, as transcriptional interference of endogenous PR and ER in MCF-7 and T47D cells has been observed (MEYER *et al*, 1989).

2.6. RECEPTOR INTRACELLULARITY.

Initially steroid receptors purified as either "cytosolic" or "nuclear" species, which led to the Gorski-Jensen two-step model of hormone (oestrogen) action (GORSKI *et al*, 1968; JENSEN et al, 1968). The term cytosolic was generally interpreted as cytoplasmic (CLARK, 1984; LEAKE, 1976), and it was presumed that hormone-binding induced receptor-activation followed by translocation to the nucleus. However, knowledge of the intracellular location of steroid receptors greatly advanced when receptor-specific antibodies became available, as these facilitated immunocytochemical location of receptors in both tissues and cultured cells.

Immunocytochemistry was first used to identify ER in the nuclei of target cells (KING & GREENE, 1984) casting doubt on the original Gorski-Jensen two-step translocation model. Work from Gorski's own laboratory confirmed this data (WELSHONS *et al*, 1984) suggesting that the ER resides permanently in the nucleus and that its affinity for DNA is increased upon ligand-binding. These results have been confirmed in the human breast cancer cell lines MCF-7 and T47D (WELSHONS *et al*, 1988) and also in human breast tissue (WALKER *et al*, 1988), where again oestrogen-binding failed to influence ER distribution.

Baulieu and colleagues showed exclusive intranuclear residence of PR independent of hormanal administration (GASC *et al*, 1984). PR has since been located using electron microscopy to visualise immunogold detected anti-PR monoclonal antibodies (PERROT-APPLANAT *et al*, 1986). These studies confirmed that PR resides within the nuclei of target cells

revealing that it associates directly with condensed chromatin after progesterone administration. Similarly, the AR (HUSMANN *et al*, 1990) and VD3R (CLEMENS *et al*, 1988) have both been shown to be predominantly nuclear proteins whether in the presence or absence of ligand. Hormone binding is again thought solely to increase the affinity of the receptor for DNA, rather than promoting any change in subcellular localisation.

The situation for GR is less well understood. There is strong evidence to suggest that GR coexists between the cytoplasm and the nuclei of target cells (WILKSTRÖM *et al*, 1987). These workers observed a hormone-dependent shift in the proportion of the two species: ligand withdrawal achieved by adrenalectomy resulted in a loss of nuclear GR, while dexamethasone significantly increased the proprotion of nuclear GR, although some cytoplasmic GR was always present. It has been suggested that the distribution of GR represents different stages in receptor recycling rather than an observation of an activation-translocation type of mechanism (QI *et al*, 1989).

Most immunocytochemical studies address the localisation of the hormone-/DNA-binding subunits of steroid receptors, and very few comment on the distribution of the nonsteroid-binding subunits, however these reports are very relevant to the work described in this thesis and will be discussed in INTRODUCTION section 4.8.

3. Heat Shock and Heat Shock Proteins.

3. Heat Shock and Heat Shock Proteins.

3.1. HEAT SHOCK ?

The terms *heat shock* and *heat shock proteins* are misnomers used to describe the phenomenon occurring during the cellular response to some environmental onslaught. These elderly terms have evolved from studies observing the physiological response to hyperthermia, the classical form of stress induction. Better terms would be *stress response* and *stress proteins,* as we now know there are many other environmental variants to heat. Unfortunately, however, convention has adopted the former terms; and so they will be used here.

3.2. THE HEAT SHOCK RESPONSE.

3.2.1. OBSERVING THE PHENOMENON.

Cellular response to heat was first visualised in *Drosophila* embryos about thirty years ago, though it was not until 1978 that the effect of heat shock on mammalian and avian protein expression was reported (KELLEY & SCHLESINGER, 1978). The response is now known to be universal; occurring in every organism tested, from archebacteria to man (LINDQUIST, 1986). The heat shock response is found in nearly all cell- and tissue-types of multicellular organisms; including cultured cells, and is characterised by the synthesis of a small number of highly conserved proteins. These are the heat shock proteins (hsp's), and their expression is the sole quantifiable parameter of the heat shock response. (For a review see LINDQUIST, 1986). However, it should be noted that although

the levels of hsp's are elevated by stress; many hsp's also exist in normal, unstressed cells. Hsp 70 has been purified from unstressed cells and hsp 90 can comprise as much as 0.5-1.0% of the protein in cytosol extracts (TOFT *et al*, 1987).

3.2.2. STRESS INDUCERS.

By definition, cellular exposure to elevated temperatures is the classic environmental stress inducing the heat shock response and hence hsp synthesis. These supraphysiological temperatures vary among different cells and species but are generally no more than 4-8°C above the normal physiological temperatures. Other known inducers of mammalian hsp's include ethanol, arsenite, heavy metals, amino-acid analogues, glucose deprivation-refeeding cycles and anoxia (BURDON, 1986). However, not all inducers are effective in all cells tested. Similarly there are some inducers of hsp synthesis in *Drosophila* and other invertebrates that are not active in mammalian cells; such as phorbol esters, ouabain or sodium azide (BURDON, 1986; CRAIG, 1985).

It is interesting that so many diverse agents can effectively substitute for one another. This raises the question as to whether there could exist a common mechanism of hsp induction, action and regulation. It is possible that the damaging effects of all these agents could be directed against either existing proteins, or more probably nascent polypeptides; thus producing abberant proteins. The latter would be especially susceptible to the sorts of stressing agents listed above. Damaged proteins are thought

to signal induction of the heat shock response and this has been confirmed using microinjection of denatured protein to induce hsp synthesis in *Xenopus* oocytes (ANANTHAN *et al*, 1986).

3.2.3. HEAT SHOCK PROTEINS.

Schlesinger defined heat shock proteins by two criteria: firstly, "their synthesis is strongly stimulated by an environmental stress, in particular, that resulting from a change in temperature a few degrees centigrade above the normal physiological one" and secondly that "their gene contains a conserved sequence of 14 base pairs in the 5' noncoding region, the Pelham box. This sequence serves as the promoter for hsp mRNA transcription." (SCHLESINGER, 1986).

Hsp's are named according to their molecular weight in kiloDaltons (kDa.). They can be subgrouped into four families of different molecular weights; the first three are hsp 83-90, hsp 70, and hsp 20-30. The sole member of the fourth subgroup is ubiquitin, an 8 kDa. protein involved in nonlysosomal protein degradation (CIECHANOVER *et al,* 1984). In eukaryotic cells each of these subgroups represents a gene family encoding closely related protein isoforms regulated by different gene promoters (CRAIG, 1985).

Molecular weights are also vital for hsp detection, as this is the only biochemical parameter available to identify hsp's other than by immunological methodology. Hsp's consistently fail to show any catalytic activity; presumably their function is achieved by their association with

target molecules and structures, and so antisera must be used to readily identify cellular hsp. It is probably for this reason that demonstration of a definite function of hsp's has evaded researchers for so long.

3.2.4. REGULATION OF THE HEAT SHOCK RESPONSE.

In mammalian cells, heat shock results in the shut down of normal protein synthesis coupled with the induction of hsp's. The translation of hsp mRNA in preference to preexisting mRNA is in part responsible for the altered protein expression profile.

The control of protein expression appears firstly at the translational level; as mRNA coding for non-heat shock proteins is not translated, although it remains translationally competent if tested in cell-free extracts (THOMAS *et al*, 1982). It has been suggested that this effect is due to an increase in phosphorylation of the α-subunit of eukaryotic initiation factor 2, eIF-2α (BURDON, 1986). This factor associates with a 90 kDa. phosphoprotein which has recently been confirmed as hsp 90 (ROSE *et al*, 1989). The activity of eIF-2α is governed by its phosphorylation state, which is in turn dependent on the phosphorylation state of the associated hsp 90; implying that hsp 90 may play a pivotal role in the regulation of normal protein synthesis during the stress response.

Transcription of hsp mRNA has been shown to be immediate; increasing in concentration from one copy to 1000 copies of mRNA per cell within the first hour (BIENZ, 1982). Pelham discovered a common promoter region 66 nucleotides upstream of the RNA start site of all *Drosophila* hsp genes

(PELHAM, 1982). Consisting of a TATA box and a short conserved sequence, it was found indispensable for hsp transcription. Further cloning work confirmed these small consensus sequences as heat shock regulatory elements (HSE) having the ability to confer heat inducibility on previously insensitive genes (PELHAM & BIENZ, 1982). Two proteins were then identified which bound to the TATA box and the HSE (WU, 1984). Tight binding of the one of these, the heat shock transcription factor (HSTF), to the HSE can be correlated with transcriptional activity, the TATA box factor seems to be permanently bound.

Bienz has proposed a hypothetical model of hsp induction whereby heat shock activates HSTF; either directly or through the actions of some other component, the activated HSTF is then able to tightly bind the HSE thus inducing rapid hsp transcription (BIENZ, 1985). It has also been suggested that ubiquitin could be involved in the regulation of HSTF activity as levels of this protein are influenced by the intracellular levels of denatured proteins (BURDON, 1986).

3.2.5. UBIQUITIN.

Ubiquitin is a highly conserved, yet abundant protein of Mr. 8000 which has latterly been termed an hsp as it demonstrates heat inducibility (BOND & SCHLESINGER, 1985). Much of the research on ubiquitin function however, has been concerned with its role in nonlysosomal proteolysis (For a review see CIECHANOVER *et al,* 1984). Several molecules of ubiquitin are conjugated to proteins through isopeptide bonds to ε-NH_2 groups of

lysine residues, forming target proteins for proteolytic degradation. This highly selective degradation system removes abnormal proteins in an energy dependent manner; as both the conjugation and the proteolysis are ATP-dependent. In addition it has been shown that conjugation is in part dependent on the presence of tRNA (FERBER & CIECHANOVER, 1986). Ubiquitin has been proposed as a regulator of hsp transcription acting as a modulator of HSTF activity (BURDON, 1986). Pelham suggested that inactive HSTF may, like histones, be normally maintained in a ubiquitinated form; and that the sequestering of ubiquitin for conjugation to stress induced damaged proteins would lower the pool levels of ubiquitin and result in the activation of HSTF (MUNRO & PELHAM, 1985). This hypothesis remains untested yet seems unlikely; as the pool ubiquitin, itself an hsp, would also increase with stress.

3.3. A ROLE FOR THE HEAT SHOCK RESPONSE.

The role of the heat shock response and the function of heat shock proteins has been the subject of many reviews (SCHLESINGER, 1986; LINDQUIST, 1986; & CRAIG, 1985) and although there is much speculation, there are very few conclusions. Thermotolerance is the best documented, and most obvious function of hsp's, although there are numerous other possible roles emerging. However, the universal demonstration of the response and the extremely high conservation shown among the hsp subgroups mean that they remain of fundamental importance to life.

3.3.1. THERMOTOLERANCE.

Thermotolerance can be defined as the acquisition of cellular resistance to lethal levels of heat by previous exposure to lesser, sub-lethal doses. However this definition should be broadened to include the other stressing agents as heat is not the only inducer of the (thermo)tolerance phenomenon. There is much indirect evidence to suggest that the protective mechanism of thermotolerance is mediated through hsp's. This evidence is well reviewed by Lindquist and can be summarised as follows:

- Agents inducing hsp synthesis also induce thermotolerance, and one agent can confer (thermo)tolerance to another.
- The synthesis and degradation of hsp's mirror the kinetics of the thermotolerance induction.
- Stages of development which do not facilitate hsp synthesis render the organism extremely sensitive to heat or other stress agents at that time.
- Mutants selected for their ability to withstand exposure to high temperatures have been shown to constitutively express hsp's, and correspondingly mutants known to be thermo**in**tolerant have been shown to posses a defective hsp synthesis.
- Finally, thermotolerance can be blocked if cycloheximide is added to cells immediately prior to heat shock.

The first direct evidence that implicates hsp's as the mediators of

thermotolerance has come from Welch and coworkers (RIABOWOL *et al*, 1988). They were able to show that heat shock was lethal to fibroblasts microinjected with monoclonal antibodies against hsp 70, whereas cells injected with non-specific antiserum survived the heat shock.

3.3.2. PROTEIN DEGRADATION.

Another possible function of hsp's which has already been raised (See INTRODUCTION section 2.4.) is that of mediators of nonlysosomal protein degradation. This line of postulation is mainly derived from the ubiquitin mediated, ATP-dependent proteolytic system (CIECHANOVER *et al,* 1984). Interestingly it has been shown that this system is in part dependent on the presence of tRNA (FERBER & CIECHANOVER, 1986), in particular $tRNA^{His}$. Initial suggestions that hsp's and ubiquitin are complementary methods of dealing with the common problem of protein turnover (FINLEY *et al*, 1984) adopt extra significance in the light of more recent evidence. The glucocorticoid receptor has been found to be composed of both a heat shock protein (hsp 90; HOWARD & DISTELHORST, 1988a) and $tRNA^{Arg}$ (ALI & VEDECKIS, 1987b), opening up the possibility that hsp 90 is mediating GR turnover in a manner analogous to the function of ubiquitin. (see INTRODUCTION section 2.2.)

3.3.3. STRUCTURAL ASSEMBLY.

It has been suggested that hsp 70 participates in the organisation of multi-subunit complexes, such as the ribosomal machinery, acting as a

molecular scaffold to aid structural integrity (WELCH & SUHAN, 1986). Transcription is heat sensitive, and enhanced hsp 70 synthesis has been shown to increase the translational resistance to further heat shock (YOST & LINDQUIST, 1986)

3.3.4. PROTEIN TRANSLOCATION.

Hsp 70 has been shown to be part of the translocational machinery facilitating protein passage through the yeast endoplasmic reticulum and the eukaryotic mitochondrial membrane (DESHAIES *et al*, 1988). It has been suggested that hsp 70 could mediate this transfer through an *unfoldase*-type activity (VERNER & SCHATZ, 1988), although this notion fails to be substantiated.

3.3.5. PROTEIN STABILISATION.

Hsp's have been proposed as stabilisers, potentiating the activity of biomolecules, largely on the observations on the association of hsp 90 with the nonactivated forms of the steroid receptors (TOFT *et al*, 1987; discussed in introduction section 4.). In this event, hsp 90 seems to be holding the steroid receptor in a state of readiness to bind ligand and become transcription activative.

An additional line of support for this role comes from early studies on the transforming gene product of Rous sarcoma virus, $pp60^{src}$. This is a transforming tyrosine kinase made up of three subunits (HUNTER & SEFTON, 1980) and regulated by tyrosine phosphorylation (PIWNICA-WORMS *et al*,

1987). One of these subunits immunoprecipitating with pp60^{src} was originally shown to be hsp 90 (OPPERMANN *et al*, 1981) and this was later confirmed by Toft and colleagues using immunocytochemical staining (SCHUH *et al*, 1985). In addition, it was nonactivated pp60^{src} that was shown to associate with hsp 90, suggesting that the hsp was acting as a regulator of enzyme activity. For both these two sets of biomolecules then, hsp 90 seems to be acting as a mediator of stability.

3.3.6. PROTEIN TARGETING.

It has been suggested that hsp's may participate in the targeting of important cell proteins, such as steroid receptors or pp60^{src}, to an appropriate intracellular location. The main thrust of this argument derives from 1) the discussed association of hsp's with other important molecules, and 2) the discovery that hsp's are associated with the protein components of the cytoskeleton.

Japanese workers initially identified both hsp 90 and hsp 100 as actin-binding proteins; having the ability to cross-link actin filaments (KOYASU *et al*, 1986). They later went on to show that both hsp 90 (NISHIDA *et al*, 1986) and hsp 100 (KOYASU *et al*, 1989) bound actin in a Ca^{2+}-calmodulin-dependent manner; the presence of Ca^{2+} causing calmodulin to bind the hsp's and prevent them associating with the actin filaments. These workers imagined a scenario whereby a nonmuscle actomyosin system could provide transportational forces for hsp movement: an hsp shuttle on an actin network, transporting important biomolecules (KOYASU *et al*,

1986). This colourful hypothesis remains untested, although hsp 90 has also been shown to associate with both α- and β-tubulin (SANCHEZ *et al*, 1988). This group used immunofluorescence microscopy to visualise the association in intact cells. However, hsp 90 only bound to actin when purified preparations were used, but did not associate in intact cells; suggesting that the *in vitro* work of the Japanese group could be artifactual.

4. Hsp 90 & Steroid Hormone Receptors.

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4.1. THE 90 kDa. HEAT SHOCK PROTEIN (hsp 90).

Hsp 90 is a heat inducible protein (CATELLI *et al*, 1985) whose gene promoter contains the necessary heat shock responsive element (REBBE *et al*, 1989; see INTRODUCTION section 3.2.4.) although it accounts for between 1 and 2% of the cytoplasmic protein in unstressed HeLa cells (LEES-MILLER & ANDERSON, 1989a). This presence represents 9 million monomers per cell; and as such hsp 90 is an exceptionally abundant protein. Biochemical data suggest that hsp 90 has an elongated shape, more akin to that of a structural protein than a globular protein (ROSE *et al*, 1989).

These workers report both three-dimensional and immunological similarities between hsp 90 and the structural protein spectrin. These conclusions may be confusing earlier observations which studied the association of hsp 90 with various cytoskeletal protein components (See INTRODUCTION section 3.3.6.) The initial reports confirmed that it was hsp 90, not spectrin, however these authors also visualised hsp 90 as an elliptical protein, 25nM-long (KOYASU *et al*, 1986).

In many mammalian species there seem to be multiple forms of hsp 90 (REBBE *et al*, 1989); however only two distinct human cDNA hsp 90 clones have been identified. These have been termed hsp 89α and hsp 89β (SIMON *et al*, 1987). Originally hsp 90 was noted to consistently migrate as a doublet during polyacrylamide gel electrophoresis (MENDEL & ORTI,

1988) although this is now known to be due to the existence of the two isoforms of 732 and 728 amino acids respectively. The hsp 89α and hsp 89β isoforms are phosphorylated on serine residues at two homologous sites (LEES-MILLER & ANDERSON, 1989a), though phosphorylation seems to bear no relation to any functional regulation of steroid receptor interaction. In addition to heat and stress, hsp 90 synthesis has been shown to be regulated by both gonadotrophins (BEN-ZE'EV & AMSTERDAM, 1989) and oestrogens (RAMACHANDRAN *et al*, 1988). These results are especially interesting as it opens up the possibility of complementary regulation of the oestrogen receptor-hsp 90 association.

4.2. COMMON NONSTEROID-BINDING RECEPTOR SUBUNITS.

As discussed in INTRODUCTION section 2.2.2. steroid receptors exist in two major forms: large heteromeric complexes consisting of steroid-binding and nonsteroid-binding subunits; and smaller complexes consisting solely of steroid-binding subunits (BAULIEU, 1987a & b). It was noticed upon receptor purification that the nonsteroid-binding components always had a Mr of ~90 kDa. (DOUGHERTY *et al*, 1984; ATRACHE *et al*, 1985). This protein was confirmed as a common component of ER, AR, PR and GR by Baulieu and colleagues (JOAB *et al*, 1984). They raised an antibody to unactivated PR which only recognised the nonsteroid-binding subunits of PR and found that the antibody also detected the nonsteroid-binding subunits of the other three steroid receptors. This 90 kDa. protein has since been immunologically identified with the MR (RAFESTIN-OBLIN *et al*,

1989), the AhR (PERDEW, 1988) and the mouse oxysterol receptor (TAYLOR *et al,* 1989). However, there have been no reports of any 90 kDa. protein associating with T3R, VD3R, RAR or the product of *erb*-A.

4.3. THE NONSTEROID-BINDING COMPONENT IS HSP 90.

To investigate the significance of the 90 kDa. protein Toft and coworkers raised an antibody (AC 88) to a related 88 kDa. protein from the water mould *Achlya ambisexualis* (RIEHL *et al,* 1985). This organism contains a receptor for the morphogenic steroid antheridiol which exhibits the typical 8 to 4S type of transformation in cell-free experiments. The AC 88 antibody was found to be specific for the 90 kDa. component of PR, but the antibody was also found to detect the 90 kDa. protein in a wide range of mammalian tissues and species. Interestingly

Rous sarcoma virus transforming protein tyrosine kinase ($pp60^{v\text{-}src}$) was also shown to associate with the 90 kDa. protein; noticeably in its non-active form (SCHUH *et al,* 1985). Similar results were obtained when other monoclonal antibodies were raised against hsp 90 (SULLIVAN *et al,* 1985). Nonactivated GR and AR were both shown to associate with the 90 kDa. protein, which was again shown to exist in a large number of different mammalian tissues. AC 88 has since been used to show that this ubiquitous 90 kDa. protein is immunologically identical to hsp 90 (TOFT *et al,* 1987). Hsp 90 fails to be associated with any enzymatic activity, and is unable to bind hormones or DNA (HOWARD & DISTELHORST, 1988b).

Baulieu and coworkers raised a monoclonal antibody to chick 8S PR and

found that the antibody recognised only the 90 kDa. nonsteroid-binding protein of PR (JOAB *et al*, 1984). This antibody was used in conjunction with peptide mapping to show that the 90 kDa. component was both immunologically and biochemically identical to the heat inducible avian hsp 90 (CATELLI *et al*, 1985). This direct evidence was confirmed by the discovery that the 90 kDa. nonsteroid-binding protein of murine PR was immunologically identical to mouse hsp 90 (SANCHEZ *et al*, 1985). This group went on to confirm that the association of hsp 90 with GR is confined to the non-activated form of GR by first removing "free" hsp 90 and then immunoprecipitating GR using anti-hsp 90 antibodies (SANCHEZ *et al,* 1987).

4.4. STEROID RECEPTOR & HSP 90 STOICHIOMETRY.

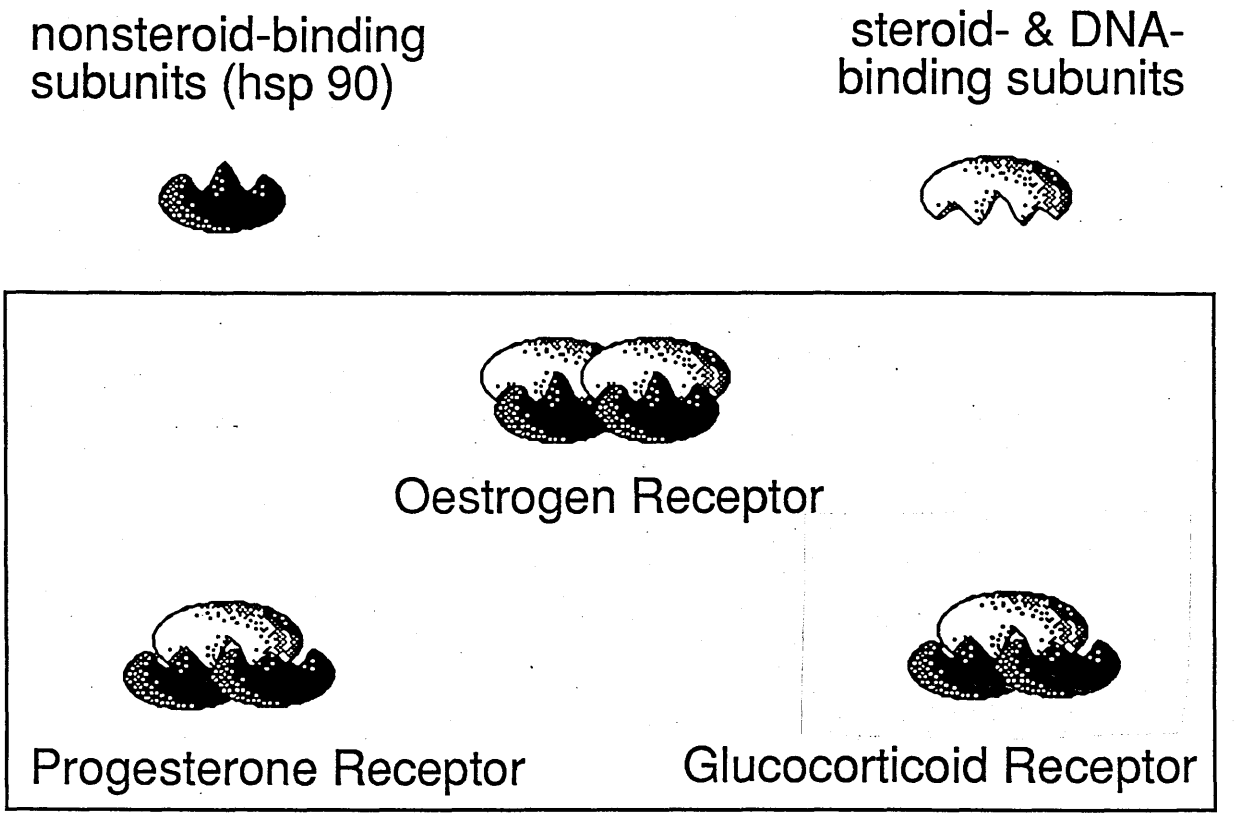
Some information regarding the stoichiometric arrangement of hsp 90 with the different steroid receptors can be gleaned from earlier studies investigating the relative amounts of steroid-binding and nonsteroid-binding subunits in different receptor preparations. Such work carried out on ER suggested that the 8-9S heteromeric form of Mr~300 kDa. was actually composed of two 65 kDa. steroid-binding subunits in a dimer complexed with two 90 kDa. nonsteroid-binding subunits (REDEUILH *et al*, 1987). Similarly, nonactivated (8-10S) GR and PR have both been shown to interact with hsp 90, however in contrast to the nonactivated ER, these proteins appear to exist as complexes consisting of only a single steroid-binding subunit associated with the two 90 kDa. nonsteroid-binding

subunits (GUSTAFSSON *et al*, 1989; RADANYI *et al*, 1989). This difference in stoichiometry is reflected functionally in that the activation of ER seems distinct from the activation of GR and PR. The DNA-binding, transformed ER exists as a dimer prior to DNA-binding; whereas the GR and PR monomers seem to bind cooperatively to DNA (discussed in INTRODUCTION section 2.2.2.). Baulieu and colleagues recently confirmed their earlier proposal showing that the two hormone-/DNA-binding subunits of ER exist as a dimer, interacting "head-to-head" through their hormone-binding domains both before and after receptor activation (SABBAH *et al*, 1989). Interestingly, this group have also demonstrated that hsp 90 is present as a dimer whether free from, or complexed to chick PR (RADANYI *et al*, 1989). Taken as a whole, the evidence seems to suggest that two hsp 90 moieties are present in nonactivated steroid receptors (ER, GR & PR; see FIGURE 2.), and these undergo synchronous dissociation upon ligand-induced receptor activation.

FIGURE 2. **The Subunit Stoichiometry of Steroid Hormone Receptors.**

This diagram is a representation of the stoichiometric ratios of steroid-binding and nonsteroid-binding subunits present in three nonactivated, 8-10S steroid receptors. These schematic structures are mainly derived from information gleaned from purification and cross-linking studies (see text for citations).

Subunit Stoichiometry of Steroid Hormone Receptors.



4.5. HSP 90 ASSOCIATES WITH NONACTIVATED RECEPTORS *in vivo*.

All observations discussed so far represent *in vitro* studies using purified receptor components. To date, no conclusive evidence has been proposed which proves that hsp 90 associates with steroid receptors *in vivo*. However, pulse chase experiments carried out using whole mouse lymphoma cells have shown that hsp 90 is associated with newly synthesised GR in a time dependent manner (HOWARD & DISTELHORST, 1988a). Furthermore, the inclusion of dexamethasone at the beginning of the pulse chase results in an inhibition of the association.

Translation of both PR and GR mRNA *in vitro* has been shown to yield nonactivated receptors (DENIS & GUSTAFSSON, 1989; CARSON-JURICA *et al*, 1989). These receptors are then activated to a DNA-binding form upon ligand-binding; suggesting that the hsp 90-associated GR represents the post-translational GR *in vivo*.

Interestingly, when different *in vitro* translation systems are used contrasting results are obtained. *In vitro* translation of GR in reticulocyte lysate results in full length GR capable of high affinity steroid-binding; while GR translation using a wheat germ extract results in a full length GR that is incapable of hormone-binding (DALMAN *et al*, 1989). Significantly, only the functional GR could be immunoprecipitated using an anti-hsp 90 antibody, which was also used to confirm the lack of detectable hsp 90 in wheat germ extract. These data again indirectly substantiate the existence of steroid receptor-hsp 90 heteromers *in vivo*.

4.6. STRUCTURAL INTERACTIONS OF STEROID RECEPTORS & HSP 90.

The sites of interaction between hsp 90 and the steroid receptors has been investigated by a variety of methods. Tryptic digestion of the 94 kDa. GR has been used to generate a 27 kDa. glucocorticoid-binding "mero-receptor" which remains immunoprecipitatable using anti-hsp 90 antibodies (DENIS *et al*, 1988). This observation has been confirmed by expression studies of GR deletion mutants. These studies found that deletion of the glucocorticoid-binding domain produced a constitutively active GR; furthermore, deletion of only three amino acid residues from within the ligand-binding domain resulted in a GR population devoid of the 9S, heteromeric GR (PRATT *et al*, 1988). Both these results suggest that the hormone-binding domain, or some part of it, is responsible for mediating hsp 90-GR interaction. These conclusions have been confirmed by similar work carried out on PR. Studies using deletion mutants located a stretch of 140 amino acids within the progesterone-binding domain which were essential for both progesterone-binding and 8S PR formation (CARSON-JURICA *et al*, 1989).

The gene sequence of chick hsp 90 has been analysed for possible interaction sites with the avian PR (BINART *et al*, 1989). These workers have identified two hydrophilic α-helical regions on the surface of hsp 90, and proposed that these regions are responsible for structural association with steroid receptors.

4.7. REGULATION OF THE RECEPTOR-HSP 90 INTERACTION.

Phosphorylation has been discussed in relation to its role as a possible regulator of steroid receptor activity (See INTRODUCTION section 2.3.). Purified hsp 90 is a serine-phosphoprotein (SANCHEZ *et al*, 1986) and it has been shown to be a co-purifying substrate for rat liver type II casein kinase *in vitro* (DOUGHERTY *et al*, 1987). Threonine phosphorylation of hsp 90 by human double-stranded DNA-activated protein kinase has also been observed *in vitro* (LEES-MILLER & ANDERSON, 1989b); however the significance of these observations remains doubtful as the affinity of hsp 90 for the enzyme was very poor and threonine-phosphorylation has never been observed *in vivo*.

As discussed in INTRODUCTION section 2.2.2., phosphorylation as a result of ligand-binding has been proposed as a method of inducing steroid receptor transformation (ORTI *et al*, 1989a). The dissociation of hsp 90 after steroid-binding could be triggered by a change in the phosphorylation state of either component (AURICCHIO *et al*, 1990). However this seems unlikely in the light of observations of GR cycling which demonstrated that the net phosphorylation of hsp 90 remained unchanged before and after glucocorticoid-binding (ORTI *et al*, 1989b). No net change was observed in the phosphate content of the glucocorticoid-binding subunits either, although the authors do not reject the possibility of phosphate exchange.

Ligand-binding is thought to be the most potent effecter of the steroid

receptor-hsp 90 dissociation. It has been suggested that the main difference between the action of hormones and anti-hormones is that anti-hormones may act to prevent dissociation of hsp 90 and so form inactive receptor species (BAULIEU, 1987a & b). This theory will be discussed elsewhere (see *Part 1* DISCUSSION section 5.)

The steroid receptor-hsp 90 complex cannot currently be reassembled *in vitro* from purified components. The only "man-made" 8-10S receptors have been formed from the *in vitro* translation of receptor mRNA in the presence of hsp 90 (DENIS & GUSTAFSSON, 1989; CARSON-JURICA *et al*, 1989). One possible problem may be that there is an extra, hitherto undiscovered component(s) which aid in the assembly of steroid receptor-hsp 90 complexes *in vivo*, but are lost or inactive in cell-free preparations. Two such proteins proposed as missing components are hsp 70 and a 59 kDa. protein termed p59 (SMITH *et al*, 1990). The hsp 70 association seems hormone-independent, however the p59 protein was seen to dissociate from PR with similar kinetics to hsp 90 in response to progesterone exposure. Further work (RENOIR *et al*, 1990) has confirmed that p59 is associated with PR, ER, AR & GR, and in addition p59 is bound to hsp 90 rather than the steroid-binding subunits. This confirms the similar steroid-induced dissociation of p59 and hsp 90 reported by Toft and colleagues (SMITH *et al*, 1990) and furthers the "missing-component" hypothesis regarding the inability of researchers to construct nonactivated receptor *in vitro*.

4.8. FUNCTIONAL INTERACTIONS OF STEROID RECEPTORS & HSP 90.

Baulieu has suggested that the function of hsp 90 is to act as a cap, "masking" the DNA-binding domain of steroid receptors until hormone-binding induces hsp 90 dissociation (BAULIEU, 1987a & b). These ideas are based on the original observations of this group that the 90 kDa. component was only found to associate with nonactive steroid receptors (CATELLI *et al*, 1985; JOAB *et al*, 1984). They remain in agreement with many of the current observations discussed in the preceding paragraphs (See FIGURE 3.).

The "masking" scenario at first seems contradictory to the profusion of data suggesting that hsp 90 interacts with the hormone-binding domain of steroid receptors. However, the larger/longer hsp 90 could span both the DNA- and the hormone-binding domains of the ER; indeed, a dual interaction would seem necessary for ligand-regulation of DNA-binding ability.

O'Malley and colleagues have produced an antibody to a fragment of PR which represents a region of the PR located between the hormone- and DNA-binding domains and is predicted to be on the surface of the protein (WEIGEL *et al,* 1989). Interestingly the antibody only recognises activated PR and not 8S nonactivated PR, suggesting that hsp 90 could be masking the DNA-binding domain. This observation parallels results from other previous experiments where purified hsp 90 was used to elute activated GR from a DNA-cellulose column (HOWARD & DISTELHORST, 1988b).

FIGURE 3. **A Schematic Model of Steroid Hormone Action.**

This diagram attempts to summarise the current understanding of steroid receptor activation in terms of ligand-binding and DNA-binding. (Based on the oestrogen receptor)

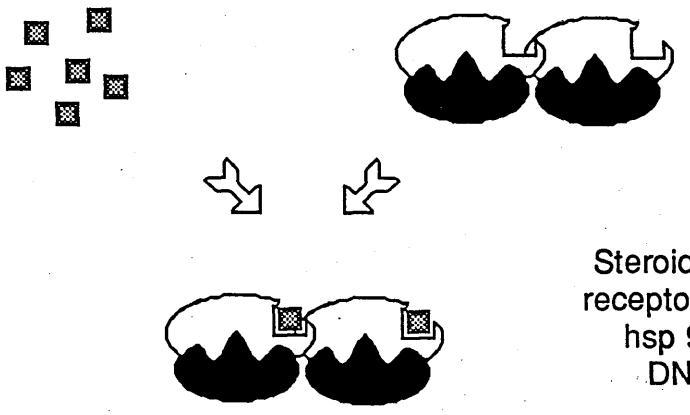
In this simplified scheme, the nonsteroid-binding subunits are shown to interact with the DNA-binding domains of the steroid-binding subunits - the "masking" effect. Steroid-binding induces the dissociation of the subunits into homodimers (activation). The steroid-binding dimer then binds to palindromic hormone response elements encoded within the DNA of steroid-responsive genes. The nonsteroid-binding subunits (hsp 90) may then be recycled.

For the sake of simplicity, other effecters (eg. phosphorylation) are omitted from FIGURE 3., as are any indications of the intracellular compartmentalisation of the various processes.

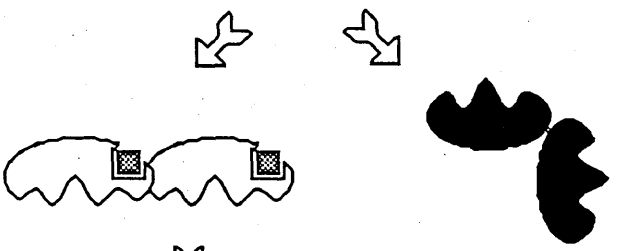
Schematic Model of Steroid Hormone Action.

Steroid enters the cell and binds to Steroid Receptors.

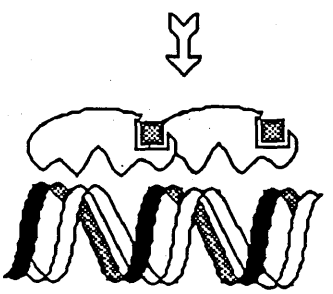
Nonactivated Steroid Receptors are present as multimers of Steroid-binding Subunits (white) and hsp 90 (black).



Steroid binding to receptor results in receptor activation; possibly because hsp 90-dissociation "unmasks" the DNA-binding site of the receptor.



The nonsteroid-binding subunits (hsp 90) remain in dimeric form whether free or released. (Recycling ?)



Activated receptor dimers bind to palindromic DNA sequences within hormone response elements.

(This model is based on the observations of ER activity cited within the text.)

Although there is an ever increasing understanding of the structural-functional relationship held by the hsp 90-steroid receptor complex *in vitro*, the significance of hsp 90 presence and its relation to steroid receptor activity *in vivo* has largely been overlooked. Early work from Baulieu and coworkers (GASC *et al,* 1984) made some tentative suggestions of the action of PR in the chick oviduct: they carried out an immunohistochemical study of both the DNA-binding subunits and the 90 kDa. (hsp 90) subunits and found that progesterone-binding altered the intracellular distribution of these two components. Apart from these studies, there are relatively few *in vivo* investigations on the effect of ligand-binding on the hsp 90-steroid receptor complexes.

Correspondingly, no researchers have investigated the possibility of a relationship existing between the actions of steroid receptors and the heat shock response. There is much direct and indirect evidence supporting the stabilising role of hsp 90 during normal steroid receptor function; yet the possibility that steroid receptor activity could then be modulated by the heat shock response remains uninvestigated. It is hoped that the work included in this thesis may answer some of these questions and address this fascinating association of two very distinct families of proteins.

METHODOLOGY.

1. Buffers and Solutions.

1.1. BUFFERS.

1.1.1. PHOSPHATE BUFFERED SALINE (PBS), pH 7.2

17OmM NaCl; 1mM Na_2HPO_4 and 2mM KH_2PO_4.

1.1.2. TRIS BUFFERED SALINE (TBS), pH 7.6

137mM NaCL and 5mM Tris-HCl adjusted to pH 7.6 using 1M HCl.

1.1.3. BICARBONATE BUFFER, pH 9.0

250mM Na_2CO_3 and 100mM NaCl adjusted to pH 9.0 using 1M HCl.

1.1.4. ETN BUFFER, pH 7.0

10mM EDTA; 10mM Tris-HCl and 100mM NaCl adjusted to pH 7.0 using 1M HCl.

1.1.5. NP-40 SOLUTION, pH 7.3

100mM NaCl and 10mM Tris-HCl adjusted to pH 7.3 using 1M HCl and made 0.5% with Nonidet P-40 (NP-40).

1.2. CELL CULTURE SOLUTIONS.

1.2.1 DULBECCO'S BUFFERED SALINE (PBS-A), pH 7.2

17OmM NaCl; 3.4mM KCl; 10mM Na_2HPO_4 and 2.0mM KH_2PO_4

1.2.2. VERSENE SOLUTION, pH 7.4

125mM NaCl; 2.7mM KCl; 6.3mM Na_2HPO_4; 3.2mM KH_2PO_4 and 0.5mM EDTA adjusted to pH 7.4 using 1M HCl.

1.3. RECEPTOR ASSAY SOLUTIONS.

1.3.1. HED-BUFFER, pH 7.4

20mM HEPES; 1.5mM EDTA and 0.25mM dithiothreitol (DTT) made 10% glycerol and adjusted to pH 7.4 using 1M NaOH. DTT is only added on the day of use.

1.3.2. DEXTRAN COATED CHARCOAL.

A solution of 20mM HEPES and 1.5mM EDTA (pH 7.4; 100 ml) was made 0.5% in sieved, pre-washed charcoal and 0.005% in dextran before mixing for 30 minutes at room temperature. The resulting solution is stored at 4°C only up to 8 weeks and must be well mixed before use.

2. Cell Culture Methods.

2.1. CELL LINES.

Three established cell lines were used; the human breast cell line MCF-7, and two human prostate cell lines LNCaP and DU-145. The MCF-7 cell line was purchased from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, England; while both LNCaP and DU-145 cells were a gift of Dr. C. Eaton, Tenovus Institute for Cancer Research, University of Wales College of Medicine, Cardiff.

MCF-7 is a stable epithelioid cell line originally obtained from the pleural effusion of a female patient with breast cancer whose disease responded to endocrine therapy (HORWITZ *et al*, 1975b). Ligand binding assay has shown this cell line to contain significant levels of ER, PR, AR and GR, and so it has been used as an in vitro model for breast cancer and its treatment by endocrine therapy.

The LNCaP cell line was established from a metastatic lesion of human prostatic adenocarcinoma, the second most frequent tumour of males in the USA (HOROSZEWICZ *et al*, 1983). This cell line is androgen-dependent and androgen-responsive, containing both AR and ER. DU-145, however, is androgen-independent. It was also originally isolated from a metastatic tumour (STONE *et al*, 1978).

2.2 ROUTINE GROWTH AND SUBCULTURE.

2.2.1. CULTURE CONDITIONS.

All cell lines were routinely grown at 37°C in an air atmosphere enriched to 5% CO_2. A humidified atmosphere was generated during prolonged growth in multiwell-style plates to prevent media evaporation. Flasks of various capacities (surface areas) were used depending on the mass of cells required: 25cm^2 containing 5ml of medium, 75cm^2 containing 10ml of medium and 150cm^2 containing 25ml of medium. Fresh culture media were generally renewed every 48 hours. All manipulations were carried out aseptically within the confines of a clean, laminar air flow cabinet.

2.2.2. GROWTH MEDIA.

Initially, Dulbecco's modification of Eagles medium (DMEM) was used for routine subculture (DULBECCO & FREEMAN, 1959). This is available as a phenol red-free powdered medium (Sigma) which can be reconstituted and sterilised in the laboratory, although an 18mM HEPES supplement is still required. Unfortunately, it did not prove possible to maintain any long-term, contamination-free cell culture using this preparation of phenol red-free DMEM. This was due principally to mycoplasma contamination as confirmed by Hoechst staining (see METHODOLOGY section 2.3.3.). Cells grown in this medium seemed particularly prone to mycoplasma contamination, the source(s) of which were never established. As a result, routine growth was established using RPMI-1640, a commercially available phenol red-containing liquid preparation (Gibco BRL Ltd.). This

medium requires no nutritional or buffering supplements as it incorporates 18mM HEPES (MOORE *et al*, 1967).

2.2.3. SUPPLEMENTS AND ANTIBIOTICS USED.

During routine subculture, all growth media was supplemented with 10% foetal calf serum (FCS). However, during hormone experiments, heat inactivated, dextran coated charcoal-stripped, dialysed FCS (HIDCCFCS) was used at 2.5% (LEAKE *et al*, 1987a).

Initially, cells were exposed to different combinations of penicillin, gentamycin, streptomycin and nystatin when DMEM was being used. However no antibiotic supplements were used in conjunction with the RPMI-1640 medium hopefully preventing any proliferation of microbial resistance often associated with long term antibiotic inclusion.

2.2.4. DRUG AND HORMONE SUPPLEMENTS USED.

Drugs and hormones were dissolved in absolute alcohol to form 1×10^{-4}M stock solutions which were stored at -18°C. These solutions could then be added aseptically to RPMI-1640 to provide working solutions of minimal alcohol content. (see individual protocols for details of the ligands used).

2.2.5. SUBCULTURE TECHNIQUE.

Cells were deemed ready to undergo passage when the substrate surface area was estimated 80-90% confluent. The cells were washed using PBS-A (c.10ml), and then 0.05% trypsin in a versene solution was

added to each flask (1ml for a 25cm^2 flask; 3ml for a 75cm^2 flask, and 5ml for a 150cm^2 flask). To neutralise the trypsin, fresh culture medium (2-3 volumes) was added and rapid pipetting was employed to form an even cell suspension. This cell suspension was then dispensed into new culture flasks containing an appropriate amount of fresh culture medium so that the ratio between the donor and recipient surface areas was between 1:3 and 1:5. All solutions were prewarmed to 37°C.

2.3. MANAGEMENT OF CELL LINES.

2.3.1 CRYOPRESERVATION OF CELL LINES.

Whenever possible, frozen stocks of cells were prepared while the cell line was still at an early passage number. This meant that all experiments could be carried out using cells of a similar, early passage number. It was found that when one confluent 150cm^2 flask (approximately 22×10^6 cells) was harvested and the cells frozen as suspension (1.0ml) then there was efficient recovery of a large number of cells after thawing from storage.

Prior to freezing, cells were grown in numerous 150cm^2 flasks using RPMI-1640 medium. The cells were washed and harvested as described in METHODOLOGY section 2.2.4. but in this case the cell suspension was transferred to a sterile universal tube; and centrifuged for 3 minutes at 1000g (4°C). After discarding the supernatant, the pellet was re-suspended in a 10% DMSO in FCS solution (1ml). The cells were then transferred to a sterile 'Biofreeze' vial (Nunc Inc.) and frozen overnight at -70°C, before ultimate long term storage under liquid nitrogen.

2.3.2. GROWTH OF CELLS FROM FROZEN.

The frozen vials were removed from liquid nitrogen and thawed in the palm of the hand for between 5-15 minutes; allowing some ice to remain in order to minimise the cytotoxic action of DMSO. After sterilising the outside of the vial using 70% ethanol, the semi-frozen cell suspension was added to a sterile universal tube containing fresh, prewarmed (37°C) culture medium. This was then centrifuged for 3 minutes at 1000 g before discarding the supernatant and resuspending the cells in 37°C medium and transferring the cell suspension to a new tissue culture flask ($25cm^2$ or $75cm^2$) also containing 37°C medium.

2.3.3. MYCOPLASMA TESTING OF CELL LINES.

Cell lines were routinely screened for the presence of contaminating mycoplasma using a modification of an established method (CHEN *et al*, 1977). This involved growing cells on sterile microscope slides under standard conditions for 72 hours, before discarding the medium, washing the cell monolayer with PBS-A, and fixing the cells as described in METHODOLOGY section 6.2.1. The cells were then incubated with 100ng/ml Hoechst 33258 for 10 minutes at room temperature, before the washing and dehydration steps described in METHODOLOGY section 6.4.2. Fluorescent microscopy was used to determine the presence of extra-nuclear DNA.

3. Steroid Receptor Assays.

3.1. LIGAND BINDING ASSAYS.

3.1.1. ASSAY PRINCIPLE.

These assays can determine the total specific steroid receptors present in a variety of tissues (LEAKE, 1981). The principle used is based on the calculations of Scatchard (SCATCHARD, 1949): cytosol is incubated over a concentration range of radioactive hormone with or without a 100-fold excess of unlabelled hormone to determine specific receptor binding. The soluble hormone-receptor complex can be separated from any free hormone by the process of dextran coated charcoal (DCC) adsorption; free steroid is adsorbed to the DCC whereas receptor-steroid complexes are not.

The scientific principle, radioactive standard preparation and assay methodology are identical for both oestrogen and androgen receptor assays.

3.1.2. RADIOACTIVE STEROID STANDARDS.

The ten standards are prepared so that there are seven radiolabelled standards within the stated range (Standards 1-7). An additional three duplicate standards of the highest three concentrations are prepared and to these 100-fold excess of non-labelled steroid (in ethanol) is added (Standards 8, 9 & 10). Unlabelled steroid is added first to tubes 8, 9 & 10 and then the solvent evaporated using compressed air. The appropriate volumes of ethanol containing [^{3}H]-steroid was then added to each of the

ten vials before equalising the alcohol content of each vial by adding ethanol alone. HED-buffer was then added in order to bring all standards to a volume of 3.0ml. These were stored for only two months at 4°C. Aliquots (50µl) of each standard were counted to obtain a value of the "totals".

The concentrations and ligands used for each of the receptor assay standards were as follows:

- For ER assay: 0.4, 0.6, 1.0, 1.5, 2.0, 3.0 & 4.0nM [^{3}H]-oestradiol-17β was used as the radiolabel with a 100-fold excess of diethylstilboestrol (DES) as the competitor in the highest three concentrations. (After addition of "cytosol" the final concentrations of steroid were 0.1 to 1.0nM.) The [^{3}H]-oestradiol-17β had a specific activity of 98Ci/mmol.
- For AR assay: 1.0, 1.5, 2.0, 4.0, 6.0, 8.0 & 10nM [^{3}H]-mibolerone was used as the radiolabel with a 100-fold excess of mibolerone as the competitor in the highest three concentrations. (After "cytosol" addition the final androgen concentrations were 0.25 to 2.5nM.) The [^{3}H]-mibolerone had a specific activity of 76Ci/mmol. In addition, the AR-assay standards were made 100nM with Org 2058 and 100nM with triamcinolone acetonide in order to prevent nonspecific-binding to PR or GR (LEAKE & HABIB, 1987).

3.1.3. LIGAND BINDING ASSAY (LBA) PROTOCOL.

Accurate ligand binding assay relies on the correct preparation of a suitable extract from tissues or cell cultures. (See specific EXPERIMENTAL sections). All solutions and conditions were maintained at 4°C.

The standards (50µl) were added to each of ten 60×6mm polypropylene test-tubes (RT-30) and mixed with 150µl of "cytosol" extract. Steroid-binding took place by incubating either for 18 hours at 4°C (overnight) or for 2 hours at 20°C. After incubation, dextran coated charcoal solution (DCC; 200µl) was added in order to remove unliganded (free) steroid. This treatment continued on ice for 15 minutes; vortexing every five minutes, before centrifuging for 5 minutes at 1000g to pellet the DCC-adsorbed steroid. Aliquots (200µl) of each supernatant were transferred to scintillation vials and 4ml of Ecoscint A (National Diagnostics) added. The vials were counted for tritium, each for 3 minutes, and the cpm used to carry out computerised Scatchard calculations.

Scatchard analysis (SCATCHARD, 1949) of ligand binding assay data was routinely carried out using a BBC microcomputer programmed as described by LEAKE *et al*, (1987b).

3.1.4. "TWO-POINT COMPETITION" STEROID RECEPTOR ASSAY

Ten-point LBA of cell culture extracts is not always possible as sufficient cytosol is seldom available, and so the abbreviated two-point assay is used. This assay is basically a straightforward competition between the

tritiated ligand and the excess of unlabelled competitor (LEAKE & HABIB, 1987). Carried out at least in duplicate, the assay incubates cytosol (150µl) with both the standards containing the highest concentration of tritiated ligand (standards 7 & 10). All timings and manipulations are similar to the ten-point LBA allowing receptor concentrations to be calculated per ml of extract.

3.2. ENZYME IMMUNO ASSAY (EIA).

3.2.1. ASSAY PRINCIPLE.

This assay system relies on direct antibody recognition of the oestrogen receptor rather than detection based upon its steroid binding ability; it is a solid phase enzyme immunoassay based upon the "sandwich" principle. Beads coated with anti-ER (rat monoclonal) antibody are incubated for 18 hours (8°C) with specimens or standards or controls. During this incubation, ER present in the specimen is bound to the solid phase, unbound materials present in the specimen are then removed by aspiration of fluid and washing of beads. A second anti-ER antibody conjugated with horseradish peroxidase is then incubated with the beads for 60 minutes (37°C) and the conjugate becomes bound to any ER on the beads. Unbound conjugate is removed by aspiration when the beads are washed. The beads are then incubated for 30 miutes (20°C) with an enzyme substrate solution (hydrogen peroxide and *o*-phenylenediamine) to develop a colour which is a measure of the amount of bound ER-conjugate. The enzyme reaction is stopped by the addition of 0.5M

H_2SO_4 and the absorbance at 492nm determined. The intensity of the colour formed by the enzyme reaction is proportional to the concentration of ER in the sample within the working range of the assay. A standard curve is obtained by plotting the ER concentration of the standards against their absorbance. (For a full description of the methods see the technical instructions (Ref.: 83-4281/R4) provided by the kit manufacturers: Abbott Laboratories, Diagnostic Division, North Chicago, Illinois 60064.)

4. Protein and DNA Assays.

4.1. BRADFORD PROTEIN MICROASSAY.

4.1.1. ASSAY PRINCIPLE.

This assay is based on the principle that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465nm to 595 nm when binding to protein occurs (BRADFORD, 1976).

4.1.2. PROTEIN STANDARDS.

Protein standards were created for the range of 0-15µg/ml protein.

Commercial albumin standard No. 23210, (Pierce Inc.) which is supplied as 2.0mg/ml, was appropriately diluted in order to form standards of 2, 4, 6, 8, 10, & 15µg/ml. These were stored for only two weeks at 4°C.

4.1.3. ASSAY PROTOCOL.

The sample to be assayed was diluted into the approximate assay range using distilled water or an appropriate buffer solution. Where reagents were used that slightly altered the assay standard curve (eg. SDS or NP-40), an alternative set of assay standards was created which incorporated the interfering agent. A duplicate set of standards (0.8ml) and triplicate diluted samples (0.8ml) were added to 60×6mm poly-propylene test-tubes. Bradford assay reagent (200µl) was then added and the tubes vortexed. The tubes were left to stand at room temperature for 30 minutes, vortexing after 15 and 30 minutes, before measuring the absorbance of each solution at 595nm. The protein content of each sample was calculated from the standard curve. (See sample standard curve, FIGURE 4.)

4.2. HOECHST DNA ASSAY.

4.2.1. DNA STANDARDS.

Standards were prepared from a 100µg/ml solution of calf thymus DNA dissolved in ETN buffer. The concentration of this solution was verified using the formula that 1 unit of absorbance at 260nm is exactly 50µg/ml.

The standards prepared were: 0, 5, 10, 15, 20, 25, 30, 40 & 50µg/ml; producing a reproducibly linear standard curve up to 50mg/ml. These were stored for only one month at -18°C.

4.2.2. ASSAY PROTOCOL.

The method used was modified from the original Leake and Habib methodology (LEAKE & HABIB, 1987) and used exclusively for the DNA assay of cultured cell monolayers; usually grown on multiwell plates. The assay is dependent on the intercalation of the Hoechst dye with solubilised DNA; the resulting complex is fluorescent with a maximum emission at 450nm.

Samples were solubilised by incubating for 30 minutes (37°C) in 0.2% SDS in ETN buffer. Aliquots (100µl) of each solubilised specimen were transferred to RT-30 test tubes to which 3.0ml of ETN buffer containing Hoechst 33258 (100ng/ml) and RN'ase (5.0µg/ml) was added. The tubes were mixed thoroughly by vortexing and incubated at room temperature for 30 minutes in the dark. The fluorescence enhancement at 450nm was then measured using a Hitachi Perkin-Elmer MPF-2A fluorescent spectrophotometer with an excitation wavelength of 360nm and both slit widths at 5nm. (See sample standard curve, FIGURE 5.)

FIGURE 4. **Bradford Protein Assay Calibration Graph.**

The reproducibility of this assay is shown in this typical calibration curve. The assay was carried out in duplicate as described in METHODOLOGY section 4.1. and used to construct a calibration graph. The equation of the line was derived using the computer program "Cricket Graph" on an Apple Macintosh PC, and used to calculate the values of protein present in unknown samples.

Sample "Bradford" Protein Assay.

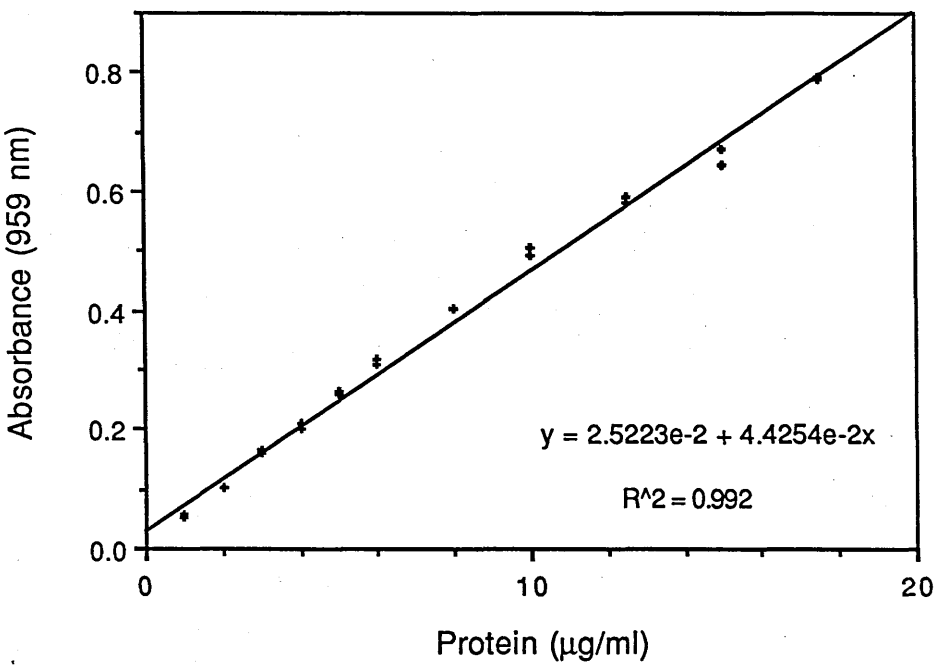
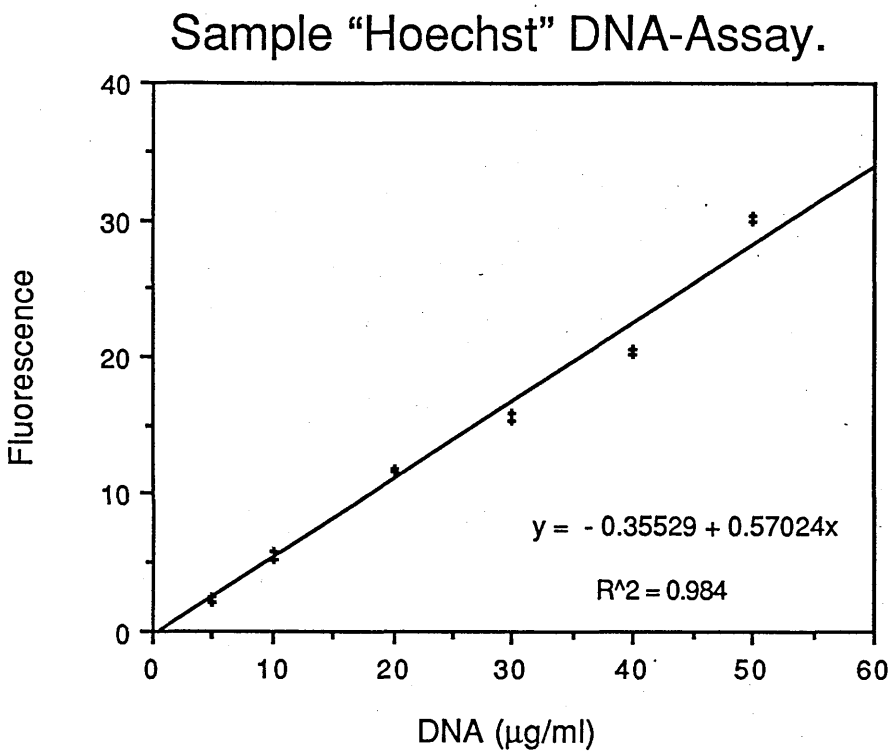


FIGURE 5. **Hoechst DNA Assay Calibration Graph.**

The reproducibility of this assay is shown in this typical calibration curve. The assay was carried out in duplicate as described in METHODOLOGY section 4.2. and used to construct a calibration graph. The equation of the line was derived using the computer program "Cricket Graph" on an Apple Macintosh PC, and used to calculate the values of DNA present in unknown samples.


Sample "Hoechst" DNA-Assay.
Fluorescence
DNA (µg/ml)
y = - 0.35529 + 0.57024x
R^2 = 0.984
0
10
20
30
40
50
60

5. Electrophoretic Methods.

5.1. POLYACRYLAMIDE GEL ELECTROPHORESIS.

The methodology is a modification of that of LAEMMLI (1970).

5.1.1. STOCK GEL SOLUTIONS.

The stacking buffer used was 0.125M Tris-HCl (pH 6.8) containing 0.4% SDS; the resolving buffer was 0.375M Tris-HCl (pH 8.8) containing 0.8% SDS. Both buffers were stored at 4°C for no longer than 8 weeks. A 30% electrophoresis grade acrylamide and 0.8% N,N'-methylene bis-acrylamide solution was prepared and filtered using Whatman no.1 filter paper before storing for no more than 4 weeks at 4°C. A solution of 1.5% ammonium persulphate was prepared 15-30 minutes prior to use, then discarded after each polymerisation.

5.1.2. GEL CONSTITUENTS AND THEIR POLYMERISATION.

The molecular weights of the proteins under investigation meant that a 7.5% slab gel was used. This should resolve proteins within the range of 45-300 kiloDaltons (kDa.).

The constituents of the resolving gel were; distilled water (23ml), resolving buffer (5ml), acrylamide (10ml) and ammonium persulphate solution (2ml). The constituents of the stacking gel were; distilled water (11ml), resolving buffer (5ml), acrylamide (2ml) and persulphate solution (1ml).

To initiate polymerisation TEMED (25µl) was added last to each mixture and the solution poured between clean glass plates (200x200mm). A slab

gel was formed so that there was a 25mm deep stacking gel on top of a 7.5% resolving gel.

5.1.3. ELECTROPHORESIS TANK BUFFER.

The tank buffer was a 0.025M Tris, 0.192M Glycine (pH 8.3) buffer containing 0.1% SDS. A ×10 stock solution was prepared and stored at 4°C for no longer than 8 weeks.

5.1.4 RUNNING BUFFER.

Samples were dissolved in running buffer to an approximate concentration of 0.1-1.0mg/ml protein. This consisted of 0.0625M Tris-HCl (pH 6.8) containing 10% sucrose; 5% β-mercaptoethanol; 2% SDS and 0.002% bromophenol blue (HAMES, 1981). Between 10µl and 30µl of sample buffer was applied per well.

5.1.5. MOLECULAR WEIGHT STANDARDS.

To assess the molecular weight of observed proteins, calibration standard proteins were applied to the gel in conjunction with unknown samples. These standards, supplied as a kit (Boehringer Mannheim GmbH), were dissolved in running buffer and used to construct calibration curves for molecular weight based on their relative mobility. The standards used were: *lactate dehydrogenase* (Mr 36,400); *glutamate dehydrogenase* (Mr 55,400); *β-galactosidase* (Mr 130,000) and *α2-macroglobulin* (Mr 170,000).

5.1.6. ELECTROPHORETIC CONDITIONS.

Whenever possible gels were run overnight in a cooled incubator (4°C) to make most efficient use of laboratory time. A constant current of 20mA and 75V maintained for 9 hours was sufficient for complete protein separation.

5.2. STAINING AND PRESERVATION OF GELS

5.2.1. COOMASSIE BLUE STAINING AND DESTAINING.

A 0.1% coomassie blue staining solution was used to stain gels by soaking the gel for 2 hours in 500ml of stain. 0.1% Coomassie brilliant blue R250 was dissolved in 500ml of a 42% methanol and 16% acetic acid solution. The gel was destained by five 60 minute soakings at 4°C in a destain solution of 30% methanol and 10% acetic acid.

5.2.2. SLAB GEL DRYING.

All gels were dried on to Whatman 3MM chromatography paper using a layer of clingfilm to form an upper protective layer. Gels were dried for 2 hours at 80°C under constant vacuum unless a fluorographic agent was included (See METHODOLOGY section 5.3.2.) in which case 4 hours at 60°C was the drying conditions chosen.

5.3. AUTORADIOGRAPHY.

5.3.1. STANDARD AUTORADIOGRAPHY.

Autoradiography was carried out after first drying gels as described in METHODOLOGY section 5.2.2.; if there was a delay between the initial drying of the gel and any subsequent autoradiography, then the gel was redried for 20 minutes at 80°C to remove any absorbed moisture. For [^{35}S]-methionine labelled gels, Kodak X-OMAT S film was used. Dried gels were brought into contact with the film and sandwiched between two sheets of Whatman 3MM chromatography paper, all operations being carried out in a dark room. The sandwich was placed inside an autoradiography cassette, which was wrapped in black polythene and placed in a -70°C refrigerator. The individual exposure times depended on the cpm available, estimated by scintillation counting. X-ray films were developed using a Kodak X-OMAT processing machine.

5.3.2. FLUOROGRAPHY.

A fluor was introduced into the gels to enhance autoradiography when there was only a very small number of cpm. This involved soaking the gel for 2 hours in a solution of 14% salicylic acid dissolved in 1M NaOH. This step was incorporated after the coomassie blue destaining stage (METHODOLOGY section 5.2.1.) but an extra distilled water prewash was also included at this stage as any excess acetic acid results in salicylate precipitation. Fluorographic gels were then dried for 4 hours at 60°C as described in METHODOLOGY section 5.2.2.

5.4. WESTERN BLOTTING.

Immunoblotting methodology was a modification of previously published methods (TOWBIN *et al*, 1979).

5.4.1. ELECTROPHORESIS.

Electrophoresis was carried out using 7.5% gel as previously described (see METHODOLOGY section 5.1.). Previously prepared samples (15-30µl per well) were added in duplicate so that the gel was in two identical halves. This meant that after transblotting the nitrocellulose blot could be cut in two; one half was used for immunoblotting and the other half for amido black protein staining.

5.4.2. ELECTROPHORETIC TRANSFER.

Transblotting was carried out using specific apparatus (BioRad); which consisted of a cassette and pads for mounting the gel, and a tank which allowed the cassette to stand vertically between two electrodes immersed in buffer. When mounting the gel and the nitrocellulose film within the cassette, each layer was added separately, immunotransfer buffer being added from a wash bottle to prevent any air bubbles. At this point, small cuts were made on the nitrocellulose to mark where the corresponding gel lanes would be and where it could be halved. Electrophoretic transfer was carried out using a constant current of 200mA (45V) for 5 hours at 4°C. The transfer (tank) buffer was 200mM glycine, 25mM Tris and 6mM SDS dissolved in a 20% methanol solution.

5.4.3. PROTEIN FIXATION TO THE NITROCELLULOSE.

After transfer and safe removal from the cassette, the nitrocellulose was carefully cut into the two identical sections and both sections washed for 15 minutes using TBS at room temperature. Fixation of the proteins to the nitrocellulose was achieved by soaking in 0.2% gluteraldehyde in TBS for 45 minutes at room temperature under constant gentle agitation. After fixation the nitrocellulose was washed in fresh TBS for 15 minutes.

5.4.4. PROTEIN TRANSFER VERIFICATION.

Protein transfer was verified in two ways. Firstly by conventional coomassie blue staining of the gel after the transfer to show that no significant bands remained; and secondly, by amido black staining of one of the nitrocellulose halves. 0.1% Amido black dissolved in a 45% methanol and 10% acetic acid solution was used to stain the nitrocellulose for 5 minutes. The excess stain was then poured off, and the nitrocellulose destained using a 90% methanol and 2% acetic acid solution.

5.4.5. IMMUNOBLOTTING.

The remaining nitrocellulose section to be blotted was first incubated at 8°C overnight, using constant gentle agitation, with a solution of 5% Marvel® dissolved in TBS. This was in order to minimise any non-specific antibody binding.

After this initial blocking step, proteins of interest were visualised using a

blotting system of mouse monoclonal primary antibodies, detected using horse radish peroxidase (hrp) labelled sheep anti-mouse secondary antiserum (METHODOLOGY section 6.1.2). All the subsequent operations were carried out at room temperature using freshly made 5% Marvel® in TBS solution. Constant gentle agitation was induced by placing the nitrocellulose inside a sealed, clean jar which was mounted between two rollers.

After aspirating off the excess 5% Marvel® solution, primary antibody, appropriately diluted using more 5% Marvel® solution, was incubated for 120 minutes at room temperature. The nitrocellulose was then washed five times using 5% Marvel® solution for a total of 60 minutes. Secondary hrp-antibody (SAPU), diluted 1:50 using 5% Marvel® was then incubated for 90 minutes, again at room temperature. Next, after three 5% Marvel® washes of 10 minutes each, there was a further three 10 minute washes only this time using TBS alone. (The developer solution was prepared at this point, see below.) The developer solution was added for 10 minutes or until bands were obvious; no increased staining being noted after 15 minutes. The nitrocellulose was washed in distilled water and dried between two sheets of filter paper.

5.4.6. IMMUNOBLOT DEVELOPER SOLUTION.

Prepared immediately prior to usage; a 0.3% chloronaphthol in methanol solution was added to TBS and mixed. To this solution was added 4.5% hydrogen peroxide solution (150µl) to form the developer solution.

6. Immunocytochemistry.

6.1. ANTISERA.

6.1.1. PRIMARY, MONOSPECIFIC ANTISERA.

All antisera used were aliquoted into 25µl lots and stored at -70°C to prevent cyclical freeze-thawing antibody deactivation. The monospecific antibodies used were as follows:

- Anti-ER monoclonal antibody:

ERP-31 is a mouse monoclonal antibody produced from murine ascitic fluid and was a gift of Dr. B. Westley, University Department of Pathology, Royal Victoria Infirmary, Newcastle upon Tyne. ERP-31 was used for immunoprecipitation of ER.

- Anti-AR monoclonal antibody:

AN1-15 is a rat monoclonal antibody raised against human AR and kindly donated by Professor S. Liao, Department of Biochemistry and Molecular Biology, The Ben May Institute, Chicago, Illinois.

- Anti-AR polyclonal antibody:

A rabbit polyclonal IgG antibody to AR was also donated by Professor S. Liao and used for AR detection by three-stage immunohistochemistry (see METHODOLOGY section 6.3).

- Anti-hsp 90 monoclonal antibody:

AC 88 is a mouse monoclonal antibody raised against an 88kDa. protein purified from the water mould *Achlya ambisexualis* (RIEHL *et al,* 1985). This antibody is specific for hsp 90 of a variety of tissues and species

(SCHUH *et al*, 1985), and was obtained as a gift of Dr. David Toft, Dept. of Biochemistry and Molecular Biology, Mayo Clinic and Medical School, Rochester, Minnesota.

6.1.2. SECONDARY, SPECIES-SPECIFIC ANTISERA.

The species-specific antibodies used were all obtained from the Scottish Antibody Production Unit (SAPU), Law Hospital, Carluke, Strathclyde. All antisera were supplied as a liquid preparation and were aliquoted and frozen on receipt.

- Anti-mouse antibody:

Sheep anti-mouse IgG was used in the production of fluorescent antiserum capable of detection of mouse monoclonal antibodies (see METHODOLOGY section 6.4.1.)

- Anti-rabbit antibody:

Donkey anti-rabbit IgG was used in the production of fluorescent antiserum capable of detection of rabbit polyclonal antibodies (see METHODOLOGY section 6.4.1.)

- Anti-rat antibody:

Sheep anti-rat gamma-globulin was used in the production of fluorescent antiserum capable of detection of rat monoclonal antibodies (see METHODOLOGY section 6.4.1.)

- Hrp-anti-mouse antibody:

Sheep anti-mouse IgG was used in the detection of mouse monoclonal antibodies during immunoblotting (see METHODOLOGY section 5.4.5.)

6.2. FIXATION.

6.2.1. ACETONE-METHANOL FIXATIVE.

A solution of acetone and methanol (1:1 (v/v), Analar reagents) was prepared and precooled to -18°C before use.

Fixation was achieved by placing the microscope slide on top of an ice pack and covering the section or cell monolayer in 200µl of fixation solution for 30 seconds. The excess acetone-methanol was then aspirated and the sections allowed to air dry for 5-6 minutes, when no remaining moisture was evident.

6.2.2. ACETONE FIXATION.

The whole slides were dipped into a coplin jar containing acetone and allowed to soak for 5 minutes before air drying the sections. If immuno-cytochemical staining was to be carried out at a later date; slides were fixed for only 1 minute, dried and stored at -70°C. After thawing to room temperature, the slides were refixed for 5 minutes and again air dried prior to staining.

6.3. ANDROGEN RECEPTOR IMMUNOHISTOCHEMICAL ASSAY.

Tissue sections cut in the Dept. of Pathology, Western Infirmary, Glasgow, and cell monolayers were fixed as described in the preceding section 6.2.2. Nonspecific-staining was abolished by a 5 minute incubation with 5% BSA in TBS. After this time, all test slides had the TBS solution removed and were incubated with rabbit polyclonal anti-AR antibody (see

METHODOLOGY section 6.1.1.) diluted 1:200 in 5% BSA in TBS. After a 60 minute incubation at room temperature, excess serum was removed by TBS washings and replaced by mouse anti-rabbit serum diluted 1:40 with 5% BSA in TBS. This secondary incubation was again carried out at room temperature, this time for 30 minutes, before adding alkaline phosphatase-labelled rabbit anti-mouse serum dilluted 1:25 (See METHODOLOGY section 6.1.2.). This final serum was incubated for 60 minutes at room temperature.

After washing off excess serum, the staining was visualised using Histomark® (Kirkegard Perry, Inc.) which utilises fast violet stains to produce either red or blue staining as required. The slides were sealed using Aquamount (BDH, Ltd.)

6.4. FLUORESCENT IMMUNOCYTOCHEMICAL STAINING.

Monospecific antibodies directed against hsp 90 and AR were detected using various species-specific antisera which were fluorescein labelled and therefore visible by fluorescent microscopy.

6.4.1. FLUOROCHROME LABELLING OF ANTISERA.

Species specific antiserum (25ml), obtained from SAPU was dialysed for 48 hours against 1l of pH 9.0 bicarbonate buffer at 4°C, fresh buffer being added after 24 hours. After dialysis the serum was removed and the protein concentration estimated in order to optimise the IgG to

fluorochrome ratio for conjugation. The appropriate amounts of fluorochrome were weighed out and dissolved in bicarbonate buffer (1.0ml; 4°C) so that there would be 0.05mg of fluorochrome present for every 1.0mg of protein during conjugation. Fluorescein isothiocyanate (FITC) was used; this emits green visible light (λ = 515nm) when exposed to excitatory ultra violet light.

The antiserum (0.25ml) and fluorochrome solution (0.25ml) were brought together in a polytube and left to react overnight at 4°C under constant mixing. After conjugation, the excess unbound fluorochrome was separated from the dye conjugated antiserum using a column of Sephadex G-25 (300×16mm). The column was first calibrated using 50µl of a 1:1 (v/v) mixture of 1% blue dextran and 0.1M potassium dichromate. The whole reaction mixture (0.5ml) was added to the column and the eluting fractions collected (0.922ml; 20 drops) using an LKB Redirac 2112 fraction collector. The labelled IgG conjugate eluted between fractions 14-18; the excess free label eluting after some 50 fractions.

The obviously fluorescent fractions were assayed for labelling efficiency by estimating the molar ratio of fluorescence to protein (JOHNSTON & THORPE, 1983). This was found by measuring the absorbance of the conjugate at 280nm and 495nm. The molar ratio should be between 2 and 4 and was calculated using the following formula:

$$\text{MOLAR RATIO} = \frac{2.87 \times \text{Ab.}_{(495nm)}}{\text{Ab.}_{(280nm)} - [0.35 \times \text{Ab.}_{(495nm)}]}$$

Fractions of molar ratio ≈ 3 were selected and stored at -70°C prior to usage.

6.4.2. FLUORESCENT IMMUNOCYTOCHEMICAL STAINING.

This method is modified from that of CRAWFORD *et al.* (1985) which exploits the fluorescent detection of a monoclonal antibody using a species specific fluorochrome conjugated secondary antiserum.

Cell monolayers were grown on 8-chamber slides (see FIGURE 14.). Excess culture medium was aspirated and the cells washed using PBS-A before acetone methanol fixation (METHODOLOGY section 6.2.1.).

Non-specific binding of the secondary antibody was eliminated by a 20 minute incubation of the tissue using 3% sheep serum (SAPU) in PBS. The excess serum was then removed and primary antiserum, diluted appropriately* using 0.1% BSA in PBS, was added and incubated for 2 hours at room temperature in a humidified box. (*See individual protocols for antibody dilution.)

After incubation, the primary antiserum was aspirated and the cells washed five times by adding 0.1% BSA in PBS and agitating slides for three minutes. Fluorescent secondary antiserum diluted 1:500 using 0.1% BSA in PBS was added and incubated for 2 hours again in the humidified box. After incubation, excess serum was removed and the cells washed five times as before. Nuclear counterstaining was achieved by a 5 minute incubation with 100ng/ml Hoechst 33258, followed by similar washing

steps.

After removing the plastic upper chambers, the slide was dehydrated. This involved sequential 60-second dips in 50%; 75%; 100%, and 100% alcohol. The slide was then cleared by two dips in xylene before being sealed using a xylene-based mountant (DPX-mountant; BDH).

Immunofluorescence was visualised within 24 hours using a Lietz "Orthoplan" fluorescent microscope.

Part 1.

Experimental

The Association of hsp 90 and ER in the MCF-7 Human Breast Cancer Cell Line.

CHAPTER 1.

The Effect of Heat Shock on MCF-7 Cell Growth.

INTRODUCTION.

In order to assess the role of heat shock proteins, and hsp 90 in particular, various experiments were carried out which exploited the overproduction of hsp's - the heat shock, or stress response (LINDQUIST, 1986). Initially it was decided to characterise the normal range of thermal tolerance exhibited by the MCF-7 human breast cancer cell line; as this would provide a threshold level to calibrate future experiments investigating the thermotolerance response displayed by these cells.

The experiment was designed so that cells would receive a measured dose of supraphysiological heat. The exposure time was kept constant over the range of temperatures between (37°C and 45°C) used to heat shock the cells. Cell viability was measured by assaying cell growth 24 hours and 48 hours after completing heat shock.

METHODS.

MCF-7 cells were subcultured as described in METHODOLOGY section 2.2. so that a 20% confluent monolayer was formed within ten 6-well (15.7cm^2/well) plastic tissue culture plates. The cells were allowed to "plate-down" for 24 hours and fresh culture medium was added immediately prior to commencing the experiment.

Heat shock was achieved using a carefully controlled and monitored water-bath (FIGURE 6.). The plates were sealed watertight using plastic film (NescoFilm®), before being floated on the water surface for two hours. The temperature of the culture medium was verified using a thermometer placed within a similarly mounted plate containing only growth medium. After subjecting the cells to the various temperatures for two hours, the cells were allowed a recovery period of 24 hours. After this time the contents of three wells were washed using PBS-A and solubilised by a 30 minute incubation with ETN buffer containing 0.2% SDS (4ml) at 37°C (METHODOLOGY section 1.1.4.). Aliquots (100µl) of solubilised cells were then assayed for DNA using the Hoechst DNA assay described in METHODOLOGY section 4.2. After another 24 hours the remaining three wells of each plate were washed, harvested and assayed for DNA as before.

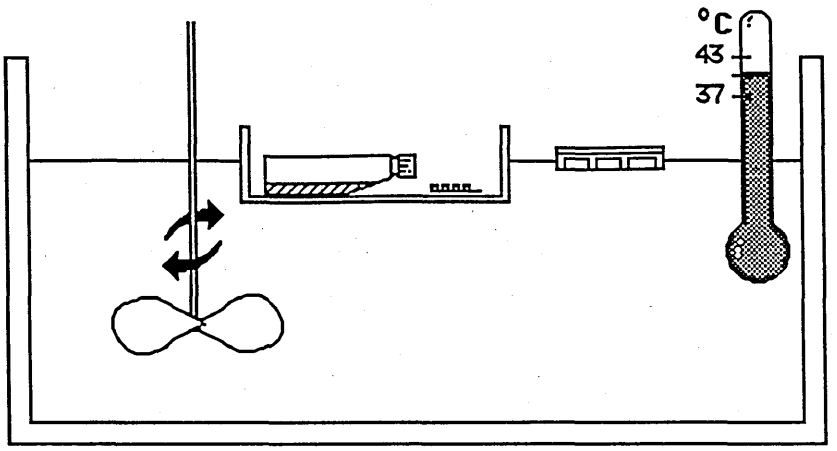
RESULTS.

The results shown in FIGURE 7. represent the mean of two experiments, each experiment consisting of 3 wells of cells for each temperature at each time. The parameter being measured is cell growth and so results are expressed as DNA (µg/ml); the amount of DNA present in the cell monolayer.

FIGURE 6. **Physical Induction of Heat Shock.**

The technique used to induce heat shock is shown in FIGURE 6.; this simple apparatus was used to ensure reproducible temperature selection.

Flasks and microscope slides were placed within a glass trough containing a layer of glycerol 1mm deep; whereas multi-well plates were sealed water tight and allowed to float on the surface of the water. The efficacy of heat transfer from the water to the cells was assessed by measuring the temperature inside vessels containing only culture medium.



Flask of Growing Cells.

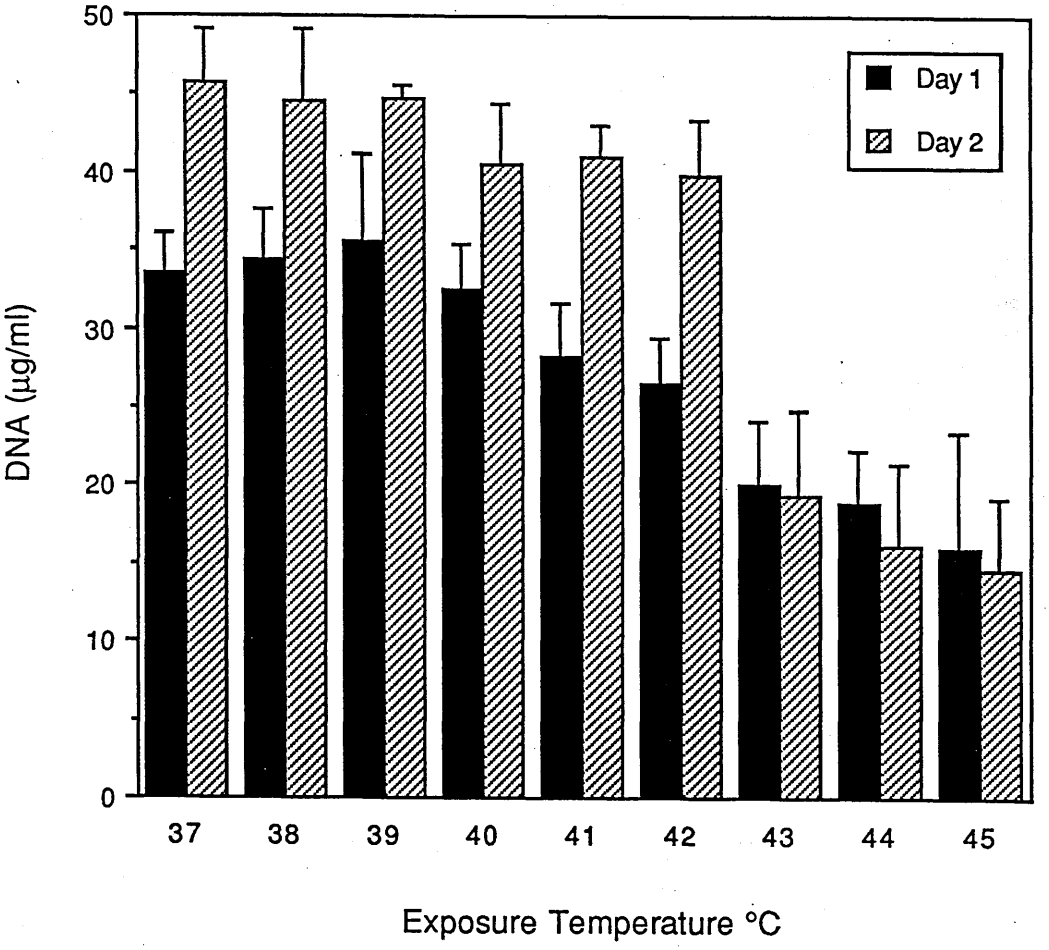
8-Chamber Microscopy Slide.

6-Well Plate.

FIGURE 7. **The Effect of Temperature on MCF-7 Cell Growth**
MCF-7 cells were subjected to two hours exposure to the temperatures shown and then their growth, in terms of the amounts of DNA, was measured after 24 and 48 hours. (Day 1 black, and Day 2 striped, respectively)

Error bars represent the Standard Deviation of the data.

The Effect of Temperature on MCF-7 Cell Growth.



CONCLUSIONS.

For most cell lines in culture, a short period after death (24 to 48 hours), cells tend to detach from the lower surface of the chosen culture vessel. However, MCF-7 cells attach very firmly and so tend to remain in contact with the substrate until some form of mechanical abrasion is used. In addition, the majority of these "cells" remain intact. For these two reasons it is impossible to correlate absolute values of DNA with cell viability and so it was decided to assess cell DNA at both 24 hours and 48 hours after heat shock, as the difference between the two values should indicate whether or not cell growth is sustained.

The effectiveness of thermal killing depends on two factors: exposure time and exposure temperature. Earlier work (not shown here) suggested that varying the length of heat shock altered the effectiveness of a single temperature. Thus, it was decided to maintain a constant exposure time of 2 hours in order to make best use of laboratory time and to simplify future experimental protocols.

As can be seen from FIGURE 7., the lethal temperature for MCF-7 cells is between 42°C and 43°C; this represents an approximate 6°C increase on the normal physiological temperature.

The effect on temperature is best visualised by comparing the DNA values of the 24 hours and 48 hours recovery periods; the difference between these two values is an estimate of the cell growth. A constant cell growth rate over the second 24 hour period is displayed among the cells

subjected to temperatures up to 42°C. This growth rate corresponds to an increase in cellular DNA of between 25-30% over the 24 hour period. However, in the cells subjected to temperatures greater than 42°C there is actually a net loss of DNA over the same period. These results show that cell growth is inhibited at these higher temperatures and that the loss of DNA represents cells that have died, become detached, and then lost during the pre-assay washing steps.

This experiment gives a measure of the normal threshold level of MCF-7 cell thermal tolerance. From this experiment, it was then possible to go on to establish whether sub-lethal temperatures could induce thermo-tolerance to temperatures above 42°C.

CHAPTER 2.

Thermotolerance of MCF-7 Cells.

INTRODUCTION.

Thermotolerance is the acquisition of heat resistance through previous exposure to sub-lethal temperatures (see INTRODUCTION section 3.3.1.). The intracellular process mediating this protective mechanism is presumed to depend on the production of heat shock proteins (hsp's; LINDQUIST, 1986). It was decided to investigate whether the MCF-7 cell line could acquire thermotolerance and so survive temperatures exceeding 43°C; shown to be lethal to this cell line after 2 hours exposure (EXPERIMENTAL chapter 1.).

METHODS.

MCF-7 cells were subcultured as described in METHODOLOGY section 2.2. so that a 20% confluent monolayer was formed within three 6-well plastic tissue culture plates. After 24 hours, fresh culture medium was added immediately prior to commencing the experiment.

Heat shock was again achieved using the water-bath technique (see previous chapter); however various heat shock treatments (HST) were used:

- HST 0 Cells maintained at 37°C throughout (controls)
- HST 1 2 hours exposure at 41°C followed by a 24 hour recovery period at 37°C.

- HST 2 2 hours exposure at 44°C followed by a 24 hour recovery period at 37°C.
- HST 1+2 2 hours exposure at 41°C followed by a 24 hour recovery period at 37°C; prior to a further 2 hours exposure at 44°C followed by a 24 hour recovery period at 37°C.

MCF-7 cells were exposed to either HST 0; HST 2 or HST 1+2. After these heat shock treatments the contents of three wells were washed and solubilised as described in the previous chapter. After another 24 hours the remaining three wells of each plate were washed and harvested. Aliquots (100µl) of solubilised cells were then assayed for DNA using the Hoechst DNA assay described in METHODOLOGY section 4.2.

RESULTS.

As mentioned in the preceding chapter, the critical factor to be considered is cell growth, as reflected by the change in DNA content over the 24 hour period (DNA [Day 2] - DNA [Day 1]). These values are more representative of the true level of cell viability than a value of absolute DNA.

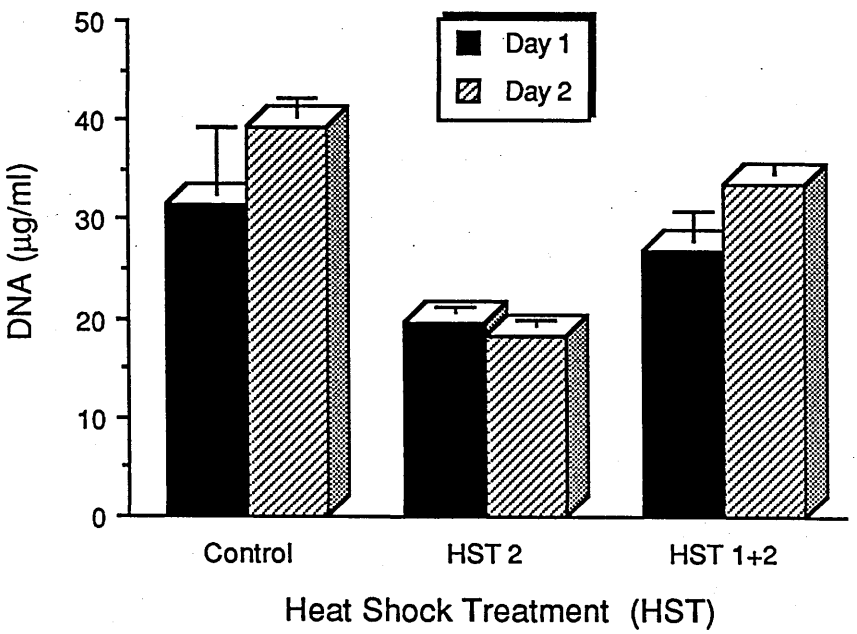
The results quoted are the mean of three experiments; each experiment consisted of three different wells of cells for each HST, at each time point. This means that the columns shown on the histogram (FIGURE 8.) represent the mean of nine individual data points.

FIGURE 8. **Demonstration of MCF-7 Cell Thermotolerance.**
MCF-7 cells exposed to HST 2 alone (44°C; 2 hours followed by 37°C; 12 hours) were compared to cells subjected to HST 0 (37°C throughout) and cells given a prior exposure to HST 1 (41°C; 2 hours followed by 37°C; 12 hours) before also being exposed to HST 2; and termed HST 1+2.
The total growth-time of all treatments was identical.

The effects of the different heat shock treatments on cell growth was assessed by measuring the amounts of DNA after 24 and 48 hours. (Day 1 and Day 2 respectively)

Error bars represent the Standard Deviation of the data.

MCF-7 Cell Thermotolerance.



*
Although the significance of the change in growth of the control cells over the 24 hour period is cast in doubt as a result of the large standard deviation incurred after Day 1, it should be noted that the patern of growth inhibition observed is consistent with these conclusions.

CONCLUSIONS.

Initial observation of FIGURE 8 reveals that the absolute values of DNA in both the HST 2 and HST 1+2 treated cells are markedly lower than the control values, though significant ($p<0.001$) only in HST 2. This altered profile is due to the thermal-killing of cells which then become detached and are lost during the pre-assay washing stages. As previously stressed, these values are not directly proportional to cell viability, though, it may be significant that the HST 1+2 treated cells fared better than the HST 2 cells in terms of total DNA.

However what is more interesting is the changes in DNA content over the second 24 hour interval. Whereas the HST 1+2 treated cells show a growth increase over this period that is comparable to the control cells maintained at 37°C; the cells treated using HST 2 alone show no growth over the 24 hours. In other words, these cells are no longer viable.

This experiment shows that transient (2 hour) exposure to the sub-lethal temperature of 41°C can induce some protective property allowing the same cells to withstand a subsequent supralethal temperature (44°C). These results are typical of the thermotolerance response and provide direct evidence that the MCF-7 breast cancer cell line demonstrates this phenomenon.

CHAPTER 3.

Hsp 90 Induction in MCF-7 Cells.

INTRODUCTION.

Having established that the MCF-7 breast cancer cell line displays the typical thermotolerance phenomenon, it was decided to assess whether the levels of hsp 90 were altered during the response. Hsp 90 seems to play a key role in the regulation of the cellular thermotolerant response to heat shock (see INTRODUCTION section 3.2.4.) although it seems to be its phosphorylation state rather than amount that is important (ROSE *et al*, 1989). Hsp 90 heat inducibility has previously been demonstrated using heat shocked chick embryo fibroblasts (CATELLI *et al*, 1985). These investigators used V8-protease digestion and two-dimensional peptide mapping to confirm hsp 90 heat inducibility. In this study, it was decided to use immunological methodology to detect the levels of hsp 90 present in MCF-7 cells subjected to different regimen of heat shock; using the mouse monoclonal AC 88 antibody (see METHODOLOGY section 6.1.1.), which is specific for hsp 90 of many species and from different tissues (RIEHL *et al*, 1985). This antibody was a generous gift from Dr. David Toft of the Mayo Clinic; although AC 88 has recently become commercially available from StressGen Biotechnologies Corp., Sidney, Canada. The experiment was designed so that the MCF-7 cells were subjected to different degrees of thermal stress, and then any proteins synthesised during recovery from heat shock were labelled using [^{35}S]-methionine incorporation. Immuno-

precipitation using AC 88 antibody, followed by autoradiography was then used to assess the different levels of hsp 90 present in the cells as a result of the various HST's.

METHODS.

1. CELL CULTURE AND HEAT SHOCK INDUCTION.

The various elevated temperatures to which the cells were exposed were again achieved using the water-bath technique described in EXPERIMENTAL chapter 1. The different HST* regimen were:

- Group A HST 1 alone
- Group B HST 2 alone
- Group C HST 1+2
- Group D HST 0 (37°C control for group A).
- Group E HST 0 (37°C control for groups B & C).

(* Details of heat shock treatments (HST) are described within EXPERIMENTAL chapter 1.)

Cells were grown in 25cm^2 flasks to minimise the amount of radioactive culture medium required. Immediately prior to commencing the various HST, the standard RPMI medium was removed and replaced with methionine-free RPMI medium (Flow Labs. Inc.) that was supplemented with only 2.5% HIDCC-dialysed FCS (METHODOLOGY section 2.2.3.). After removing the flasks from the elevated temperatures, the methionine-free

medium was made 50μCi/ml with [^{35}S]-methionine. The cells were exposed to this label for the 12 hours of the recovery period until they were harvested and processed for immunoprecipitation. Groups D and E represent control cells grown at 37°C throughout and labelled with [^{35}S]-methionine during similar recovery periods to groups A and B/C respectively.

The cells were harvested using the "rubber-policeman technique": this involved removing residual culture medium from within the flasks, washing the cell monolayers with PBS-A, and then physically scraping the cells from the substrate using an angular glass rod sheathed in PTFE-rubber. The free cells were collected in a volume of added PBS-A (3.0ml; 4°C), and the suspension transferred to suitable tubes and centrifuged to provide a pellet of whole-cells. This pellet (approximately 3.5 million cells) was solubilised on ice using NP-40 solution containing 0.5M NaCl (2.0ml; METHODOLOGY section 1.1.5.) and centrifuged at 13,000 rpm for 3 minutes using an MSE Microcentaur microfuge. The resultant supernatant was the solubilised cell extract used for hsp 90 immuno-precipitation. DNA assay (see METHODOLOGY section 4.2.) was carried out on the pellet from this centrifugation.

2. IMMUNOPRECIPITATION OF HSP 90.

To immunoprecipitate hsp 90, AC 88 monoclonal antibody was first pre-adsorbed onto a suspension of heat-killed *Staphylococcus aureus* bacteria termed Pansorbin® (Calbiochem); this formed an insoluble agent

capable of immunoprecipitating hsp 90 from the solubilised cell extract.

A 10% suspension of Pansorbin (1.0ml) was washed twice using NP-40 solution (4.0ml; 4°C) by successive vortexing and centrifugation (60 seconds at 13,000 rpm using an MSE Microcentaur microfuge). The final pellet was suspended at 10% ($^{w}/_{v}$) in NP-40 solution (1.0ml; 4°C). 10% BSA (10µl) was added to this suspension which was known as "washed Pansorbin".

To minimise nonspecific immunoprecipitation, the cell extract was "cleared" using a small amount of the washed Pansorbin. 20µl of the washed Pansorbin was added to 200µl of cell extract and mixed gently on a rotator for 60 minutes at 4°C. At the end of this time centrifugation was used as before to produce a supernatant of cleared cell extract.

Preadsorption of AC 88 onto washed Pansorbin was achieved by incubating antibody (50µl, pre-diluted 1:100 in NP-40 solution) with washed Pansorbin solution (500µl), again by mixing gently on a rotator for 60 minutes at 4°C. After preadsorption, the suspension was divided into five 100µl aliquots before centrifuging as before and discarding the supernatant. These pellets represent the five immunoadsorbant agents used to immunoprecipitate hsp 90. The remainder of the washed Pansorbin (500µl) was treated similarly, except that 1% BSA was substituted for AC 88; these five pellets being used as the controls for non-specific immunoprecipitation.

Immunoprecipitation was achieved by incubating aliquots of each cleared cell extract (100µl) with either an immunoadsorbing pellet or a control pellet for 120 minutes using constant rotation at 4°C.

After incubation, the suspensions were centrifuged as before and the supernatants discarded. The pellets were then washed three times by resuspension in successive solutions (100µl) by vortexing followed by centrifugation for 30 seconds at 13,00 rpm using the MSE microfuge. The three solutions were; NP-40 solution; NP-40 solution containing 0.5M NaCl, and NP-40 solution containing 0.1% SDS.

The final pellets were dissolved in running buffer (50µl; METHODOLOGY section 5.1.4.), boiled for 60 seconds and analysed by 7.5% polyacrylamide gel electrophoresis (see METHODOLOGY section 5.1.). Autoradiography was carried out as described in METHODOLOGY section 5.3.

The bands on the gel corresponding to Mr ≈90,000 were then excised and β-scintillation counting of the acrylamide sections was used to determine the total amount of radioactivity immunoprecipitated. The use of Ecoscint A permitted a 36% counting efficiency from the segments of undissolved acrylamide.

RESULTS.

The results of this experiment are shown in the following three figures. FIGURE 9. is a photograph of the coomassie blue-stained 7.5% PAGE gel that was used to resolve the protein products of the immunoprecipitation. FIGURE 10. is an autoradiogram developed from this gel. The histogram shown in FIGURE 11. illustrates the relationship between the different heat shock treatments and the amounts (measured as cpm [^{35}S]-methionine immunoadsorbed) of hsp 90 purifiable from the cell extracts.

The electrophoretic patterns of the nonspecific-immunoprecipitates are not shown as scintillation counting of these pellets found that they contained levels of β-radiation not significantly different from background levels. This suggests that AC 88 immunoprecipitation is highly specific.

FIGURE 9. **The Effect of Heat on the Total Amount of Immunoprecipitatable hsp 90 from MCF-7 Cells.**

Hsp 90 was immunoprecipited from cells subjected to different Heat Shock Treatments (HST). HST 1 was 2 hours exposure to 41°C followed by 12 hours recovery at 37°C. HST 2 was 2 hours exposure to 44°C followed by 12 hours recovery at 37°C.

The five lanes of coomassie blue stained protein on the gel represent immunoprecipitated hsp 90 from cells subjected to different regimen of HST. Reading lanes 1-5 from left to right the regimen were:

1. HST 1
2. HST 2
3. HST 1+2
4. HST 0 (Control for HST 1)
5. HST 0 (Control for HST 2 & HST 1+2)

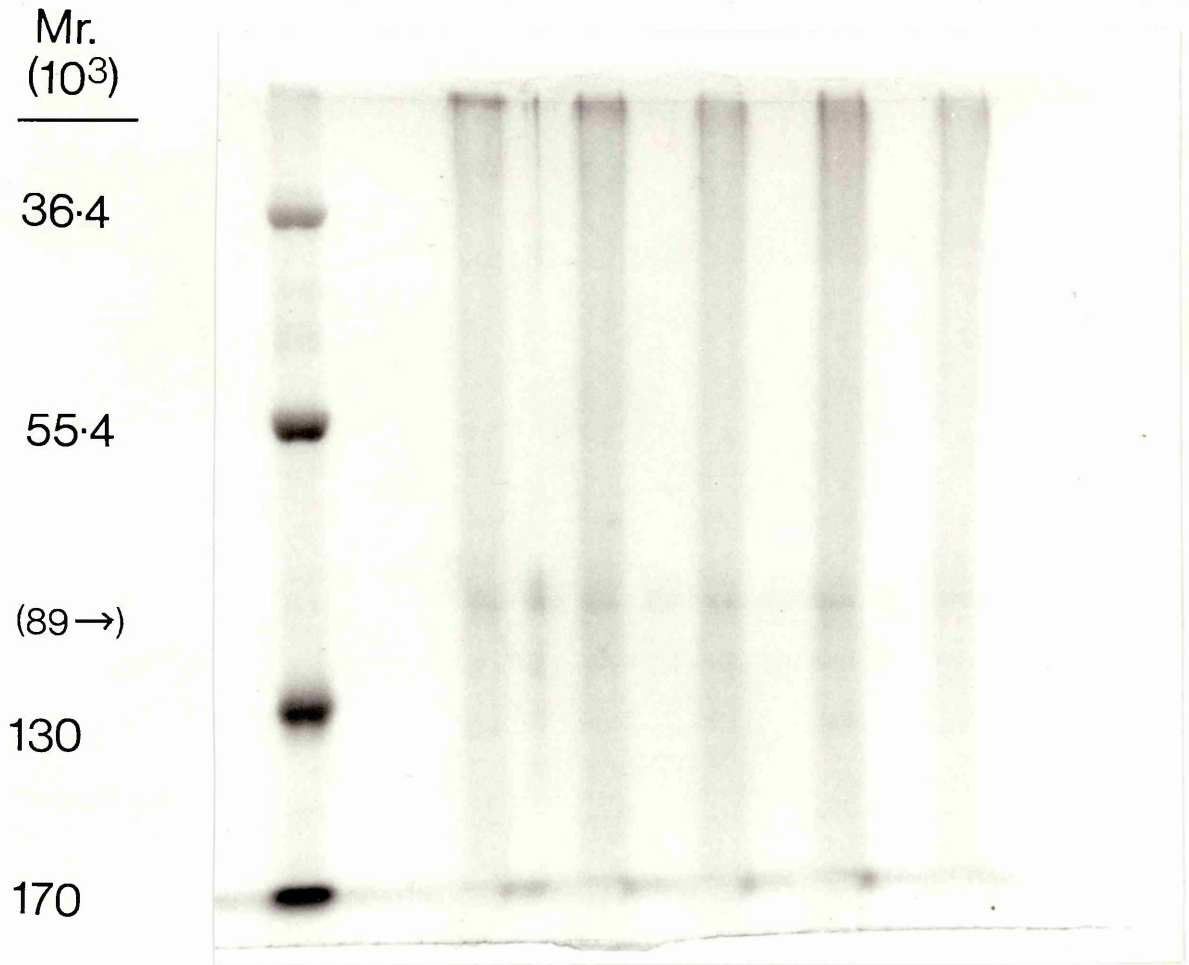

Mr.
(10³)
36·4
55·4
(89→)
130
170
1 2 3 4 5
Lane Nº

FIGURE 10. **Heat Shock Induction of MCF-7 Cell hsp 90 Assessed by [^{35}S]-labelled hsp 90.**

This autoradiogram was developed from the gel shown in FIGURE 9. This then represents the level of [^{35}S]-methionine incorporated into AC 88 immunoprecipitated material during the 12 hour recovery period subsequent to heat shock, and prior to hsp 90 immunoprecipitation. The five lanes represent the different regimen of HST. Reading lanes 1-5 from left to right the regimen were:

1. HST 1
2. HST 2
3. HST 1+2
4. HST 0 (Control for HST 1)
5. HST 0 (Control for HST 2 & HST 1+2)

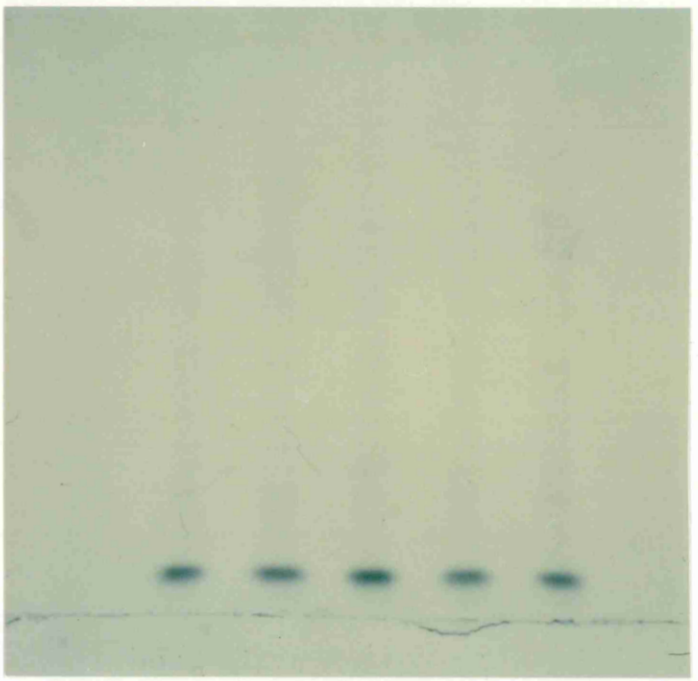
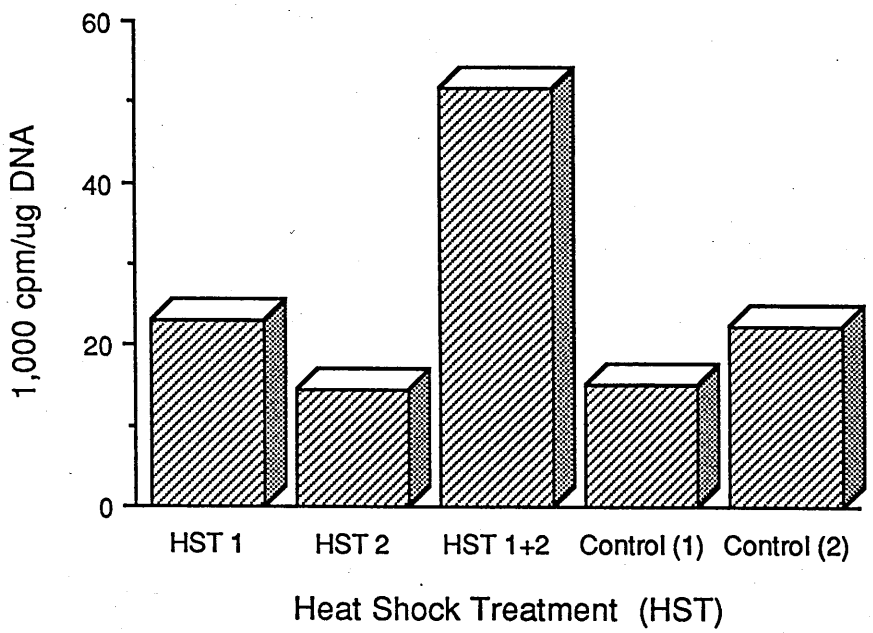

MR. ≈89
1 2 3 4 5
Lane Nº

FIGURE 11. **Quantitation of Heat Shock Induction of MCF-7 Cell hsp 90.**

This histogram shows the relative amounts of hsp 90 synthesised during MCF-7 cell recovery from heat shock. The protein bands corresponding to the molecular weight of hsp 90 were excised from the gel shown in FIGURE 9. and the [^{35}S]-methionine incorporated quantified by β-scintilation counting. The results were expressed in terms of the DNA present in the original cell extract used for hsp 90 immunoprecipitation.

Data represents the mean of two experiments for each condition. Each value is the cpm. recovered from the 90kDa. region of the gel after immunoprecipitation and PAGE.

The Effect of Heat Shock on MCF-7 Cell hsp 90 Levels.



*Please note that to assess the level of hsp 90 induction, lanes 2 & 3 should be compared to the control (HST 0) forthese lanes which is lane 5. The control for lane 1 is lane 4.

When lanes 2 & 3 are compared to lane 5, there is a significant difference in the observed level of immunoprecipitated hsp90 - lane 3 showing the most increased levels.

CONCLUSIONS.

The low amounts of protein resolved by the gel shown in FIGURE 9. suggest either that there is relatively little hsp 90 present in the original cell extract, or alternatively that AC 88 immunoprecipitation is inefficient. As hsp 90 is a highly ubiquitous protein, present in significant concentrations in most cell types, it is unlikely that the former is the case. More probably, it seems likely that in our hands AC 88 is an inefficiently immunoprecipitating antibody. However, the fact that there is only a single band in each of the five lanes demonstrates that AC 88 immunoprecipitation is nonetheless very specific.

When a calibration curve of molecular weight versus mobility is calculated it is found that the predominant band of all five lanes has a Mr ≈87,500. This is assumed to be hsp 90 and is further confirmation of the specificity of antibody AC 88.

FIGURE 9. shows that the greatest amount of hsp 90 is recoverable from the extract of cells treated by HST 1+2 (lane 3): the cells displaying thermotolerance*. In addition, FIGURE 10. demonstrates that these cells contain the greatest amount of [^{35}S]-methionine labelled-hsp 90, suggesting that hsp 90 synthesis was greatest in this cell group during recovery from heat shock. Together, FIGURES 9. and 10. show that there is a significant amount of hsp 90 both present, and synthesised during recovery from heat shock. This is in accordance with other workers who have found that hsp 90 is present in abundant levels even in unstressed cells (LEES-

MILLER & ANDERSON, 1989b).

Since heat shock is known to reduce the overall level of cell viability it is impossible to make a direct comparison of the immunoprecipitates with each other. This is because the hsp 90 purified from heat shocked cells (lanes B and C) was purified from extracts of fewer cells due to the elevated temperatures. For this reason the DNA content of each extract was estimated prior to immunoprecipitation in order to equate the amount of immunoprecipitated hsp 90 with the original number of cells.

The results shown in the histogram in FIGURE 11. represent the cpm present in the excised bands from the gel shown in FIGURE 9. expressed in terms of the amount of DNA present in the original cell extract. This allows a more relevant assessment of the effect of the different heat shock treatments on hsp 90 synthesis. As can be readily observed, there is an enhanced amount of hsp 90 present in the cells exposed to the double heat shock (HST 1+2). This is the cell group that shows the greatest resistance to heat (see EXPERIMENTAL chapter 2): the thermotolerant group, suggesting that hsp 90 plays an integral role in the mediation of the thermotolerance phenomenon.

CHAPTER 4.

The Influence of Elevated Temperatures on the Oestrogen-Binding Capacity of MCF-7 Cells.

INTRODUCTION.

It has been established that the level of hsp 90 is modulated by heat in MCF-7 cells and that its expression is reflected in the thermotolerance status of the cells in question. As discussed in INTRODUCTION section 4., hsp 90 has been demonstrated to associate with the nonactivated form of steroid receptors; seemingly to mask the DNA-binding site of these receptors until ligand-induced dissociation results in receptor activation (BAULIEU, 1987a & b). If this is the biological role of hsp 90 in relation to steroid receptors, then, given our observations that hsp 90 is strongly influenced by heat and as such may play a role in the mediation of MCF-7 cell thermotolerance, it was decided to investigate whether temperature could have an effect on the steroid-binding capacity of heat treated cells. Temperature is known to induce receptor activation in the absence of ligand *in vitro* (SAKAI & GORSKI, 1984). However, no workers have discussed the possibility that temperature could effect the steroid-binding capacity of receptors *in vivo* or *in vitro* by thermal-modulation of the levels of receptor-associated hsp 90.

4.1. THE EFFECT OF HEAT ON MCF-7 CELL OESTROGEN - BINDING CAPACITY.

METHODS.

MCF-7 cells were subjected to similar heat shock treatments (HST's) as described in EXPERIMENTAL chapter 1 again using the same techniques. Cells were subcultured in 150cm^2 flasks 24 hours prior to commencing the experiment so that a 20% cell monolayer was present. After exposing the cells to either 37°C (controls), HST 2 alone, or HST 1+2 the medium was removed from the flasks, the cell monolayer washed twice using ice cold PBS-A and the cells harvested by freeze-thawing. This involved adding ice cold HED-buffer (3.0ml; METHODOLOGY section 1.3.1.) to each flask which was then floated on liquid nitrogen for 15 seconds. The flasks were then moved to a 37°C incubator for 10 minutes to allow rapid thawing, before transferring the contents to suitable tubes, and centrifuging at 1000g and 4°C using an MSE 'Mistral' for 5 minutes. The resultant supernatant "cytosol" extract was used to determine the oestrogen-binding capacity of the heat treated cells by assaying for ER using the ten-point method described in METHODOLOGY section 3.1.3. The protein content of each sample was determined by the method of Bradford as described in METHODOLOGY section 4.1.

RESULTS.

The results described in TABLE 1. represent the means of four separate experiments, each experiment consisting of triplicate ER assay so that the values represent the mean of 12 assays. Results are quoted in terms of femtomoles of oestrogen receptor per mg of protein.

TABLE 1. THE EFFECT OF HEAT SHOCK ON THE OESTROGEN BINDING CAPACITY OF MCF-7 HUMAN BREAST CANCER CELLS.

Treatment	ER (fmol/mg protein)	Std. Devn.	No. of Expts.
HST 0	20.09	5.64	12
HST 1+2	2.25	5.56	12

4.2. THE KINETICS OF HEAT ABOLITION OF OESTROGEN-BINDING.

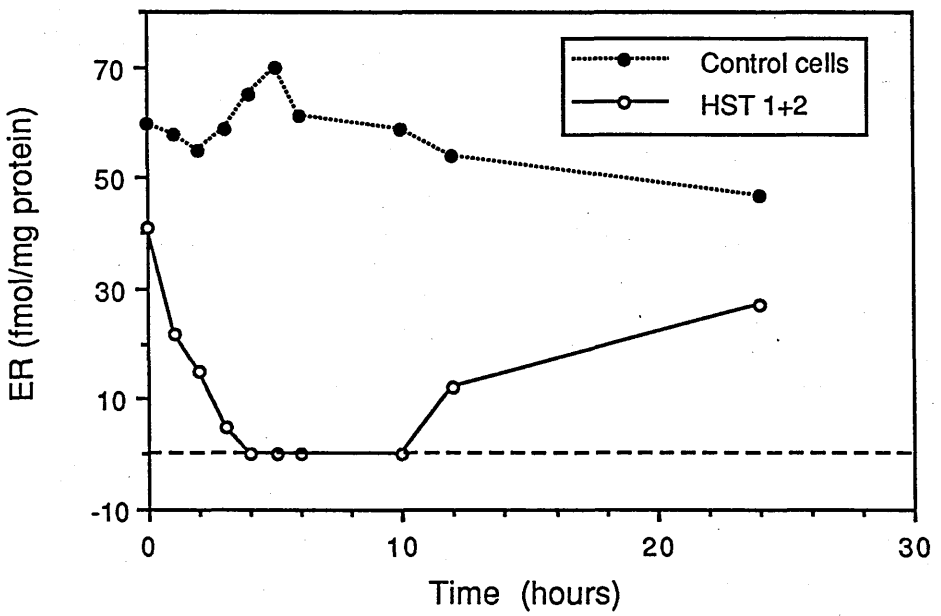
METHODS.

In order to investigate the transience of the observed abolition of MCF-7 cell oestrogen-binding capacity, the experiment was repeated in such a manner as to allow sampling of the ER-binding ability at different time points immediately after (during recovery from) heat shock. To allow sampling, cells were subcultured and allowed to form monolayers of 20% confluence in twenty 25cm^2 flasks. Ten of these were subjected to HST 1+2, while the remainder were maintained as 37°C controls throughout. Time zero (T_0) was taken as the time upon removal of the flasks from the second elevated temperature - the beginning of the second recovery period. Cells were harvested as described in the previous experiment at ten time intervals from T_0 to 24 hours later; a control flask being harvested in conjunction with each heat treated flask. The "two-point competition" ER-assay was carried out in triplicate for each flask (see METHODOLOGY section 3.1.4.). Protein was again determined by the method of Bradford as described in METHODOLOGY section 4.1.

FIGURE 12. **The Kinetics of Thermal Inhibition of MCF-7 Cell Oestrogen Binding Capacity.**

The amount of "available" ER in cells at different time points immediately commencing recovery from heat shock (T_0). Each point is the mean of three wells. Capacity to bind [^{3}H]-oestradiol was determined by the two-point competition assay.

The Kinetics of Thermal Inhibition of MCF-7 Cell Oestrogen Binding Capacity.



RESULTS.

FIGURE 12. shows the effect of heat on the oestrogen-binding capacity of MCF-7 cells. This experiment was only carried out once, although each data point represents the mean of triplicate ER assay of each set of heat shock-treated cells. This time course study would have benefited from a longer total sampling period, perhaps up to 48 hours; however this is logistically impossible as the experiment dictates that cells are growing continuously for almost 72 hours, by which time full confluence has been reached and ER content is one of many parameters which are altered in the switch from growing to confluent cells.

CONCLUSIONS.

The results of the first experiment in this chapter (4.1.) show the dramatic effect of heat on the oestrogen-binding ability of MCF-7 cells (TABLE 1.). It can be seen that the amount of ER present in the control cells slumps to a level below the detection limit of the assay as a result of heat shock. The value obtained for the observed concentration of ER in the heat shocked cells is actually less than the standard deviation of the data. This is because in ten out of the twelve assays carried out the amount of ER present was lower than the ligand-binding assay could detect; and so a value of zero was obtained. The two positive results cannot be discounted though it is possible that the large number of tissue culture flasks being used meant that not all flasks received the full heat treatment.

In view of these results it was decided to investigate the kinetics of this almost total abolition of MCF-7 cell oestrogen-binding ability by heat shock (4.2.) The results shown in FIGURE 12. illustrate the time dependent abolition of ER-binding ability by heat. Interestingly, there is a delay of approximately 3 hours between the end of thermal shock and the loss of measurable ER ligand-binding, suggesting that there is some mechanism involved that is initiated by heat, but is not dependent on the continued presence of heat.

ER only becomes redetectable by ligand binding assay 10 -12 hours after returning the cells to 37°C. Noticeably, the cells in experiment 4.1. were assayed after 12 hours at 37°C, suggesting that we may have been observing the cells at the point where the inhibitory effect was in remission. This is another possible explanation for the two positive values that appear with the ten zero values of ER for the heat shocked cells in experiment 4.1.

The concentration of ER present in the control cells seems to tail-off after approximately 12 hours of sampling. However, this represents the point when the faster growing control cells are approaching 100% confluence and ER are normally down-regulated.

What is most interesting about this data (FIGURE 12.) is that the kinetics of ER-binding inhibition mirror the synthesis of hsp's (CRAIG *et al*, 1985). This tentatively suggests that the effect of heat could be an indirect inhibition of ER-binding ability through its stabilising-association with excess hsp 90.

CHAPTER 5.

The Influence of Elevated Temperatures on Oestrogen Receptor Levels in MCF-7 Cells, as Determined by Enzyme-immunoassay.

INTRODUCTION.

The results of the experiments described in the previous chapter suggest that heat causes a temporary abolition of oestrogen-binding capacity in MCF-7 human breast cancer cells. Furthermore, the effects of temperature are time dependent and mimic the kinetics of hsp synthesis (CRAIG *et al*, 1985). It was decided to investigate how the transient increase in temperature could modulate ER concentration determined by immuno-assay rather than by ligand-binding ability.

This is because among potential mechanisms of heat-induced regulation of oestrogen-binding; temperature could have effects on 1) the amount of ER, or 2) on the binding ability of ER.

To distinguish between these two pathways it was decided to use an immunological assay to detect the levels of ER after heat shock. This type of assay is independent of either the DNA- or ligand-binding functions of steroid receptors and so is ideally suited to answer the questions raised by the previous experiments. (Assuming that the antibody is not directed against an epitope involved in the steroid-binding process).

METHODS.

This experiment was designed so that the cells being investigated were treated exactly as those of Experiment 1 of the previous chapter; that is to say that control cells and cells subjected to HST 1+2 were both assayed for ER. In addition, a set of cells were grown in the presence of 50nM cycloheximide during the second recovery period so that the normal turnover of ER could be taken into account.

The assay used to immunologically detect ER was a commercially available enzyme-immunoassay (EIA) from Abbott Laboratories (see METHODOLOGY section 3.2.). This is a relatively simple sandwich assay used routinely to determine the levels of ER in breast tumour biopsies. Protein values were again estimated by the method of Bradford (METHODOLOGY section 4.1.).

RESULTS.

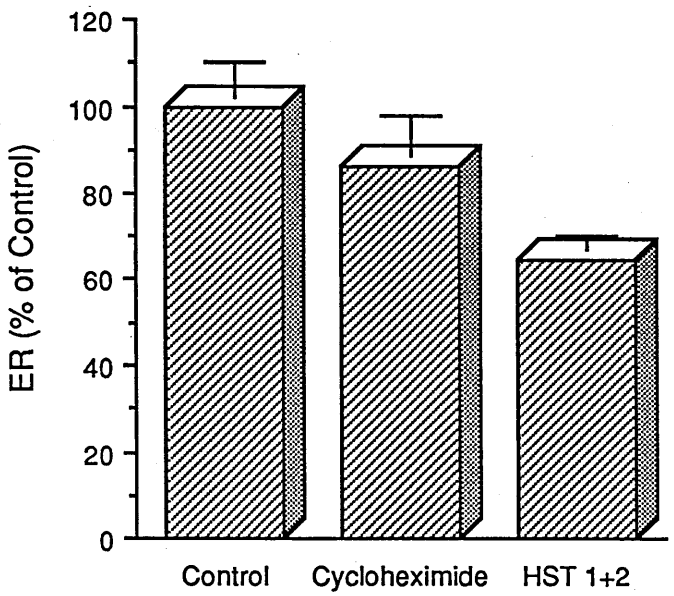
The results of the ER-EIA are shown on FIGURE 13. These results represent the mean of three experiments (three wells for each condition in each experiment), each of which was assayed in duplicate by Abbott Laboratories ER-EIA kit.

FIGURE 13. **The Effect Heat Shock on MCF-7 Cell Oestrogen Receptor.**

This FIGURE shows the effect of heat shock on MCF-7 cell ER as assayed using Enzyme-immunoassay kit (Abbott). Cells were harvested after the second 12 hour recovery period.

Error bars represent the Standard Deviation of the data.

The Effect of Heat Shock on MCF-7 Cell ER.



CONCLUSIONS.

FIGURE 13. demonstrates that the effects of heat on ER-binding ability must be independent of the absolute number of ER present, as approximately two-thirds of the ER remain after heat shock. The ligand-binding assay predicts that there should be virtually no detectable ER 12 hours after heat shock; however the EIA demonstrates that at this time 67% of ER remain. The difference between these two assays is the former relies on ligand-binding ability, whereas the latter is an immunological detection of ER protein. The implication therefore is that heat shock has minimal effect on the levels of ER protein, but instead directs its inhibition at the oestrogen-binding function of the receptor.

The cells incubated with cycloheximide show a significant (16%) loss of ER protein, although not as great as the heat shocked cells. Heat shock is known to halt normal protein synthesis at the same time as enhancing hsp synthesis (BURDON, 1986; see INTRODUCTION section 3.2.4.). No doubt this explains the 16% part of the 33% reduction in heat shocked ER due to the inhibition of protein synthesis. It does seem therefore that there is some additional, more direct action of heat on ER levels; protein denaturation for example.

In conclusion, it seems that although the effect of heat shock is to partially decrease the amount of immunologically detectable ER; heat shock does not reduce the amount of ER protein by the amounts predicted by the loss of ligand binding ability described in the preceding chapter. This suggests that hsp 90 may specifically block ligand binding by ER.

CHAPTER 6.

Hsp 90 Immunocytochemical Localisation.

INTRODUCTION.

Hsp 90 is an abundant protein comprising between 1% and 2% of total soluble protein (LEES-MILLER & ANDERSON, 1989a). However there have been few immunocytochemical studies describing the intracellular distribution and localisation of hsp 90 either *in vitro* or *in vivo*. It was decided to investigate the subcellular distribution of hsp 90 and to observe any variation in the distribution occurring as a result of heat shock, as this may aid in an interpretation of the effects of heat shock on the oestrogen-binding ability of ER.

This type of immunocytochemical study has successfully been carried out for other HSP's, notably hsp 70, in different cell lines (RIABOWOL *et al*, 1988; WELCH & SUHAN, 1986). These investigators observed both an induction and also a nuclear localisation of hsp 70 in response to heat shock. Little information has been obtained for hsp 90.

Indirect fluorescent immunocytochemical staining was used to visualise hsp 90: this procedure involves detection of an appropriate monoclonal antibody with a fluorescein isothiocyanate (FITC)-labelled, species-specific, secondary antibody. When the fluorescent label attached to the secondary antiserum is exposed to the excitatory ultra-violet light generated by a fluorescent microscope, the label emits visible green light (λ = 450nm).

METHODS.

MCF-7 cells were subcultured so that a 20% monolayer was formed in the culture vessels and treated either as 37°C controls or as HST 1+2 (double heat shocked) cells. The vessels used were commercially available microscope slides that come complete with a detachable upper chamber forming eight 25mm^2 chambers (LabTek® Chamber Slides, Nunc Inc.). These slides (see FIGURE 14.) are invaluable tools to assist in the staining of cell monolayers as they allow concurrent staining of different antibodies on the same slide, and in addition require minimal amounts of antisera to ensure adequate staining.

The complete method of fluorescent immunocytochemical staining is described in METHODOLOGY section 6.4.2.; while the method used to prepare a suitable FITC-labelled secondary antibody is described in METHODOLOGY section 6.4.1. The primary serum used to detect hsp 90 was mouse monoclonal antibody AC 88, diluted 1:500 times. This was visualised using an FITC-labelled sheep anti-mouse serum also diluted 1:500 times, Hoechst 33258 being used as a nuclear counterstain.

FIGURE 14. **The 8-Well LabTek Chamber Slide.**

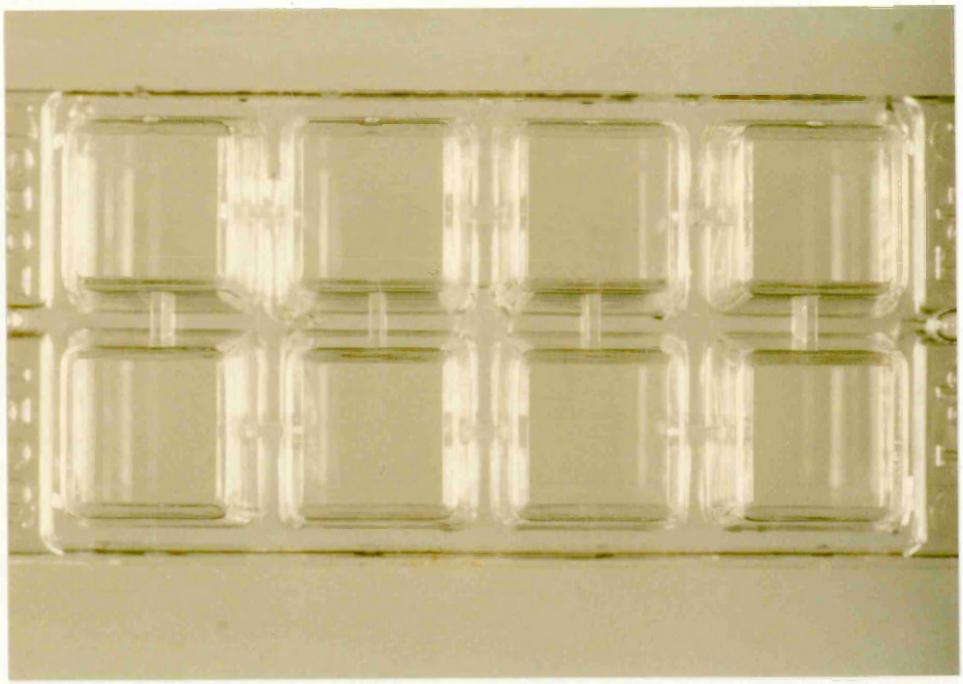
This photograph shows the piece of equipment used to grow cells for immunocytochemical assay.

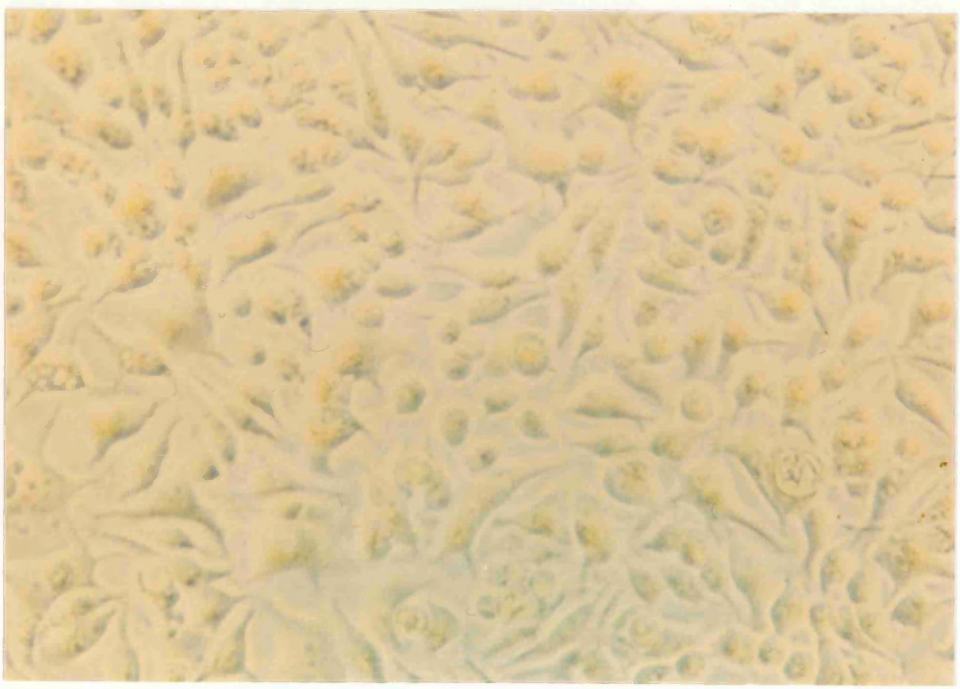
The dimensions of the glass slide are 25mm × 63mm; the individual wells had a surface area of 36mm^2 and had an optimum capacity of 0.5 ml.

FIGURE 15. **MCF-7 Cell Morphology.**

This photograph shows the typical "cobble-stone" appearance of the MCF-7 epithelioid breast cancer cell line.

These cells were photographed upon achieving 100% confluence. Phase contrast microscopy was used with the culture medium being replaced with PBS-A.





RESULTS.

FIGURE 15. shows the normal morphology of the MCF-7 breast cancer cells growing as a confluent monolayer and visualised using standard phase-contrast microscopy (120×); note the typical "cobble-stone" appearance of this epithelial cell line.

Each FIGURE is represented as a pair of micrographs, a. and b. Respectively, these show the Hoechst nuclear counterstain (blue) and the FITC-fluorescence (green) of the same microscope field. This allows assessment of the hsp 90 staining in terms of both cytoplasmic and nuclear localisation. All photographs of immunofluorescence were taken using a microscope magnification of ×400. The results can be summarised in the following figures:

FIGURE 16b. shows the nonspecific FITC staining, ie. staining in the absence of primary antibody AC 88.

FIGURE 17b. shows the specific FITC-staining of hsp 90 in MCF-7 cells cultured constantly at 37°C.

FIGURES 18b. and 19b. show the specific FITC-staining of hsp 90 in heat shocked (HST 1+2) MCF-7 cells.

Taken together these four figures indicate a pronounced effect of heat shock on hsp 90 intracellular localisation.

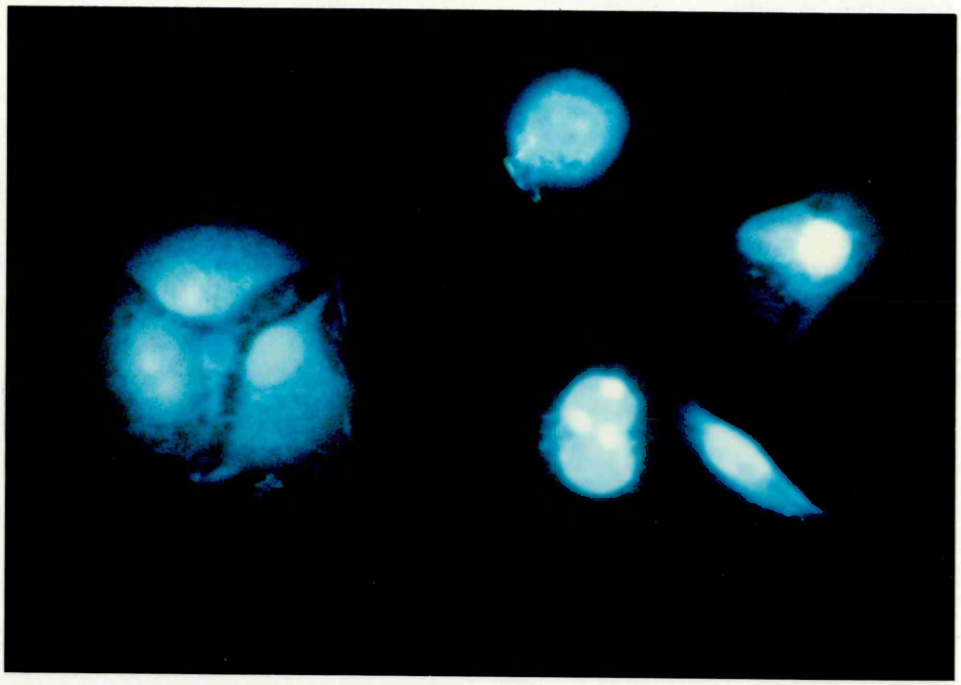
FIGURE 16. **Fluorescent Immunocytochemical Localisation of hsp 90 in MCF-7 Cells.**

FIGURE 16b. shows the FITC-fluorescence contributed by nonspecific staining. Primary anti-hsp 90 antibody was omitted from the staining proceedure.

FIGURE 16a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 16b.

(Microscope magnification ×400 in both photographs)

a



b

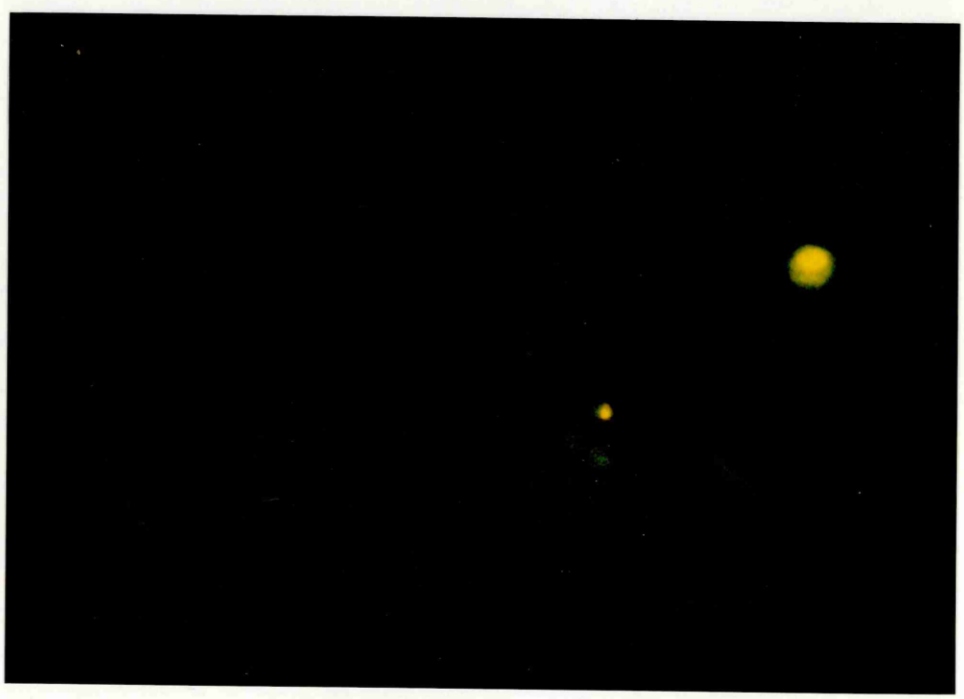


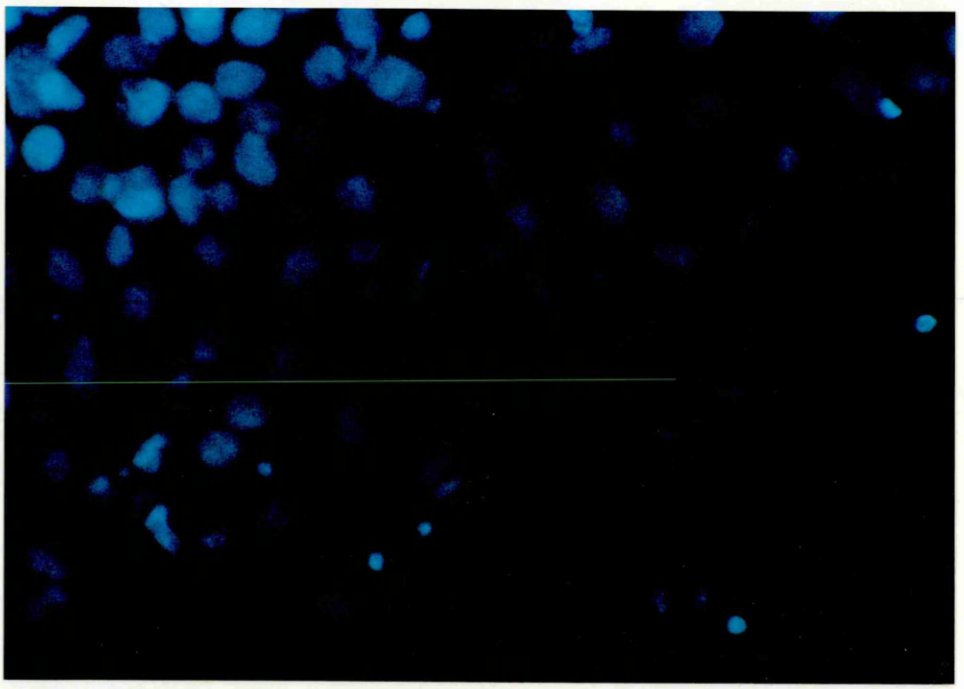
FIGURE 17. **Fluorescent Immunocytochemical Localisation of hsp 90 in MCF-7 Cells.**

FIGURE 17b. shows the specific anti-hsp 90 FITC-fluorescence of cells grown at 37°C throughout (HST 0).

FIGURE 17a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 17b.

(Microscope magnification ×400 in both photographs)

a



b

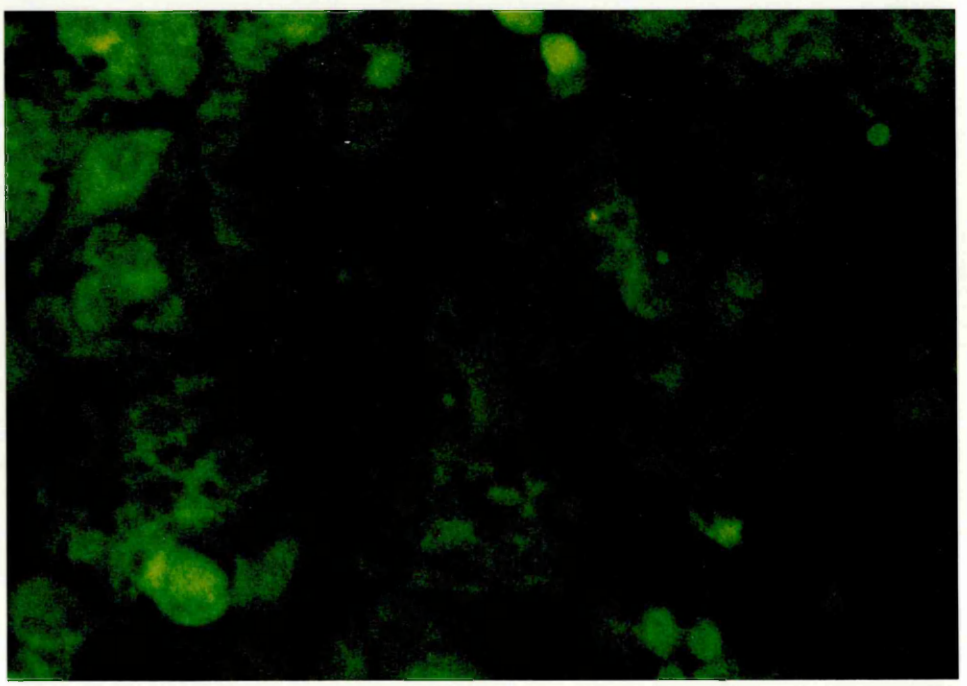


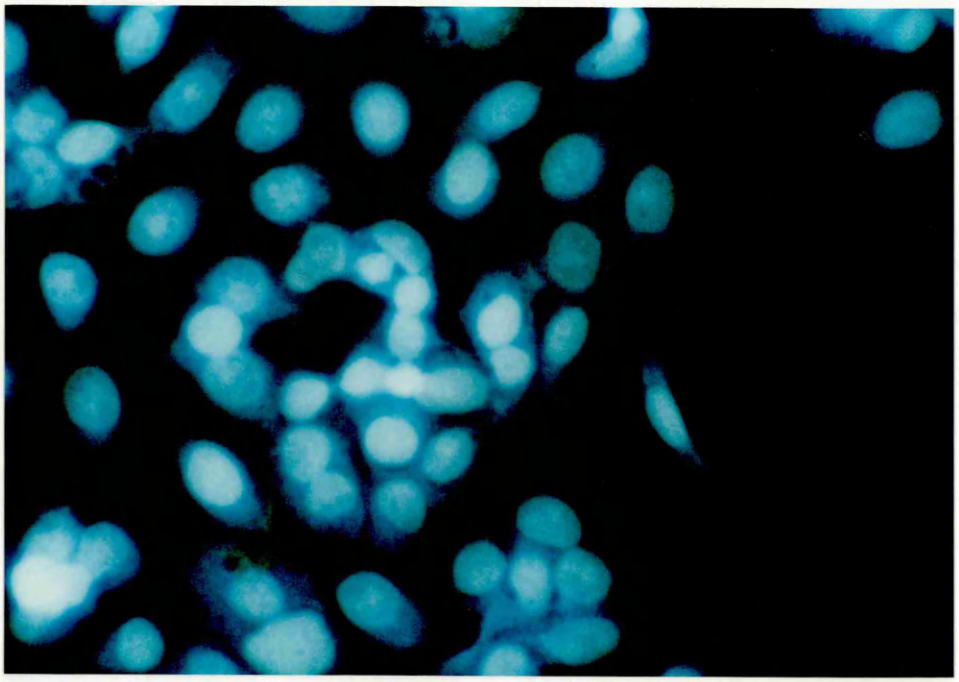
FIGURE 18. **Fluorescent Immunocytochemical Localisation of hsp 90 in Heat Shocked MCF-7 Cells.**

FIGURE 18b. shows the specific anti-hsp 90 FITC-fluorescence of cells subjected to HST 1+2 (double heat shock).

FIGURE 18a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 18b.

(Microscope magnification ×400 in both photographs)

a



b

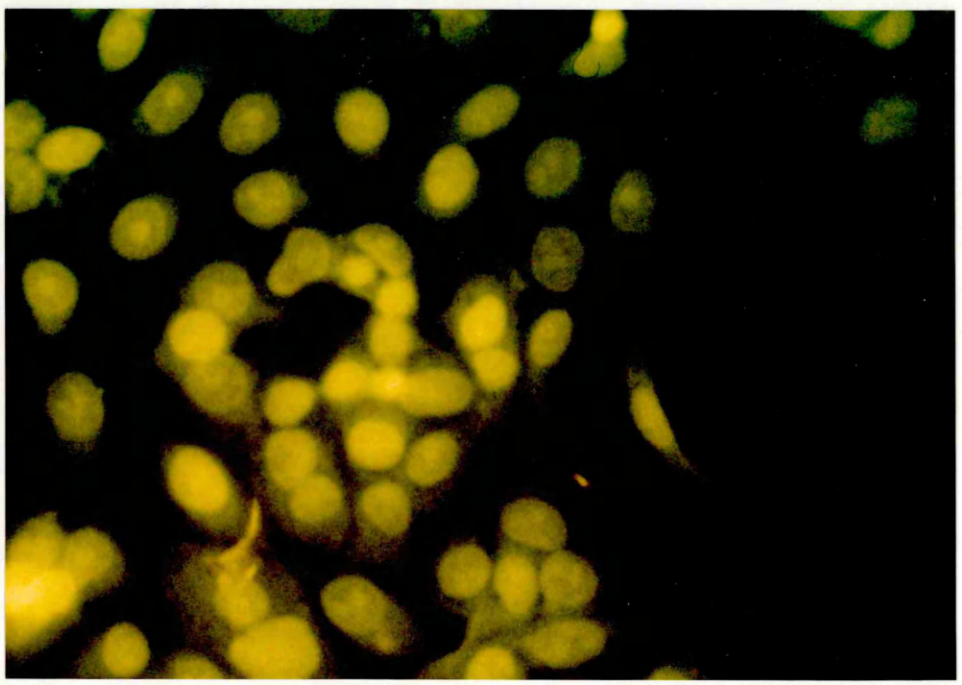


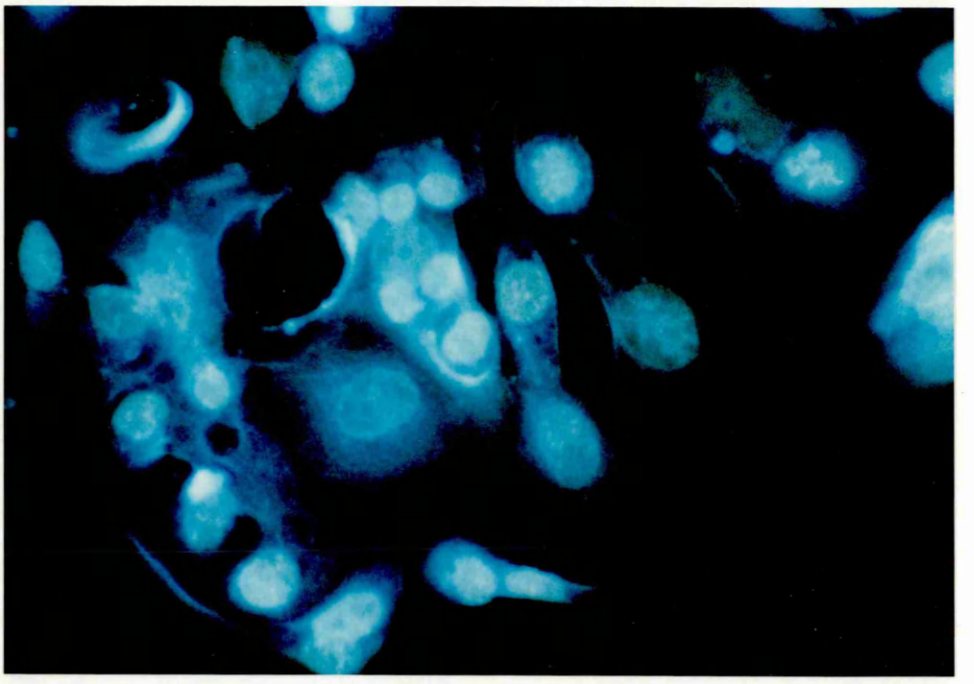
FIGURE 19. **Fluorescent Immunocytochemical Localisation of hsp 90 in Heat Shocked MCF-7 Cells.**

FIGURE 19b. is a neighbouring field from the the specific anti-hsp 90 FITC-fluorescence of cells subjected to HST 1+2 (double heat shock) as the cells shown in FIGURE 18.

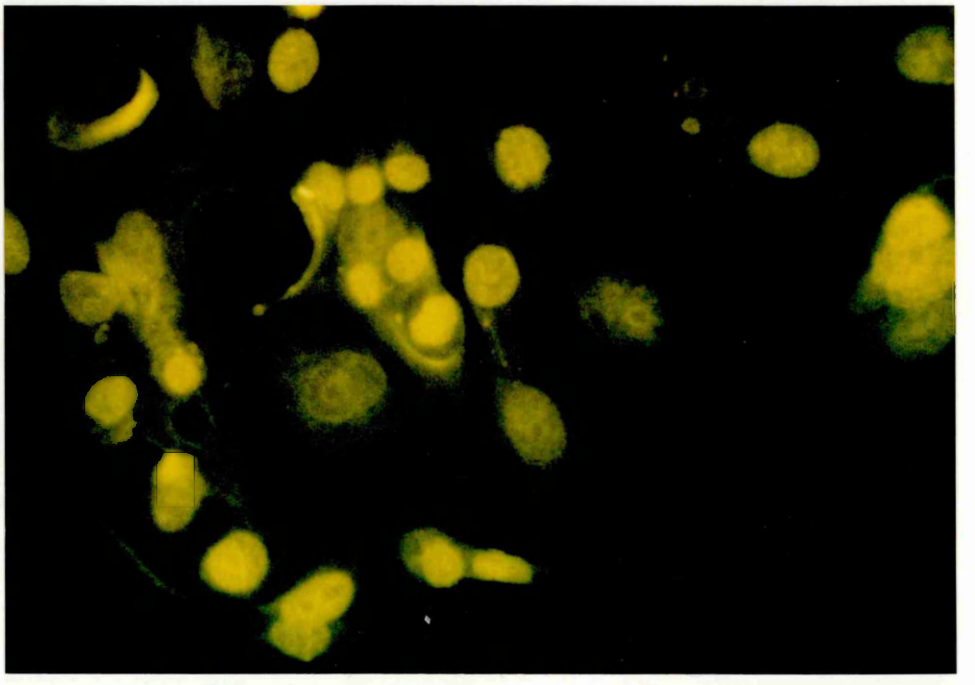
FIGURE 19a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 19b.

(Microscope magnification ×400 in both photographs)

a



b



CONCLUSIONS.

The technique of fluorescent immunocytochemical staining is a reliable method of staining cell monolayers and so is a valuable research tool. Best results were obtained when the cells were 50-75% confluent, as staining observed from clusters of cells is extremely difficult to interpret.

As can be seen looking at FIGURES 16-19. the staining is highly specific. There is virtually no fluorescence from the cells incubated with only secondary serum, the nonspecific-binding controls (FIGURE 16b.)

These photographs reveal some very interesting properties of hsp 90. At 37°C hsp 90 seems to be located in a broadly cytoplasmic environment although there is some definite nuclear staining (FIGURE 17b.) Heat shock has two main effects: firstly, the general level of fluorescence is enhanced (FIGURES 18b. & 19b.). This is not surprising, since the amount of hsp 90 present was shown to be upregulated by heat in EXPERIMENTAL chapter 3.

The second obvious effect of heat is the distinct nuclear localisation of hsp 90 in the heat shocked cells (FIGURES 18b. and 19b.). This type of heat-induced hsp nuclear localisation has been previously noted for hsp 70 in heat shocked fibroblast cells (RIABOWOL *et al*, 1988), yet this seems to be the first report of any hsp 90 nuclear translocation. There also seems to be some plasma membrane association of hsp 90 which becomes enhanced after heat shock. This type of staining has never been reported for hsp 90 although other workers have suggested that it may be present as part of the plasmamembrane superstructure in an analogous manner to spectrin (NISHIDA *et al*, 1986).

CHAPTER 7.

Coimmunoprecipitation of hsp 90 & ER.

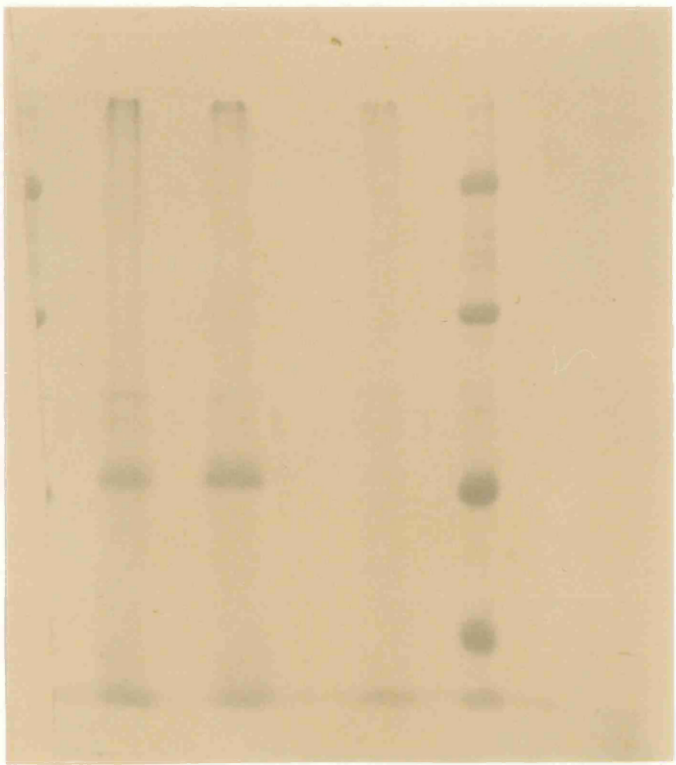
INTRODUCTION.

There are many investigations into the association between hsp 90 and steroid receptors (see INTRODUCTION section 4.) using a variety of different techniques. Many of these reports however, do not fully address the problem of nonspecific interaction of the two proteins. It was decided to attempt to immunoprecipitate ER using an anti-ER monoclonal antibody, and then use Western immunoblotting to try and identify hsp 90 present in the immunoprecipitate. If hsp 90 was detectable, it would add to the evidence that suggests that the interaction of the two proteins is a true *in vivo* complex, and not an *in vitro* artifact.

METHODS.

MCF-7 cells ($300cm^2$) were harvested as described in *PART 1* EXPERIMENTAL chapter 3. Immunoprecipitation was carried out on the extract of these cells as described in this previous chapter using anti-ER monoclonal antibody ERP-31 diluted 50 times (METHODOLOGY section 6.1.1.). The immunoprecipitate pellet was run out on a 7.5% PAGE gel as described in METHODOLOGY section 5.1. and the gel was used to transfer the protein products to nitrocellulose (see METHODOLOGY section 5.4.3.). This nitrocellulose was subjected to immunoblotting (METHODOLOGY section 5.4.5.) using anti-hsp 90 monoclonal antibody AC 88.

FIGURE 20. **Nitrocellulose Blot of Immunoprecipitated ER.**
This photograph shows the nitrocellulose blot stained using amido black to reveal protein transferred from an SDS-PAGE gel used to resolve immunoprecipitated ER.


MR.≈64

RESULTS.

FIGURE 20. is a photograph of the nitrocellulose blot of the ERP-31 immunoprecipitate stained using amido black as described in METHODOLOGY section 5.4.5. Unfortunately, no positive results were obtained when AC 88 immunoblotting of this nitrocellulose was carried out (data not shown).

CONCLUSIONS.

The lack of positive hsp 90 immunoblotting on the nitrocellulose blot of the ER immunoprecipitate could be attributed to a variety of causes; including the possibility that there is no association between hsp 90 and ER. Discounting this reason purely on the grounds that it is at odds with the overwhelming body of the literature, the most obvious reason for failure is the experimental design itself. It is possible that anti-ER antibody immunoprecipitation results in a highly purified form of ER that is devoid of any hsp 90. This is in fact reflected in the single band of Mr ≈64,000 present on the nitrocellulose blot. One possible reason for this type of immunopurification could be that the antibody is specific for activated ER, and so may recognise a site on the ER protein that is normally masked by hsp 90. This would mean that it would be impossible to immunoprecipitate any 8-10S heteromeric complexes incorporating hsp 90.

Another reason for the lack of reactivity of AC 88 is that it may not recognise hsp 90 on the nitrocellulose blot, though AC 88 has previously

been shown to be capable of efficient immunoblotting of hsp 90 during Western blotting (D.O. Toft, *personal communication*).

This experiment was repeated on numerous occasions without success. When the antibodies were reversed so that ERP-31 immunoblotting of AC 88-immunoprecipitated hsp 90 was attempted there was still no positive staining to show the presence of ER in the hsp 90 immuno-precipitate. However, these disappointing results do not distinguish between an incorrect model of ER and hsp 90 association and inappropriate experimental design. Indeed, the possibility that the antibody only recognises activated ER should be further explored. Conversely, AC 88 may have precipitated the ER-hsp 90 complex but the techniques used failed to dissociate the complex to reveal the anti-ER antibody epitope on the ER.

DISCUSSION.

The overall aim of these studies was to gain some understanding of the functional relationships existing between hsp 90 and steroid hormone (oestrogen) receptors. This association represents a link between two very distinct families of proteins, each of which is of vital cellular importance.

As one approach to this question of the association of the two proteins, it was decided to investigate the modulation of oestrogen receptor activity by heat in breast cancer cells. This represents a novel approach, as researchers have almost exclusively studied endocrine effects on the hsp 90-steroid receptor association, as opposed to the effects of thermal parameters. Yet thermal aspects of breast cancer have received recent attention (McCURDY, *personal communication*) and thermal therapy of both prostate cancer and benign prostatic hyperplasia is also gaining ground (See*Part 2* of this thesis).

1. THERMAL KILLING OF MCF-7 CELLS.

It was important to characterise the normal range of thermal-tolerance displayed by MCF-7 human breast cancer cells. This allows the definition of a titration point which can be used during the designation of either thermotolerance or thermo-*in*- tolerance of cells subjected to different temperature regimes. The conclusion drawn from the results of *Part 1*, EXPERIMENTAL chapter 1. is that MCF-7 breast cancer cell growth is inhibited by temperatures greater than 42°C.

There has been a great deal of work on the effects of temperature on the growth of both normal and malignant cells, due mainly to the intense interest in thermal-therapy of various neoplasms such as carcinoma of the colorectum, prostate and cervix (PILEPICH *et al*, 1987). Effective treatment of these tumours is possible because their accessibility allows efficient heat transfer from external apparatus. For this reason thermal-therapy has not been used extensively to suppress the growth of more deep-seated tumours, such as breast cancer (DEWHIRST *et al*, 1987). The great advantage of this approach, if successfull in a definable group of patients, is that surgery and the associated complications may be avoided in this group of patients.

This value obtained for MCF-7 cell temperature lethality is in agreement with the levels of heat lethality (42.5°C) demonstrated by a wide variety of normal tissues such as heart and liver (FIELD, 1987), but it is approximately 2°C lower than the critical lethal temperature of those malignant tissues studied. This does not fit in with the general hypothesis that reproductive cancer cells are more heat-sensitive than their normal counterparts. This may be explained by the fact that Field *et al* were using intact tissues and not monolayer cell cultures, where heat conduction is inherently poorer, and so higher temperatures may be required to "penetrate" the tumour mass. In addition, these figures are only valid when a common reference exposure time is chosen.

The problem of the time and temperature relationship during heat shock -

the "isoeffect dose" - means that it is difficult to compare the work of different authors (SAPARETO, 1987). When the log of exposure time (in minutes) is plotted versus the exposure temperature (°C) for the equivalent thermal effect (isoeffect) then a straight line is produced between 42°C and 46°C for most tissues. However, thermotolerance heating (prior exposure to sub-lethal temperatures) and step-down heating (prior brief exposure to lethal temperatures) both negate this linear relationship (FIELD, 1987). For this reason it was decided to maintain a constant time of heating throughout all exposures.

2. MCF-7 CELL THERMOTOLERANCE.

The cumulative aim of these studies was to assess the effects that varying the population of intracellular hsp 90 had on the oestrogen-binding capacity of MCF-7 cells. By definition, simple heat shock is the obvious method of upregulating the levels of hsp's; hsp 90 in particular has been demonstrated to be extremely heat inducible (CATELLI,1985). Alternatively, it has been shown that the acquisition of thermotolerance is accompanied with the overproduction of hsp's (LINDQUIST, 1986) when compared to heat shock alone. In addition, the surviving cell population is considerably more viable.

Thermotolerance is defined as the acquisition of cellular resistance to lethal temperatures by prior exposure to sub-lethal elevated temperatures (CRAIG, 1985). Mammalian fibroblasts in monolayer cell culture have been shown to become thermotolerant to lethal temperatures as a direct result

of a prior mild heat shock followed by a 12 hour recovery period (WELCH & SUHAN, 1986). Correspondingly, these double heat shocked cells accumulated hsp 70 at a faster rate and demonstrated higher overall hsp 70 levels than their single heat shocked equivalents. This double step induction has been cited as a possible cellular mechanism for ensuring efficient stress protection during cellular "siege" situations (YOST & LINDQUIST, 1986).

In order to vary the amount of hsp 90 in the MCF-7 cell-line, it was decided to exploit the double heat shock-thermotolerance reaction of these cells. As demonstrated by the results shown in *Part 1*, EXPERIMENTAL chapter 2., MCF-7 cells subjected to 44°C fail to undergo any significant growth subsequent to the exposure to this lethal temperature. This observation confirms the result of *Part 1*, EXPERIMENTAL chapter 1. when 42°C was identified as the watershed in terms of cell survival. However, cells exposed to an initial heat shock of 41°C followed by a 12 hour recovery prior to 44°C exposure show growth rates comparable with those of the cells maintained at 37°C. It is therefore concluded that the thermotolerance response was induced by the initial mild heat treatment, allowing these MCF-7 cells to withstand a temperature known to be lethal to unprimed MCF-7 cells.

3. THERMAL INDUCTION OF hsp 90.

As previously mentioned, hsp 90 has been shown to be a thermally inducible protein (CATELLI *et al*, 1985) and its gene promoter is known to contain the appropriate heat shock response element that confers

heat-inducibility on hsp 90 transcription (REBBE *et al*, 1989). The results of *Part 1*, EXPERIMENTAL chapters 1. & 2. suggested that the MCF-7 cell line demonstrates the thermotolerance response, and this response is known to be accompanied by the overproduction of hsp's (WELCH & SUHAN, 1986). This inferred that there existed a non-endocrine method of increasing the amount of hsp's present within these cells. However, it was decide to investigate whether the instigation of the thermotolerance response was accompanied by an upregulation in the levels of hsp 90 in particular.

The results of *Part 1*, EXPERIMENTAL chapter 3 demonstrate that there is a small, yet measurable increase in the levels of immunoprecipitatable hsp 90 in MCF-7 cells subjected to the thermotolerance-inducing double heat shock treatment. The amounts of protein immunoprecipitated are very low, and with hindsight a more sensitive experimental protocol should have been used. However, even with the limited amounts present, the resolution is sufficient to demonstrate the heat-inducibility of hsp 90 during thermotolerance-induction. (Assuming an equal proportion of hsp 90 was immunoprecipitated in each case.)

These results are extremely interesting in the light of a recent report suggesting that hsp 90 may play a pivotal role in the regulation of normal protein synthesis during heat shock (ROSE *et al*, 1989). Hsp 90 is involved in the shut-down, and subsequent return, of translation of normal protein mRNA during heat shock. The variation of intracellular hsp 90 during heat shock may represent both a regulator and a product of the pattern of hsp-induction.

Apart from heat, it is possible to increase the levels of hsp 90 using oestrogens (RAMACHANDRAN *et al*, 1988); although the experiment described here has shown that the levels of hsp 90 in MCF-7 cells can be upregulated in a non-endocrine manner while still retaining a viable population of cells. In addition, for the purposes of this study of the relationship of hsp 90 and ER, it would not be relevant to use oestrogen to upregulate hsp 90, since oestrogen is known to rapidly modulate ER levels in oestrogen-sensitive cells.

4. THERMAL INHIBITION OF OESTROGEN-BINDING.

The results of *Part 1*, EXPERIMENTAL chapters 4. & 5. demonstrate the contrasting effects of heat shock on both the amount of immuno-detectable ER, and on ER binding ability. Heat shock results in an almost total abolition of the oestrogen-binding capacity of the MCF-7 cells. However, since the actual amount of ER recognised by the anti-ER antibody kit was only reduced by approximately 33% it is apparent that some mechanism is reducing the oestrogen-binding capacity of ER in heat shocked MCF-7 cells.

There is only one prior report of thermal modulation of ER activity *in vivo*. This unique observation (WOLFFE *et al*, 1984) reported the abolition of oestrogen- activated transcription of vitellogenin mRNA by heat shock. Interestingly these workers also noted that heat shock resulted in a loss of biochemically detectable ER, and a corresponding accumulation of hsp's. The observations of Wolffe*et al* are remarkably similar to the data

presented within this thesis, although this group did not use immunological methods to detect ER.

Since knowledge of the association of hsp 90 with the steroid receptors was not widespread at the time of this previous report (WOLFFE *et al*, 1984) the investigators did not comment on the possibility of an hsp 90-mediated suppression of ER-binding ability. However, when these earlier observations are taken into account with those reported in *Part 1*, EXPERIMENTAL chapters 4., 5. & 6. they may be interpreted to indicate that, after heat shock, nonactivated-nuclear receptor remains although steroid-binding is in some way inhibited by excess and/or relocated hsp 90.

5. COULD hsp 90 MEDIATE A HEAT SHOCK-INDUCED SUPPRESSION OF OESTROGEN-BINDING CAPACITY?

The difference between the results of ligand-binding ER-assay and immunological-ER assay suggest some functional modification of the heat shocked ER. Therefore, the distinction must be made between the levels of total receptors and the levels of available receptors. The mechanism behind this functional modification is unclear, though the heat-induced nuclear localisation of hsp 90 as visualised by immunocytochemistry (*Part 1*, EXPERIMENTAL chapter 6.), could be responsible for some blocking mechanism. (see FIGURE 3.)

This supposition is based on the work of Baulieu, who has produced a model for antisteroid action. He proposes that the inhibitory action of

antisteroids is due to the production of nonactivated receptors; created as a result of antisteroid-binding producing a heteromeric receptor that is incapable of undergoing activation (BAULIEU, 1987a & 1987b). This functionally inactive receptor consists of steroid-binding subunits with their DNA-binding sites masked by tightly bound hsp 90 subunits; locked together as a result of antihormone-binding.

The antisteroidal theory of Baulieu does not entirely agree with the data concerning the binding of the antioestrogen tamoxifen (HAM & PARKER, 1989); the antiprogestin RU-486 (GUICHON-MANTEL, 1988) and other antisteroids. There is evidence that antisteroid-binding is able to produce an activated receptor that is capable of DNA-binding (tamoxifen-ER has partial agonist activity and can induce PR-synthesis), although it seems that RU-486 binding to PR is non-productive and incapable of initiating trans-activation (RICHARD-FOY *et al*, 1987). It must therefore be concluded that the model of Baulieu is not entirely correct as regards antisteroid activity, yet it does provide an interesting concept for theorising the function of hsp 90.

Leaving aside the action of antisteroids *per se*, it remains highly likely that hsp 90 has a role to play in the regulation of receptor activity. Applying the observations reported within *Part 1*, EXPERIMENTAL chapters 4., 5. & 6. to a modification of Baulieu's model, it suggests that heat shock may induce a significantly increased amount of nuclear located hsp 90 which may then suppress the binding ability of the remaining nuclear ER by affecting the

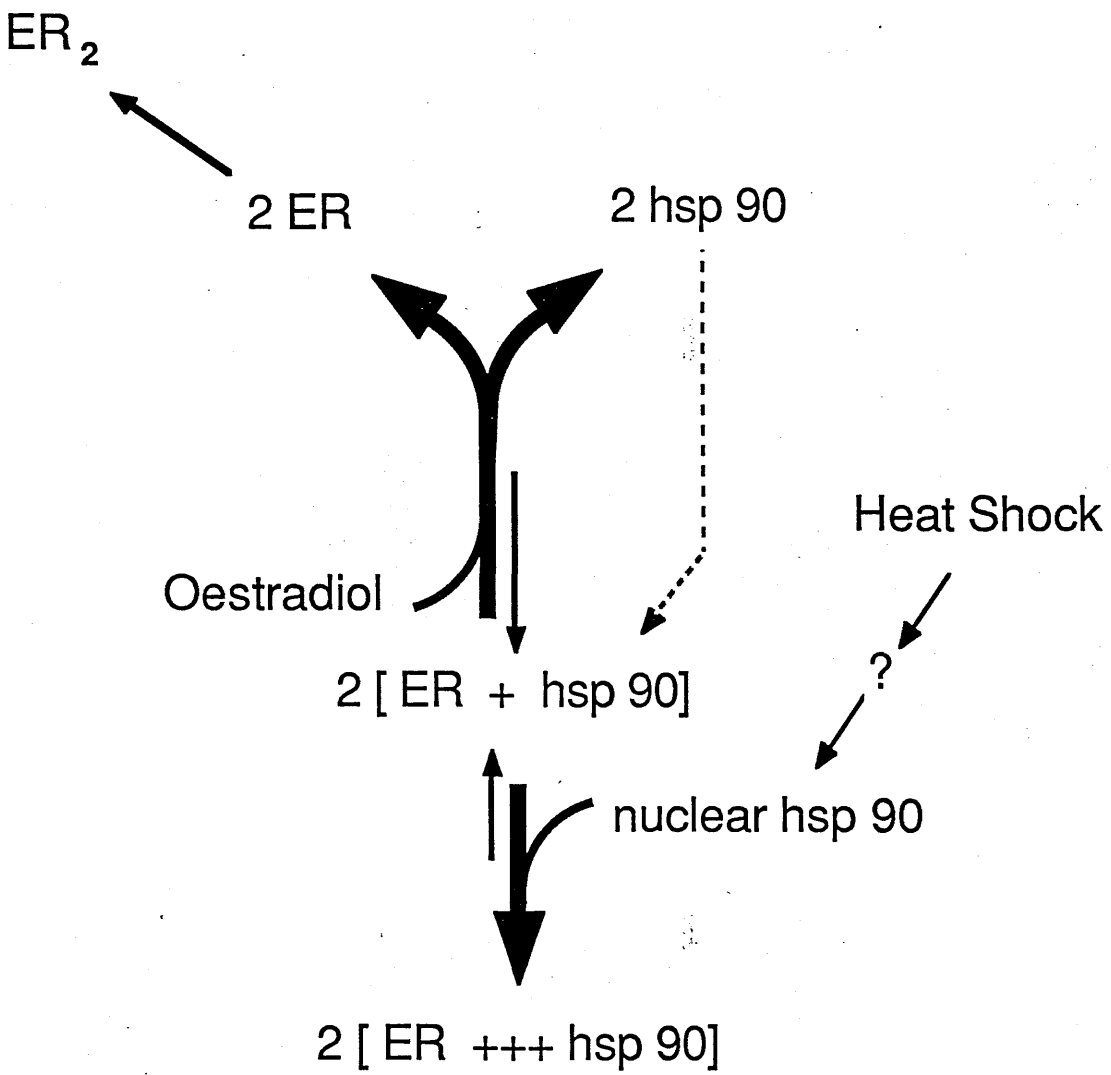
dissociation kinetics of the heteromeric nonactivated receptor. In this sense, excess nuclear hsp 90 is promoting the nonactive form of nuclear ER, in a manner analogous to the antisteroidals of Baulieu's model. (see FIGURE 40.)

It is unclear how this suppression of ER binding function could occur; although there exist a variety of possible scenarios:

Firstly, it is possible that the excess of available hsp 90 present within the heat shocked nucleus could inhibit the ligand-induced dissociation reaction during receptor activation. This argument is based on the assumption that the greater concentration of free hsp 90 would result in altered dissociation kinetics of the ER-hsp 90 complex during receptor activation; and so promote the nonactivated form of the receptor.

Alternatively, hsp 90 may compete directly with DNA for binding of activated receptors. This type of activity has been observed *in vitro.* Hsp 90 has no affinity for DNA itself although it has been shown to compete with activated glucocorticoid receptor for DNA-binding (HOWARD & DISTELHORST, 1988b). Hsp 90 inhibition was specific to GR as it had no effect on the binding of other proteins to DNA. However, only non-specific DNA-binding was investigated in this study, and not specific GR DNA-binding sites.

FIGURE 40. **Possible hsp 90-mediated suppression of MCF-7 cell ER binding capacity following heat shock.**
This figure schematically shows a possible mechanism where excess nuclear hsp 90, present as a result of heat shock, could result in a suppression of oestrogen-binding ability. (See text for details)


ER2
2 ER
2 hsp 90
Heat Shock
Oestradiol
?
2 [ER + hsp 90]
nuclear hsp 90
2 [ER +++ hsp 90]

One possible model is that hsp 90 may mediate the passage of steroid receptors from the polysome through the nuclear membrane. This type of shuttle function has been demonstrated for hsp 70 (VERNER & SCHATZ, 1988) and it is in agreement with the nuclear staining of hsp 90 seen in the fluorescent micrographs (*Part 1*, EXPERIMENTAL chapter 5.). It is possible that the upregulation of nuclear hsp 90 observed in these photographs arises as a result of hsp 90 accumulation at the sites of passage through the nuclear membrane. This could result in a block in the transfer of ER from the synthesis site to the activation-site and so an accumulation of inactive receptor.

One argument against any hsp 90-mediated type of mechanism is based on simple arithmetic. The number of hsp 90 proteins per cell is in vast excess of the total of *all* steroid receptors, even during unstressed conditions (DALMAN *et al*, 1989). Hsp 90 is present at a concentration of 1μM in L-cell cytosol, outnumbering GR, the predominant steroid receptor, by a ratio of 100:1. It is unlikely then, that the observed change in hsp 90 concentration could greatly effect the kinetics of ER-hsp 90 association. However, this assumption is derived from estimates of *cytosolic* hsp 90 concentration; but as shown in the fluorescent micrographs (*Part 1*, EXPERIMENTAL chapter 5.), there is a much more significant increase in hsp 90 levels in the *nuclei* of the heat shocked cells.

6. Hsp 90 AS A NUCLEAR PROTEIN.

There have been very few reports concerned with the intracellular localisation of hsp 90. One possible reason for this is that the highly conserved nature of hsp 90 has hindered the production of an antisera capable of immunocytochemical detection. Two earlier reports used immunocytochemistry to visualise components of the progesterone receptor; monoclonal antibodies were used to detect avian PR in chick oviduct (GASC *et al*, 1984; PERROT-APPLANAT *et al,* 1986). The various antisera were raised against purified *nonactivated*-PR. This meant that a comparison of the different binding affinities of the antibodies yielded an estimation as to the intracellular localisation of the steroid-binding and nonsteroid-binding subunits. Briefly, these reports suggested that activated PR was a nuclear species, and that hsp 90 was a predominantly cytoplasmic protein. This finding was in agreement with the purification terminology suggesting that activated steroid receptors were nuclear proteins and the nonsteroid-binding subunits cytosolic (SAKAI & GORSKI, 1984). Interestingly, these workers did observe slight staining of hsp 90 within the target cell nuclei (GASC *et al,* 1984), but they failed to apply any significance to its presence.

Further work from this group has used the anti-hsp 90 antibody produced by Toft and colleagues (AC 88) to locate hsp 90 in the nuclei of rabbit uterine cells (GASC *et al*, 1990). This recent observation represents the first report of hsp 90 localisation in the nuclei of steroid hormone target cells, and is confirmation of the results contained within this thesis when AC 88

was used to visualise hsp 90. Interestingly, other workers have studied the distribution of PR and hsp 90 in chick oviduct using a variety of monoclonal antibodies, including AC 88, observing only cytoplasmic staining of hsp 90 (PEKKI & TUOHIMAA, 1990). This continues to be an area of considerable debate.

Gasc and coworkers do not report any investigation into the localisation of hsp 90 before or after heat shock, and so the nuclear accumulation of hsp 90 reported herein remains unique. Post heat shock-nuclear accumulation of hsp 70 has been conclusively demonstrated, where it was associated with cellular resistance to supraphysiological temperatures (RIABOWOL *et al,* 1988).

The role of hsp 90 nuclear accumulation is unclear. The obvious possible function is one of protein stabilisation, in analogy to hsp 70 (see INTRODUCTION section 3.3.1.). However hsp 70 and hsp 90 show no other similarity in terms of either structure or function, and it seems unlikely that evolution would provide a parallel set of proteins to provide similar functions. Alternatively, it may be the case that the role of hsp 90 accumulation and thus suppression of oestrogen-binding capacity represents a general role in the masking of various potential gene activators, although further investigation is required.

Part 2.

Experimental

The Effect of Temperature on the Androgen Responsiveness of two Human Prostate Cancer Cell Lines.

A Role for hsp 90 in Prostatic Disease.

1. CANCER OF THE PROSTATE.

Prostatic cancer accounts for one quarter of male cancer deaths in Scotland and its incidence is second only to lung cancer in the Western World (Cancer Registration Statistics Scotland, 1984). This malignant disease must be distinguished from the very common benign prostatic hypertrophy (BPH) which occurs in most males after the age of 50. The incidence of both these diseases rises with the age of patients, and so they represent an increasingly important problem as the longevity of western populations rises.

2. PROSTATIC DISEASE AND ANDROGENS.

Both prostatic cancer and BPH respond to androgen suppression. Charles Huggins was the first to note that hormonal manipulation was an effective method to suppress a proportion of metastatic prostate cancer (HUGGINS & HODGES, 1941) and despite recent technological advances this remains the prevailing method of treatment at this time.

The significance of the androgen-responsive nature of these diseases has led to a variety of methods being devised to quantitate the presence of AR in biopsied prostate tissue. These have included AR ligand binding assay (KIRKALI *et al*, 1990), immunohistochemical localisation (TAKEDA *et al*, 1990) and AR mRNA Northern blotting (CHANG *et al*, 1989). However, there is some evidence to suggest that receptor-positivity is not in itself

sufficient to predict androgen-responsiveness of the tumour (KIRKALI *et al*, 1990).

3. ENDOCRINE THERAPY OF PROSTATIC DISEASE.

Earlier work from Huggins showed that oestrogen played a suppressive role in the formation of prostate cancer metastases (HUGGINS & HODGES, 1941); and as a result of this discovery androgen ablative orchiectomy (castration) or supplemental oestrogens were instigated as methods of treatment. However, the detrimental psychological effects of these treatments led to the search for suitable antiandrogens that were devoid of any oestrogenic properties.

Various "antiandrogens" have been used clinically: these include Cyprostat® (cyproterone acetate), Flutamide® (α-α-α-trifluoro-2-methyl-4'-nitro*m*-propionotoluidine), and Megace® (17α-acetoxy-6-dehydro-6-methyl-progesterone). Only flutamide is a nonsteroidal antiandrogen, both cyproterone and megestrol are steroid derivatives and as such have positive effects mediated by other steroid receptors.

For example, megestrol acetate has been shown to bind to AR and result in an activation, rather than inhibition, of androgenic activity (POYET & LABRIE, 1985) and so can not be defined as an antiandrogen. The metastatic suppressive action of this compound is thought to be exerted through its progestational activities; as administration can result in a decrease in the levels of both luteinising and follicle stimulation hormones and thus a decrease in the levels of circulatory androgen.

Cyproterone is an effective antiandrogen, although some androgenic activity, and significant glucocorticoid and progestational activities have also been noted *in vivo* (POYET & LABRIE, 1985). These activities have undesirable implications for the endocrine therapy of both prostatic cancer and BPH and so flutamide is the antiandrogen of choice.

Flutamide is a nonsteroidal-antiandrogen that shows no androgenic, progestational or glucocorticoid activities *in vivo* (POYET & LABRIE, 1985). However, *in vitro* both flutamide (and its activated derivative 4-hydroxy-flutamide) have been shown to result in the proliferation of androgen-sensitive human prostate tumour cells (OLEA *et al,* 1990; WILDING *et al,* 1989). Not surprisingly, this discrepancy between the *in vivo* anti-androgenic and *in vitro* androgenic dual qualities of flutamide raises profound questions as to the mechanism of its clinical efficacy.

4. THERMOTHERAPY OF PROSTATIC DISEASE.

In the last decade an alternative method of treatment has arisen for the clinical management of both prostatic cancer and BPH: localised hyperthermia (MENDECKI *et al*, 1980). This therapy involves short doses of microwave heat and is based on the observation that many types of tumour cells are more sensitive to elevated temperatures than normal cells (CRILE, 1963; see next section). The hyperthermic effect is obtained using temperatures of between 41°C and 44°C, though temperatures above 42.5°C (± 0.5°C) have also been observed to result in significant damage to normal tissues (LEIB *et al*, 1986).

Success of this treatment is mainly due to the accessibility of the malignant gland to an external heat source, which is introduced non-surgically transrectally (MENDECKI *et al*, 1980). There are various techniques capable of generating the heat but the common principles necessitate that the area of heating is defined by the size and shape of the applicator while the depth of heating is a function of the frequency and wavelength of the microwaves (MENDECKI *et al*, 1980).

In two separate studies Servadio and colleagues have investigated the use of microwave hyperthermia in the treatment of both prostate cancer (SERVADIO *et al*, 1987) and BPH (LINDNER *et al*, 1990). A marked improvement was observed in 50% of patients (146 in total) that was almost totally maintained after 12 and 24 months. These workers do not conclude whether this treatment represents a cure for either disease, however they do suggest that it is at least a very effective palliative measure, and that it is preferable whenever surgery is contraindicated. Interestingly, the most successful results were obtained when patients received adjuvant therapy in the form of cyproterone acetate in conjunction with the hyperthermia (LINDNER *et al,* 1990), suggesting that a certain synergy exists between these two treatments.

5. HYPERTHERMIA OF MALIGNANT DISEASE.

There is a variety of reasons why malignant cells seem to be endowed with thermosensitivity: in general, most tumour cells are intrinsically more

sensitive to heat than normal cells (GIOVANELLA *et al*, 1976). Malignant tissues contain hypoxic cells that are sensitive to heat (KIM *et al*, 1975). Local hyperthermia enhances the immunological response against the tumour (JANIAK & SZMIGIELSKI, 1982).

The exploitation of the thermosensitivity of tumour cells has led to different hypotheses regarding thermotherapy; one area of particular confusion is the isoeffect relationship between dose and exposure time. As an attempt to compare the work of different laboratories, a system was introduced that converted different time/temperatures to a single unit: the equivalent heating time at 43°C (EQ43; DEWEY *et al*, 1977). Unfortunately, this classification of isoeffective dose fails to correlate well with *in vitro* data. This is mainly due to nonuniform heating of tumours within deep seated tissues (OVERGAARD, 1987) and more importantly, the inability of the EQ43 to allow for the complicated kinetics of thermotolerance (SAPARETO, 1987). As a result of this, less than complete understanding of thermal quantitation it is difficult to distinguish between thermal-killing, and thermal sensitisation of neoplastic tissue (GERWECK, 1985). Simply, are increased temperatures direct effecters, or just mediators of neoplastic suppression?

In the case of prostatic cancer, the mechanism responsible for the thermal-suppression of neoplastic growth is unclear; nonetheless, treatment of prostatic cancer has greatly benefitted from the instigation of thermal-therapy (SERVADIO *et al*, 1987). Thermal-therapy involves heating

tumour tissue to between 41-45°C for up to 30 minutes: this range incorporates the regimen that were shown in *Part 1* to be capable of both hsp 90 and thermotolerance induction. It is unlikely then that exposure to temperatures elevated to 41°C-43°C is sufficient to result in tumour necrosis *per se* .

As an attempt to investigate the treatment of prostatic cancer by thermal therapy, it was decided to use two human prostate cell lines to construct an *in vitro* model of the clinical scenario.

In addition, the question of flutamide's paradoxical activity *in vivo* and *in vitro* was also investigated. This antiandrogen is used in conjunction with thermal- therapy; the combination of heat and endocrine treatments resulting in a synergistic suppression of neoplastic prostate cell growth (SERVADIO *et al*, 1987). It was therefore of interest to investigate whether the *in vitro* androgenic activity of flutamide was maintained when used in conjunction with thermal stress.

CHAPTER 1.

Immunocytochemical Localisation of AR and hsp 90 in Prostate Cells.

INTRODUCTION.

Before investigating any interrelationships between the actions of heat and antiandrogens during the treatment of prostate cancer, it was decided to verify the presence of both androgen receptor (AR) and hsp 90 in the prostate cancer cell lines LNCaP and DU-145. These cells are androgen-dependent and androgen-independent respectively (See METHODOLOGY section 2.1.).

Two different methodologies were used. Firstly, an immunocytochemical method was used in conjunction with antibodies against AR and hsp 90 to stain for these proteins in both LNCaP and DU-145 cells as well as in two sections of benign prostate disease. A second immunofluorescent procedure was used to verify the presence of AR in the two prostate cell lines, though this procedure is not suitable for immunohistochemical staining of tissue sections.

METHODS.

LNCaP and DU-145 cells were subcultured as described in METHODOLOGY section 2.2. into 8-well microscopy slides. No exogenous ligands were added to the RPMI-1640 culture medium and the cells were allowed to grow for 48 hours until approximately 80% confluence was achieved. After this period the culture medium was removed and the cell monolayers were washed using PBS-A prior to fixation. The cells to be stained using the immunofluorescent technique were fixed using the acetone-methanol process described in METHODOLOGY section 6.2.1., while all other cells and the tissue sections were fixed by the acetone process described in METHODOLOGY section 6.2.2.

Immunocytochemical localisation (see METHODOLOGY section 6.3.) of AR was achieved using a polyclonal rabbit antibody (raised against AR) diluted 400 times. Immunocytochemical localisation of hsp 90 employed AC 88 mouse monoclonal antibody diluted 1000 times. (See METHODOLOGY section 6.1. for a full list of antisera.)

Fluorescent immunocytochemistry (see METHODOLOGY section 6.4.2.) was achieved using the polyclonal anti-AR antibody diluted 500 times. This was detected using a donkey anti-rabbit serum labelled with FITC as described in METHODOLOGY section 6.4.1.

RESULTS.

The results of the immunocytochemical studies are shown in the following figures. Figure 21. is a photograph of slides of DU-145 cells stained for both AR (red) and hsp 90 (blue). FIGURES 22a. & 22b. are high power views of these slides; showing non-specific and specific staining of hsp 90 respectively. FIGURE 22c. is a high power photograph of the DU-145 cells stained for AR. (LNCaP cells showed similar but brighter staining;- data not shown.)

FIGURES 23a. & 23b. are photomicrographs of benign prostate sections stained for hsp 90; FIGURE 23a. differs from FIGURE 23b. in that the red carbalum nuclear counterstain has been omitted. (Microscope magnification ×300)

FIGURE 24b. is a fluorescent micrograph of LNCaP cells stained for AR, while FIGURE 25b. is a similarly stained micrograph of DU-145 cells. (Microscope magnification ×400). The Hoechst nuclear counterstain of each field (FIGURES 24a. & 25a.) is shown to allow assessment of AR intracellularity.

FIGURE 21. **Immunocytochemical Localisation of AR and hsp 90 in DU-145 Cells.**

This photograph shows the AR (red) and hsp 90 (blue) staining present in DU-145 cells. The 4 wells furthest from the name label represent specific-binding.

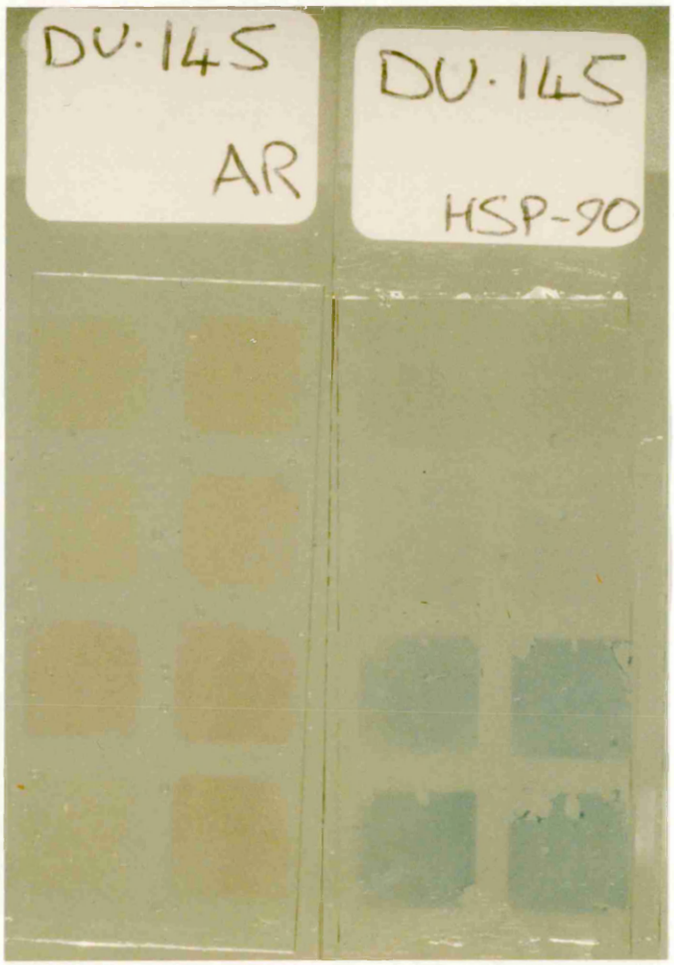

DU·145
AR
DU·145
HSP-90

FIGURE 22. **Immunocytochemical Localisation of AR and hsp 90 in DU-145 Cells.**

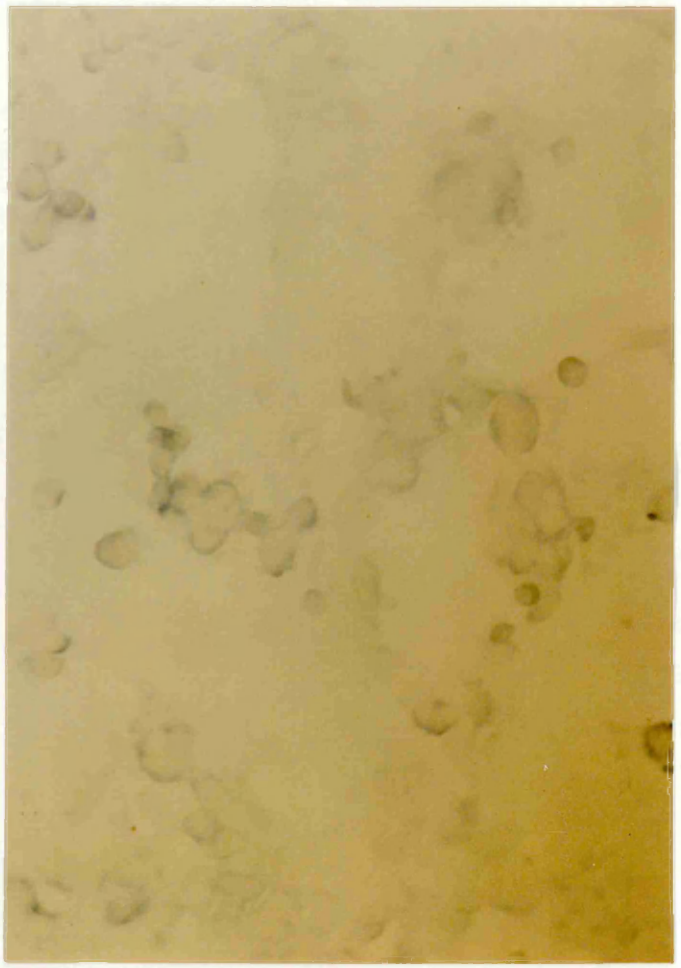
FIGURE 22a. shows the nonspecific AR staining (staining in the absence of primary anti-AR polyclonal antibody).

FIGURE 22c. shows the specific AR staining (staining in the presence of primary anti-AR polyclonal antibody).

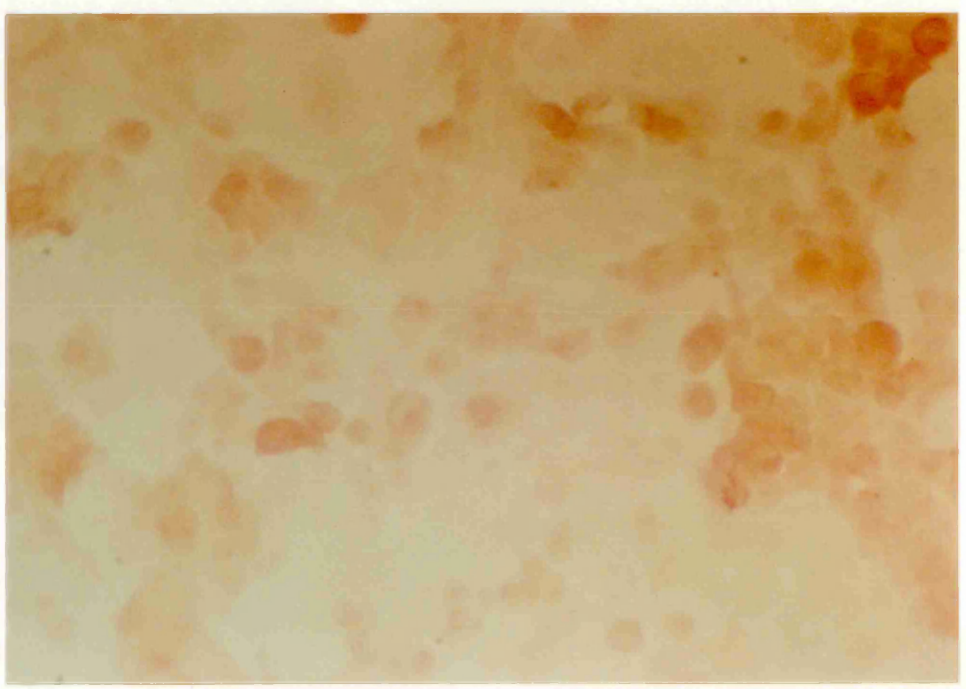
FIGURE 22b. shows the specific hsp 90 staining (staining in the presence of primary anti-hsp 90 AC 88 monoclonal antibody).

(All microscope magnification ×400)

a.



b.



c.

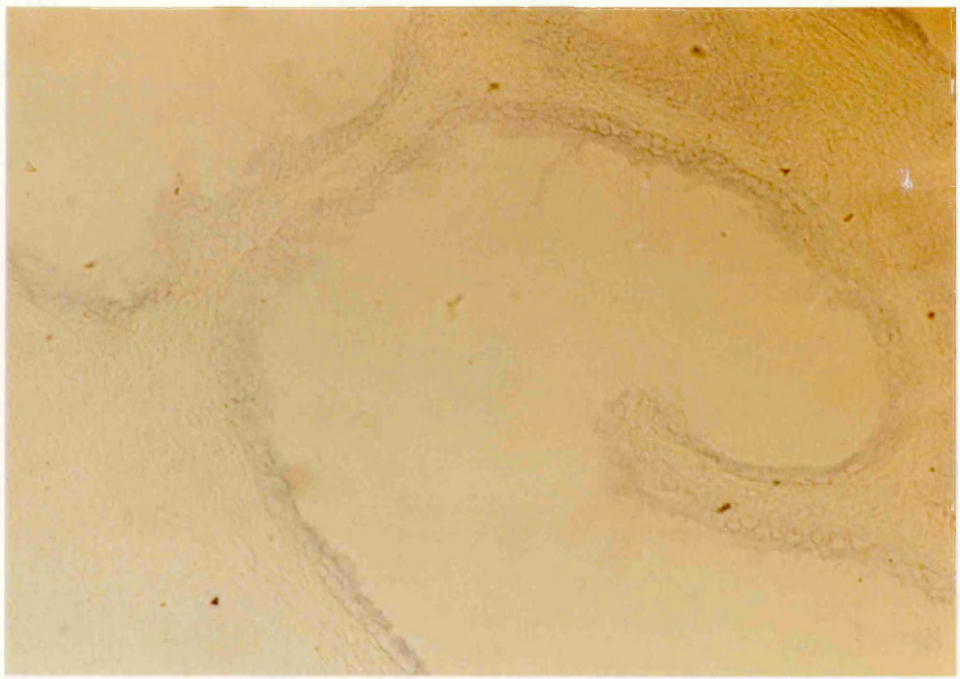
FIGURE 23. **Immunohistochemical Localisation of hsp 90 in Benign Prostate Hyperplasia (BPH).**

FIGURES 23a. & b. show a section of BPH stained for hsp 90 using monoclonal antibody AC 88.

FIGURE 23a. shows hsp 90 staining in the absence of carbalum nuclear counterstain (red; included in FIGURE 23b.) in order to emphasise hsp 90 cytoplasmic localisation.

(Microscope magnification ×300 in both photographs)

a



b

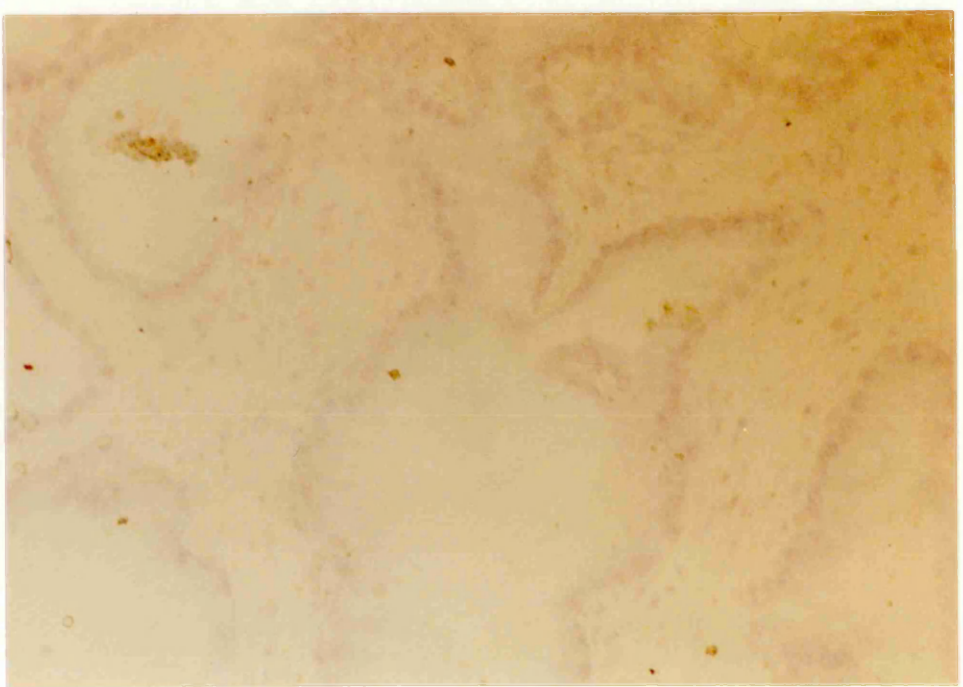


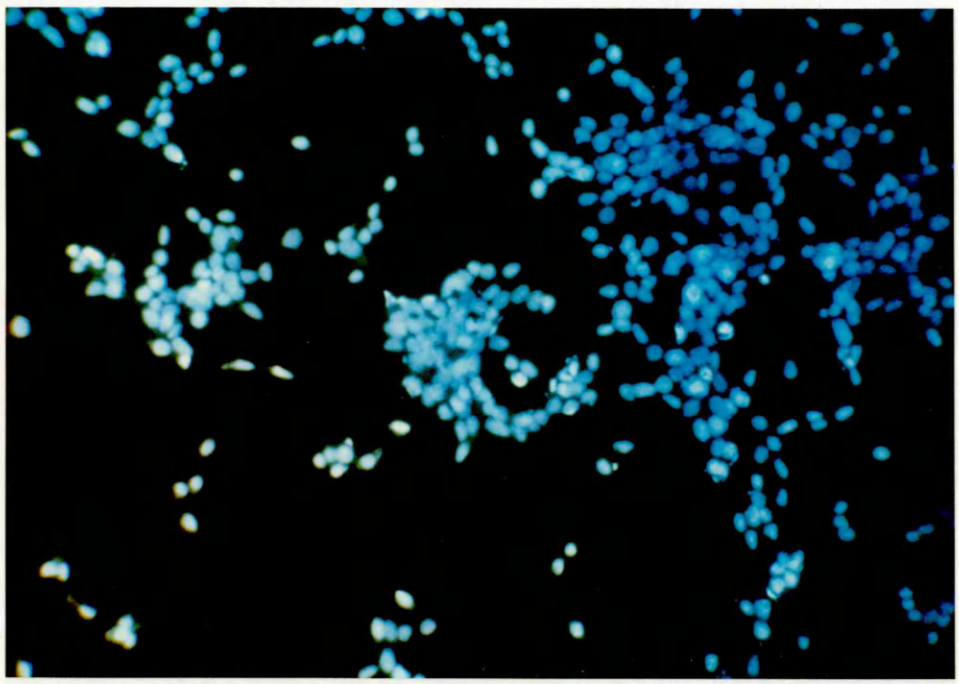
FIGURE 24. **Fluorescent Immunocytochemical Localisation of AR in LNCaP Cells.**

FIGURE 24b. shows the specific anti-AR FITC-fluorescence of LNCaP cells.

FIGURE 24a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 24b.

(Microscope magnification ×400 in both photographs)

a



b

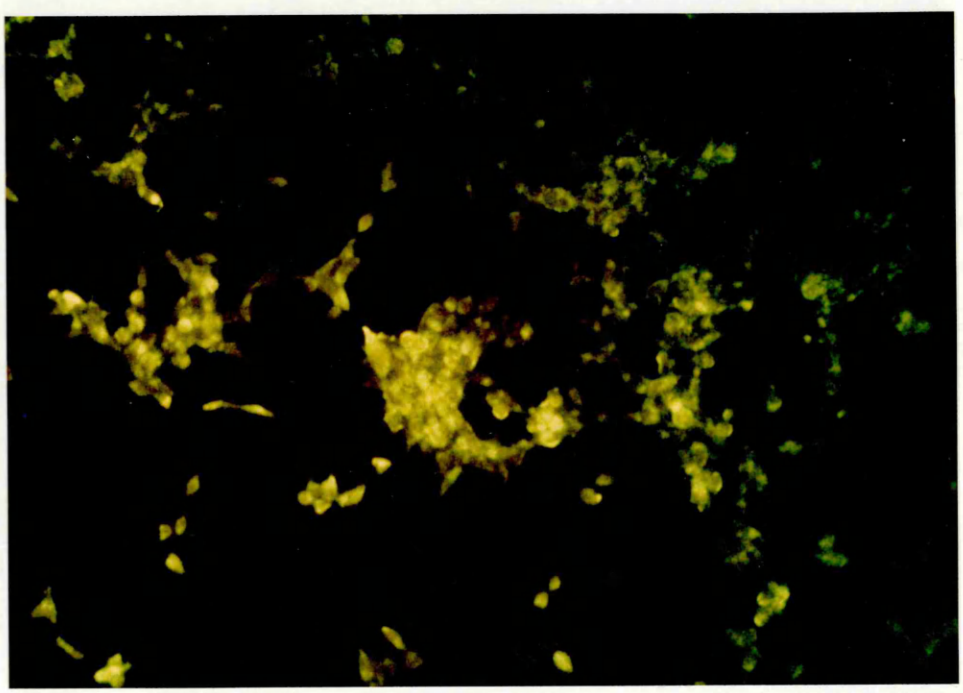


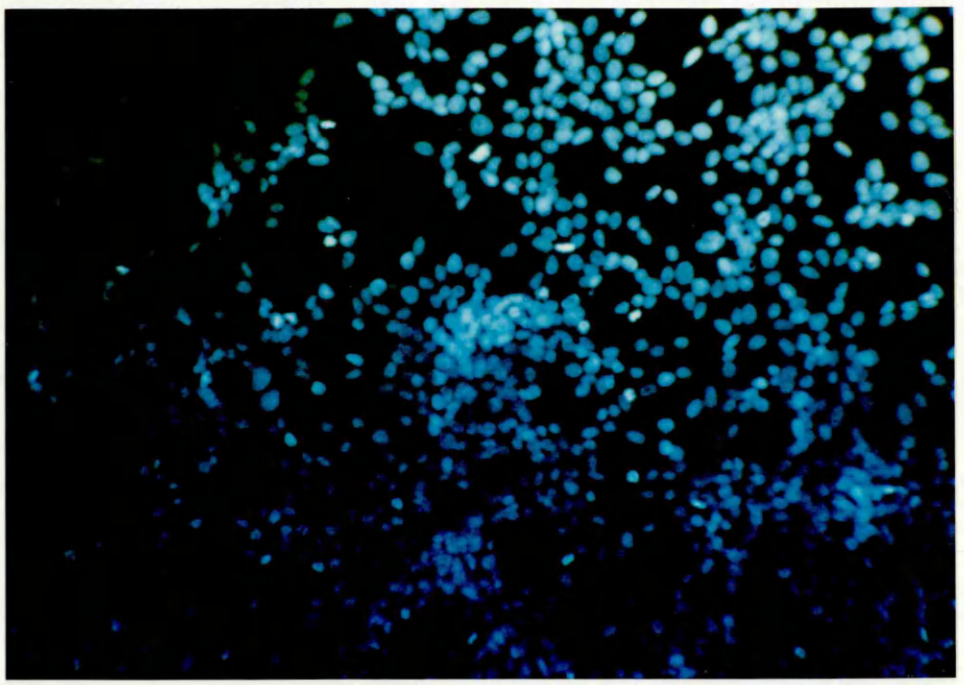
FIGURE 25. **Fluorescent Immunocytochemical Localisation of AR in DU-145 Cells.**

FIGURE 25b. shows the specific anti-AR FITC-fluorescence of LNCaP cells.

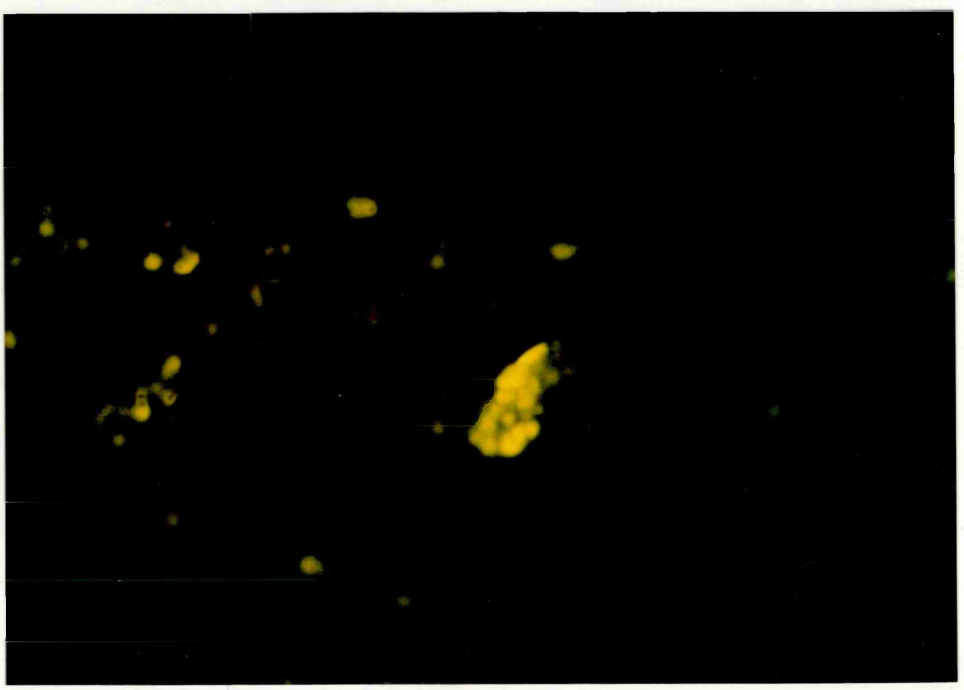
FIGURE 25a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 25b.

(Microscope magnification ×400 in both photographs)

a



b



CONCLUSIONS.

From Figure 21., showing two slides of DU-145 cells stained for both AR (red) and hsp 90 (blue) it should be noted that four of the wells were used for nonspecific-binding; staining in the absence of the anti-AR and anti-hsp 90 primary antibodies. These are the four wells nearest the name-tag, and they demonstrate both the specific nature of the staining as well as the positive identification of both AR and hsp 90 proteins in DU-145 cells. However it should be noted that DU-145 cells showed no growth response to androgen (see *Part 2.* EXPERIMENTAL section 4.)

FIGURES 22a. & 22b. are high power views of the slide stained for hsp 90; these show non-specific and specific staining respectively. Hsp 90 can be observed in all cells and is present mainly in the cytoplasm. AR, however, is present in different amounts in individual cells (FIGURE 22c). The AR staining does seem to be predominantly nuclear.

FIGURES 23a. & 23b. are photomicrographs of benign prostate sections stained for hsp 90; best assessment of hsp 90 intracellularity can be obtained from a comparison of these two photographs. FIGURE 23a. shows the tissue stained for hsp 90 in the absence of brown/red nuclear counterstain; and it is very clear that the nuclei are devoid of stain, especially in the cells surrounding the gland.

FIGURE 24b. shows the presence of significant levels of nuclear AR in LNCaP cells, while FIGURE 25b. confirms that there are also AR present in DU-145 cells. Compared to the LNCaP cells, the DU-145 AR are located in the cell nuclei although there is much less staining overall. This reflects

the lower levels of AR present in these cells (see Part 2. EXPERIMENTAL chapter 5.) as detected by ligand binding assay.

In conclusion, this work has confirmed the presence of hsp 90 in the cytoplasm of both LNCaP and DU-145 cells, as well as in prostate tissue sections. AR has also been located in the nuclei of both LNCaP and DU-145 cells, although LNCaP cells possess significantly higher levels of AR and were separately shown to grow in response to exogenous androgen. In addition the standard immunocytochemical staining procedure has been verified by the fluorescent immunocytochemical procedure to confirm these results.

CHAPTER 2.

Prostate Cell Temperature Responses.

INTRODUCTION.

It is important to establish whether or not prostate cancer cells can develop thermotolerance during the hyperthermic treatment of cancer of the prostate. To this end, studies on LNCaP and DU-145 human prostate cancer cell lines can be of great relevance (SAPARETO, 1987). Repeated low-dosage thermal stress could inadvertently create a thermotolerant population of neoplastic cells. For this reason it was decided to use the LNCaP and DU-145 cell lines as a model system to investigate the effects of heat on prostate cell growth *in vitro*.

It is important to distinguish a point of thermo-kill; the lethal temperature for a given cell line, so that the induction of thermotolerance can be assessed. Previous work (Part 1. EXPERIMENTAL chapter 1.) using the MCF-7 human breast cancer cell line suggested that 2 hours exposure to any temperature greater than 42°C was lethal to this cell line. For this reason, 41°C was initially chosen as a sub-lethal temperature for these two prostate cell lines. However, as the results show, this was an incorrect assumption, and further investigation was required.

METHODS.

LNCaP and DU-145 cells were subcultured as described in METHODOLOGY section 2.2. so that 20% confluent monolayers were formed within 6-well (15.7 cm^2/well) plastic tissue culture plates. The two cell lines were allowed to "plate-down" for 24 hours and fresh culture medium was added immediately prior to commencing the experiment. Heat shock was administered in an analogous manner to the experiments using MCF-7 cells described in Part 1. EXPERIMENTAL chapter 1. After subjecting the prostate cells to the various heat shock treatments (HST), they were harvested at 24 and 48 hours after heat shock. The harvesting methodology was identical to that described in Part 1. EXPERIMENTAL chapter 1; aliquots (100µl) of these solubilised prostate cells being assayed for DNA using the Hoechst DNA assay described in METHODOLOGY section 4.2.

RESULTS.

The results of this experiment are shown in the following four figures; these experiments were carried out only once, however each column of the histogram represents the mean of duplicate DNA assay of triplicate wells of cells.

FIGURES 26. and 27. show the effect on LNCaP and DU-145 cell viability respectively of HST 0, HST 2 alone, and HST 1+2 in combination. These heat shock treatments were identical to those used in Part 1. EXPERIMENTAL chapter 1 during the induction of thermotolerance in MCF-7 cells (ie. 41°C and 44°C for HST 1 and HST 2).

FIGURES 28. and 29. show the effect on LNCaP and DU-145 cell viability respectively of HST 0*, HST 2* alone, and HST 1*+2* in combination. These heat shock treatments were reduced in terms of the temperature magnitude so that HST 1* was a 2 hour exposure at 39°C followed by a 12 hour recovery period, and HST 2* was a 2 hour exposure to 42°C followed by a 12 hour recovery period.

It should be noted that the total time of potential cell growth was maintained as equal throughout the different heat shock treatments.

FIGURE 26. **The Effect of Temperature on LNCaP Cell Viability.**

LNCaP cells exposed to HST 2 (44°C; 2 hours followed by 37°C; 12 hours) alone were compared to cells subjected to HST 0 (37°C throughout) and cells given a prior exposure to HST 1 (41°C; 2 hours followed by 37°C; 12 hours) before subsequent HST 2, ie HST 1+2. The effects of the different heat shock treatments on cell growth was assessed by measuring the amounts of DNA after 24 and 48 hours. (Day 1 and Day 2 respectively)

Error bars represent the Standard Deviation of the data.

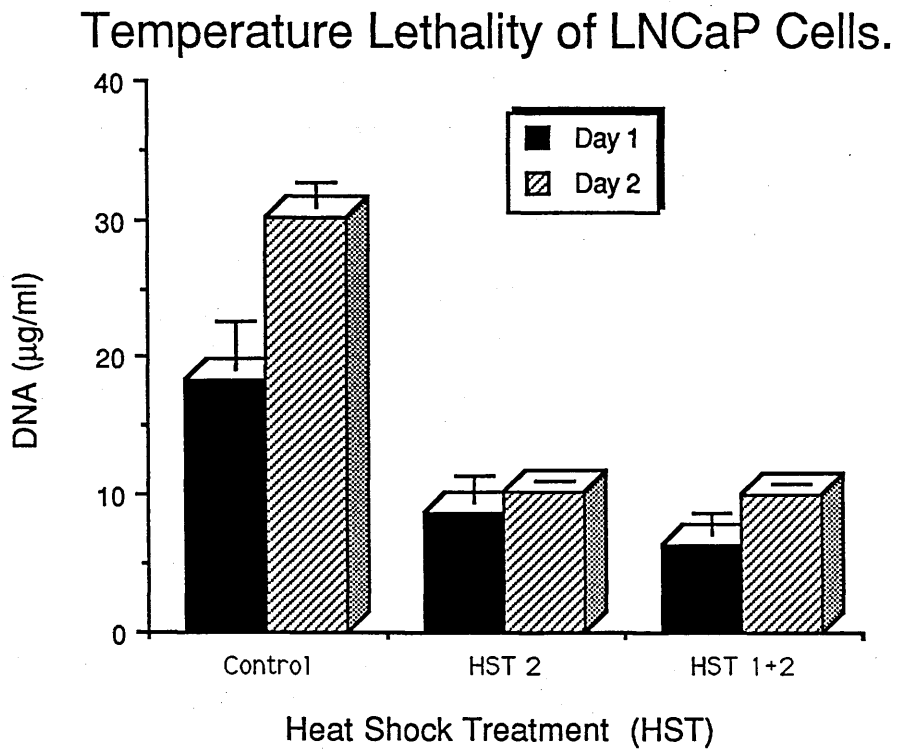

Temperature Lethality of LNCaP Cells.
40
30
20
10
0
DNA (µg/ml)
Day 1
Day 2
Control
HST 2
HST 1+2
Heat Shock Treatment (HST)

FIGURE 27. **The Effect of Temperature on DU-145 Cell Viability.**

DU-145 cells exposed to HST 2 (44°C; 2 hours followed by 37°C; 12 hours) alone were compared to cells subjected to HST 0 (37°C throughout) and cells given a prior exposure to HST 1 (41°C; 2 hours followed by 37°C; 12 hours) before subsequent HST 2, ie. HST 1+2. The effects of the different heat shock treatments on cell growth was assessed by measuring the amounts of DNA after 24 and 48 hours. (Day 1 and Day 2 respectively)

Error bars represent the Standard Deviation of the data.

Temperature Lethality of DU-145 Cells.

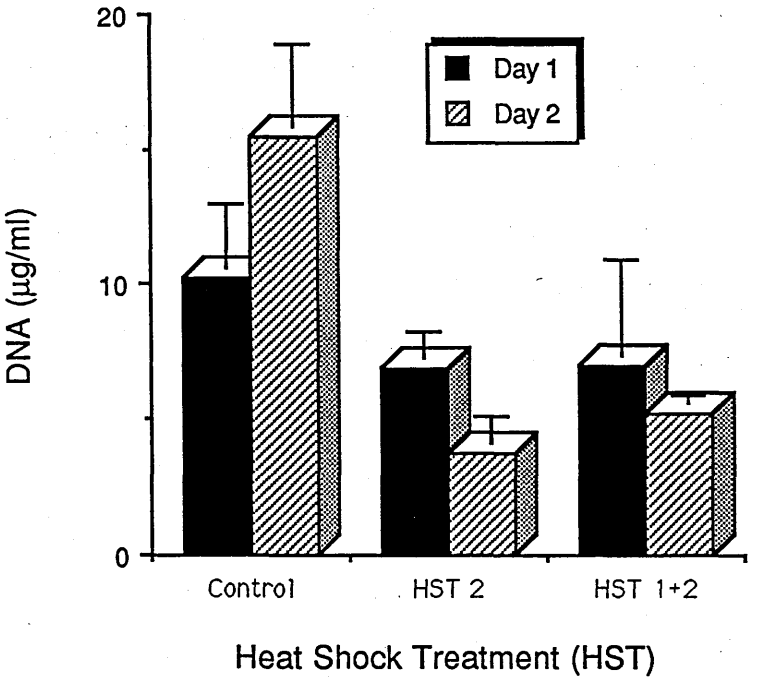


FIGURE 28. **The Induction of Thermotolerance in LNCaP Cells.**

LNCaP cells exposed to HST 2* (42°C; 2 hours followed by 37°C; 12 hours) alone were compared to cells subjected to HST 0 (37°C throughout) and cells given a prior exposure to HST 1* (39°C; 2 hours followed by 37°C; 12 hours) before subsequent HST 2*, ie. HST 1*+2*.

The effects of the different heat shock treatments on cell growth was assessed by measuring the amounts of DNA after 24 and 48 hours. (Day 1 and Day 2 respectively)

Error bars represent the Standard Deviation of the data.

LNCaP Cell Thermotolerance.

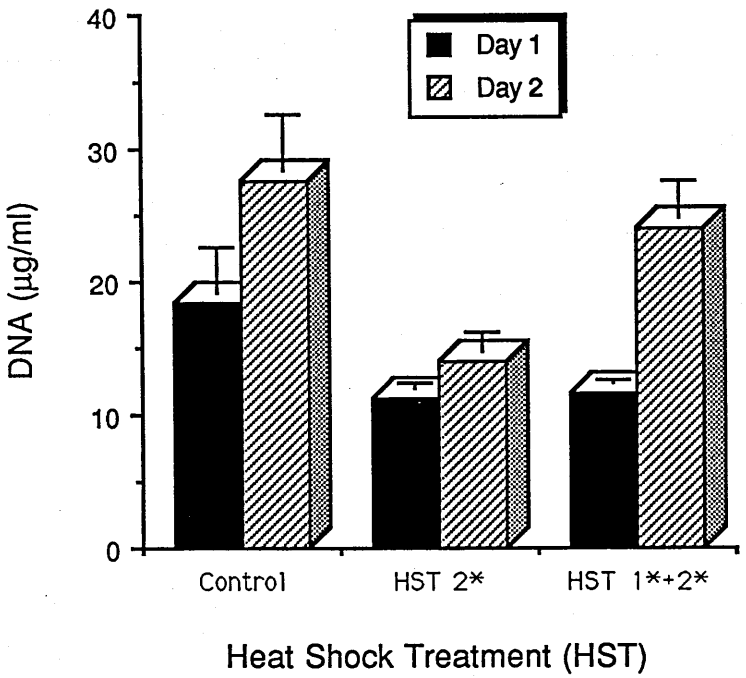


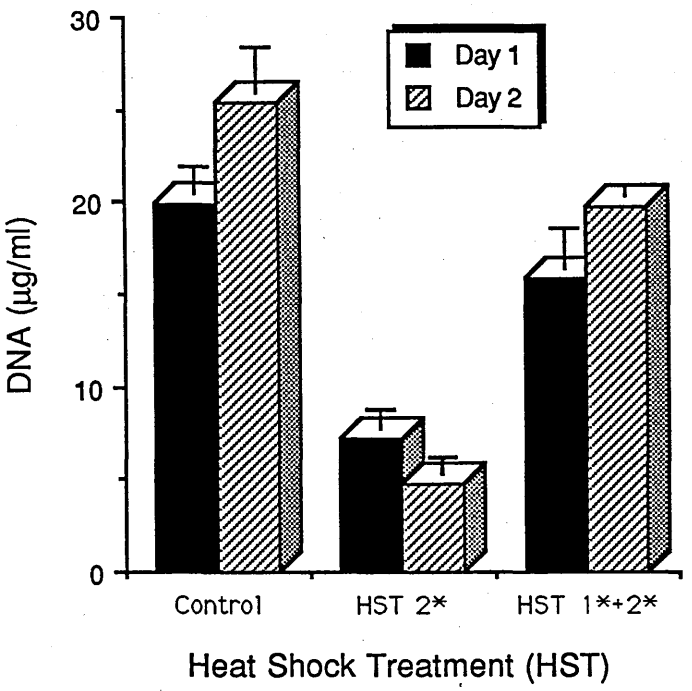
FIGURE 29. **The Induction of Thermotolerance in DU-145 Cells.**

DU-145 cells exposed to HST 2* (42°C; 2 hours followed by 37°C; 12 hours) alone were compared to cells subjected to HST 0 (37°C throughout) and cells given a prior exposure to HST 1* (39°C; 2 hours followed by 37°C; 12 hours) before subsequent HST 2*, ie. HST 1*+2*.

The effects of the different heat shock treatments on cell growth was assessed by measuring the amounts of DNA after 24 and 48 hours. (Day 1 and Day 2 respectively)

Error bars represent the Standard Deviation of the data.

DU-145 Cell Thermotolerance.



CONCLUSIONS.

The results demonstrated in FIGURES 26. and 27. show that both LNCaP and DU-145 human prostate cells appear to be susceptible to temperatures greater than 41°C.

These conclusions are drawn from the fact that in the case of LNCaP cells (FIGURE 26.), there is significantly lower levels of DNA present after either HST. Not only has the exposure to 41°C failed to induce LNCaP thermotolerance to 44°C, but it has effectively been a lethal dose, as these HST 1+2 treated cells show only minimally greater growth levels when compared to those treated to HST 2 alone. (The presence of a small number of thermotolerant LNCaP cells may be indicated by the slight increase in the 48 hour DNA levels of the HST 1+2 treated cells, although this is of questionable statistical significance.) A similar conclusion can be drawn for the DU-145 cells (FIGURE 27.); there is an overall inhibition of growth in either HST treated cell group. In addition, there is a significant loss in DU-145 DNA over the 24 hour period between assays suggesting that cell death is resulting in detachment of many cells.

With these results in mind, it was decided to reduce the temperatures of the corresponding heat shocks by 2°C (i.e. initial treatment at 39°C; second treatment 42°C). The results of these experiments can be seen in FIGURES 28. and 29.

FIGURE 28. shows that the LNCaP cell line demonstrates a very strong resistance to the lethal temperature of 42°C as a result of prior exposure to 39°C. This thermotolerance response is observed very clearly in the

maintainance of the growth rate of the HST 1*+2* treated LNCaP cells when compared to the growth rate of the control cells over the 24 hour period.

A similar phenomenon is observed in the DU-145 cell line. FIGURE 29. shows that the HST 1*+2* treated DU-145 cells not only contain significantly higher levels of DNA than the HST 2* alone treated cells; but in addition the growth rate is, like in the LNCaP cell line, comparable with that of the control cells. Once again, the HST 2* alone treated cells show a loss of DNA over the 24 hour period emphasising that unprepared exposure of DU-145 cells to 42°C results in cell death and detachment.

In conclusion, both LNCaP and DU-145 cells are unable to withstand 2 hours exposure to 42°C, unless primed by a 2 hour exposure to 39°C. Presumably, this phenomenon is occurring as a result of heat shock instigation of the thermotolerance response.

CHAPTER 3.

Immunocytochemical Localisation of Hsp 90 in Heat-Shocked Prostate Cells.

INTRODUCTION.

In view of the results reported in Part 1 EXPERIMENTAL chapter 6. it was decided to investigate if the observed nuclear accumulation of hsp 90 in heat shocked MCF-7 cells was a phenomenon confined to this breast cancer cell line. Similar hsp 90 accumulation in either LNCaP or DU-145 cells could implicate a thermal modulation of nuclear AR in an analogous manner to the proposed suppression of oestrogen-binding ability observed in the heat shock breast cells. Any thermal modulation of the endocrine response to either androgens or antiandrogens would represent an important factor in the design of any combined antiandrogen and hyperthermic therapy for prostatic cancer and might shed light on the mechanism of heat-induced tumour cell kill.

METHODS.

The protocol for heat shock induction, cell processing and hsp 90 immunofluorescent staining was identical to the methods used for hsp 90 staining in MCF-7 cells (Part 1 EXPERIMENTAL chapter 6.) with the exception of the reduced HST temperatures. Both LNCaP and DU-145 cells were stained for hsp 90, comparing 37°C control cells with heat shocked (HST 1*+2*) cells.

RESULTS.

The immunofluorescent photomicrographs in FIGURES 30-33. are shown as pairs (termed a. and b.), to allow comparison of the Hoechst nuclear counterstaining and the FITC-hsp 90 staining (respectively blue and green). All photographs were taken using a microscope magnification of ×400.

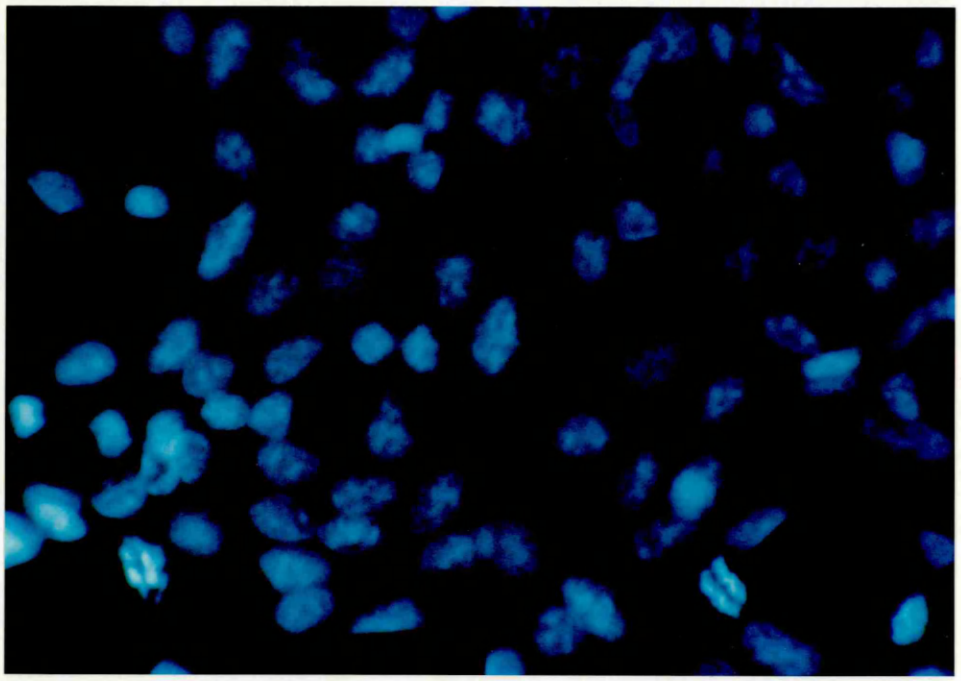
FIGURE 30. **Fluorescent Immunocytochemical Localisation of hsp 90 in LNCaP Cells.**

FIGURE 30b. shows the specific anti-hsp 90 FITC-fluorescence of cells grown at 37°C throughout (HST 0).

FIGURE 30a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 30b.

(Microscope magnification ×400 in both photographs).

a



b

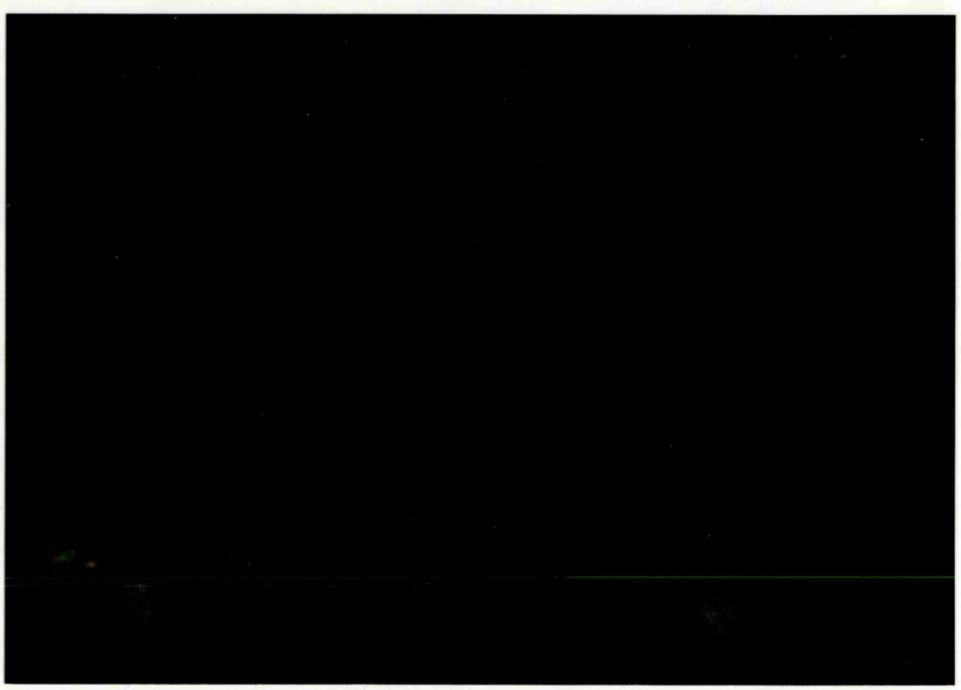


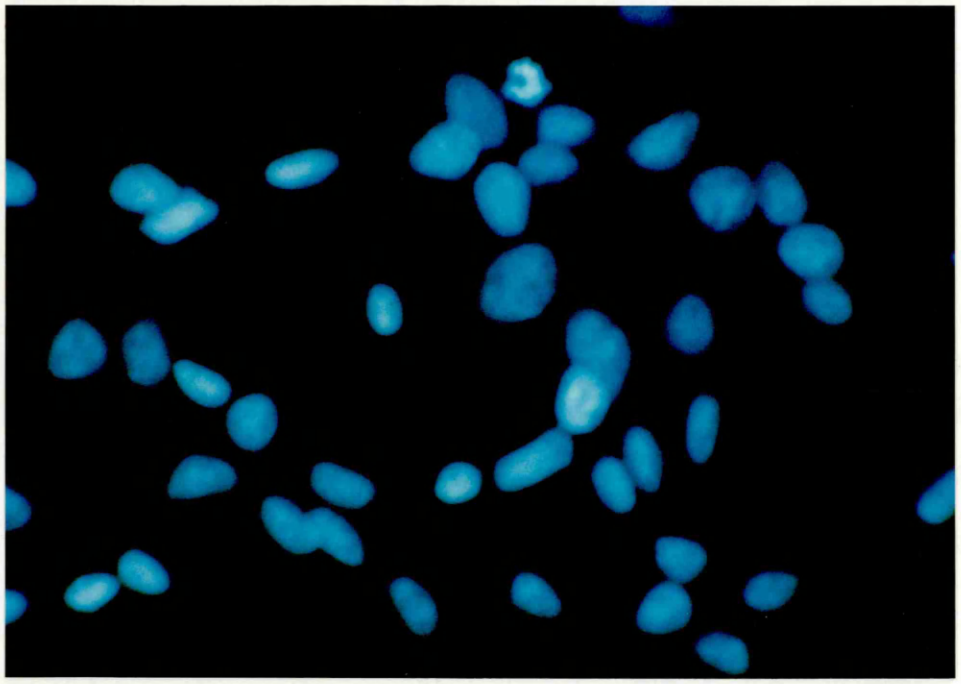
FIGURE 31. **Fluorescent Immunocytochemical Localisation of hsp 90 in Heat Shocked LNCaP Cells.**

FIGURE 31b. shows the specific anti-hsp 90 FITC-fluorescence of cells subjected to HST 1*+2* (double heat shock).

FIGURE 31a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 31b.

(Microscope magnification × 400 in both photographs)

a



b

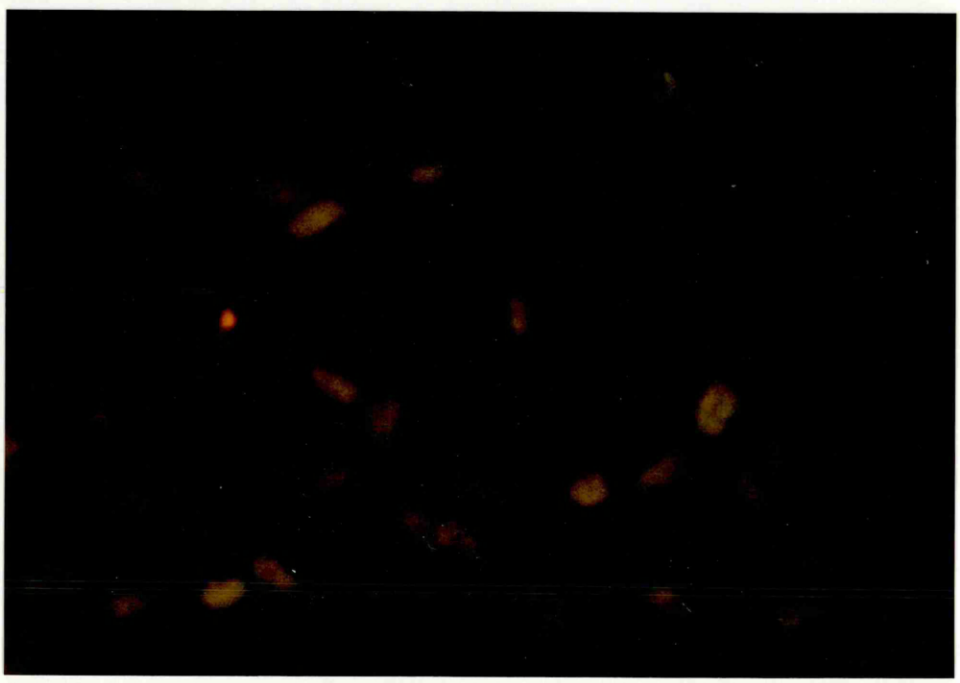


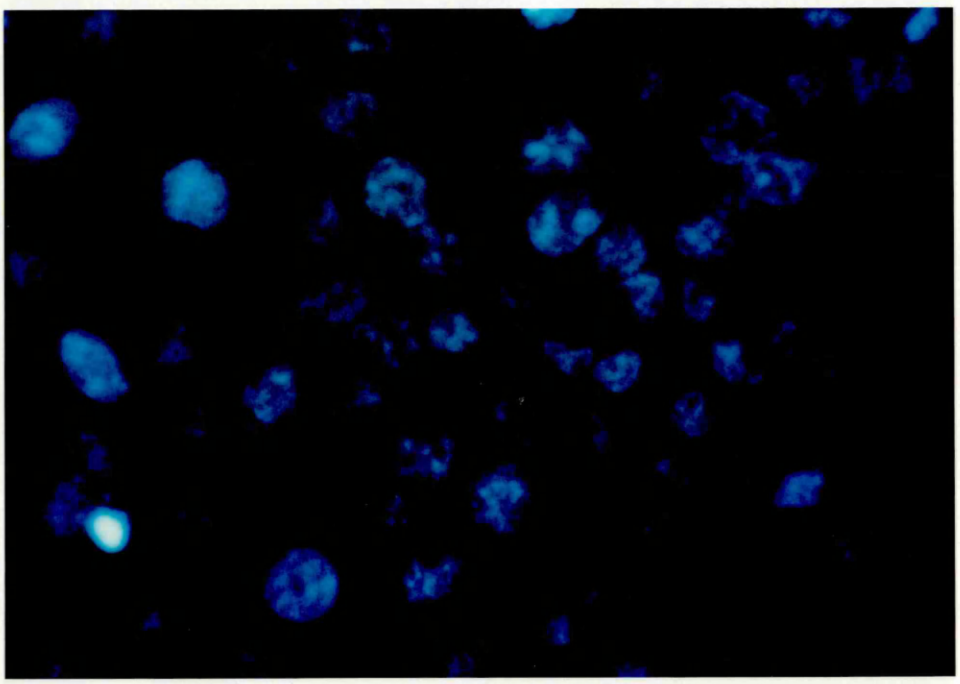
FIGURE 32. **Fluorescent Immunocytochemical Localisation of hsp 90 in DU-145 Cells.**

FIGURE 32b. shows the specific anti-hsp 90 FITC-fluorescence of cells grown at 37°C throughout (HST 0).

FIGURE 32a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 32b.

(Microscope magnification ×400 in both photographs).

a



b

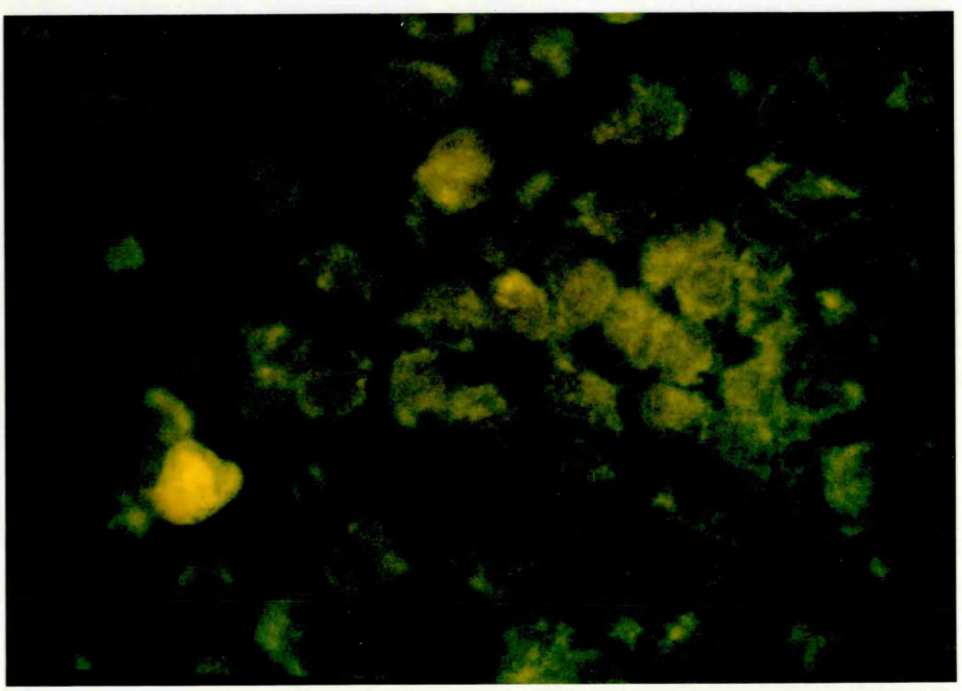


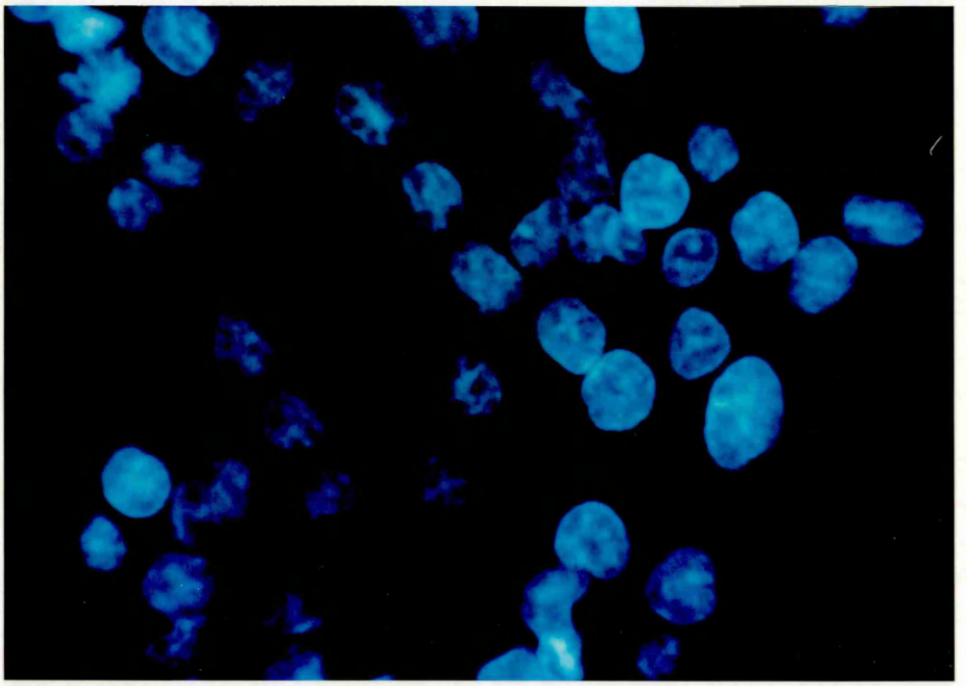
FIGURE 33. **Fluorescent Immunocytochemical Localisation of hsp 90 in Heat Shocked DU-145 Cells.**

FIGURE 33b. shows the specific anti-hsp 90 FITC-fluorescence of cells subjected to HST 1*+2* (double heat shock).

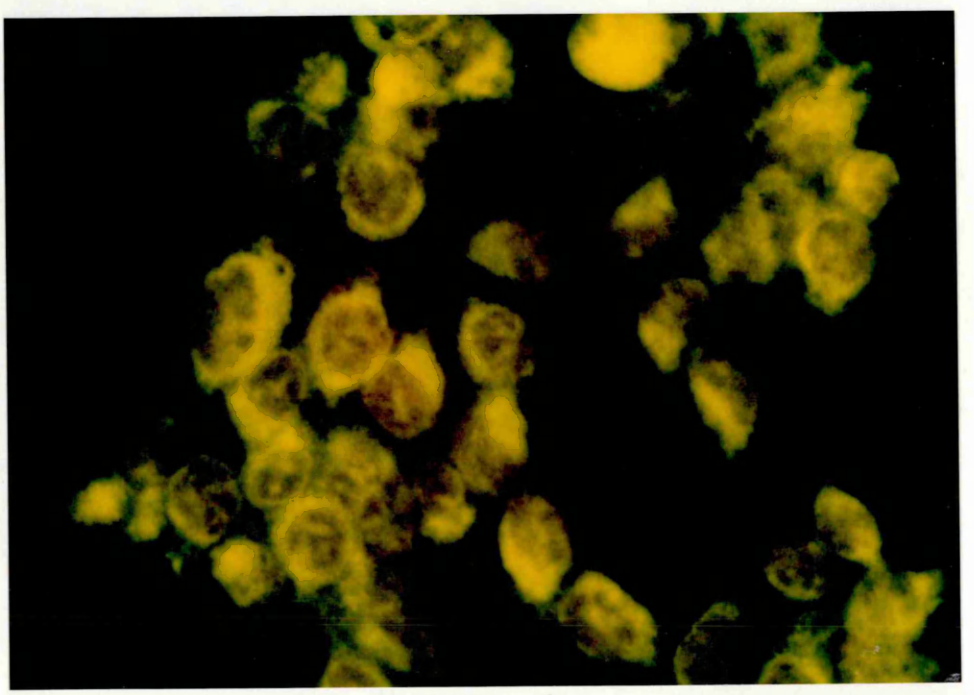
FIGURE 33a. shows the corresponding Hoechst nuclear counterstain of the same field shown in FIGURE 33b.

(Microscope magnification ×400 in both photographs)

a



b



CONCLUSIONS.

A studied comparison of FIGURES 30b. and 31b. shows that heat shock results in an apparent overall increase in LNCaP hsp 90 staining, though the difference is relatively minor. There is a more distinctive nuclear staining in the heat shocked LNCaP cells shown in FIGURES 31b. than the control LNCaP cells shown in FIGURE 30b. This is similar to, but not as prominent as the change in staining pattern observed when heat shocked MCF-7 cells were compared to their 37°C controls. This suggests that the nuclear accumulation of hsp 90 is not a phenomenon peculiar to the MCF-7 breast cell line.

There is resounding confirmation of the more general nature of this observed effect when the heat shocked DU-145 cells (FIGURE 33b.) are compared with their 37°C equivalents (FIGURE 32b.). There is evidence of distinctive nuclear accumulation of hsp 90 and of association with the nuclear membrane in particular. This pattern of hsp 90 localisation is previously unreported. Hsp 70 has been observed to accumulate in the nucleus of heat shocked fibroblasts, but this seemed to represent nucleolar localisation (WELCH & SUHAN, 1987), and not nuclear-membrane association.

CHAPTER 4.

Prostate Cell Responses to Exogenous Agonists and Antagonists.

INTRODUCTION.

Hyperthermic therapy of prostate cancer is usually administered in conjunction with some form of radio-/endocrine-therapy (LINDNER *et al*, 1990; HOLMES *et al*, 1990). Having assessed the effects of temperature on the growth of the two human prostate cell lines LNCaP and DU-145 it was important to characterise the growth response of these cell lines to exogenous ligands. In order to simplify the understanding of these investigations, the cells were maintained at 37°C throughout.

METHODS.

The ligands of interest were the androgen 5-α-dihydrotestosterone (5αDHT), and the nonsteroidal antiandrogen α-α-α-trifluoro-4-hydroxy-2-methyl-4'-nitro*m*-propionotoluidine or hydroxy-flutamide (OH-Flut.).

These were both dissolved in absolute alcohol to form stock solutions of 1×10^{-4}M which could then be diluted into culture medium to produce working concentrations of negligible alcohol content.

LNCaP and DU-145 cells were subcultured so as to form 20% monolayers within 24-well multiwell tissue culture plates. After 24 hours establishment, the RPMI-1640 medium was removed, the cells washed

using PBS-A, and the medium replaced with RPMI containing 2.5% HIDCC-dyalised-FCS (METHODOLOGY section 2.2.3.). Either OH-Flut. or 5αDHT was then added to duplicate wells to cover the range 1×10^{-6}M to 1×10^{-14}M. Quadruplet control cells were included for each cell line.

After a 48 hour incubation at 37°C, the culture medium was removed and the cell monolayers washed using PBS-A. The cells were then harvested and assayed for DNA as described in the Part 1. EXPERIMENTAL chapter 1. However, due to the smaller surface area only 1.0ml per well of 0.2% SDS in ETN solution was used to solubilise the contents of each well, and only aliquots of 20µl were assayed for DNA by the Hoechst method (METHODOLOGY section 4.2)

RESULTS.

The results are expressed in the form of dose-response curves. FIGURE 34. shows the growth response of LNCaP cells to both OH-Flut. and 5αDHT, whereas FIGURE 35. illustrates the dose response curves exhibited by DU-145 cells.

The data points are obtained from the triplicate DNA assay of duplicate ligand tests.

FIGURE 34. **Dose Response Curve for 4-hydroxy-flutamide and 5α–dihydrotestosterone in LNCaP Cells.**

LNCaP cells were exposed to 4-hydroxy-flutamide (OH-Flut) and 5α–dihydrotestosterone (5αDHT) over a range of concentrations from 1×10^{-6}M to 1×10^{-14}M for 48 hours.

Data points represent duplicate wells of cells assayed for DNA in triplicate.

The Effect of Androgen & Antiandrogen on LNCaP Cell Growth.

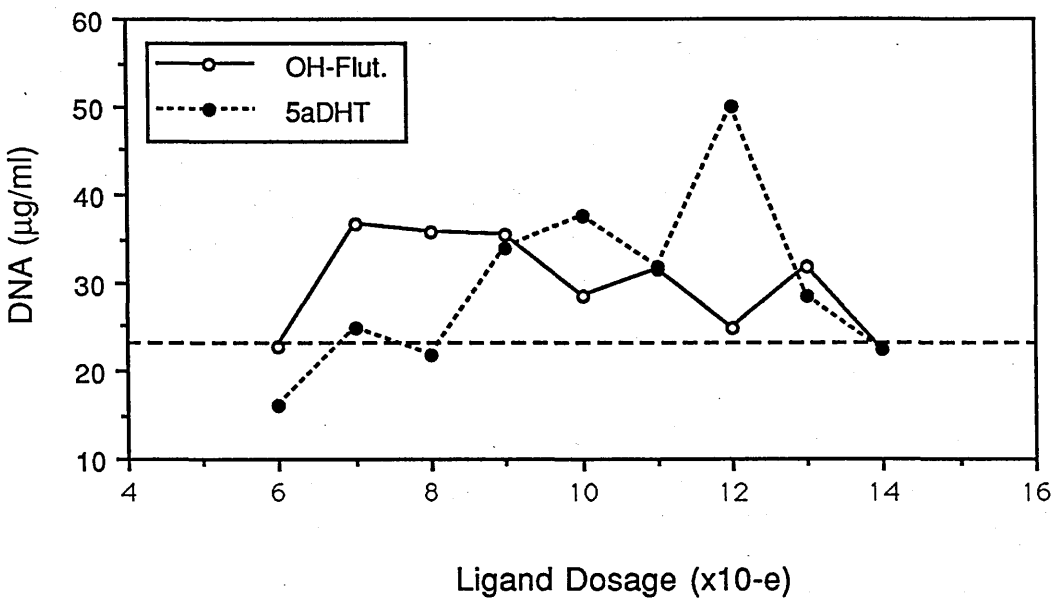
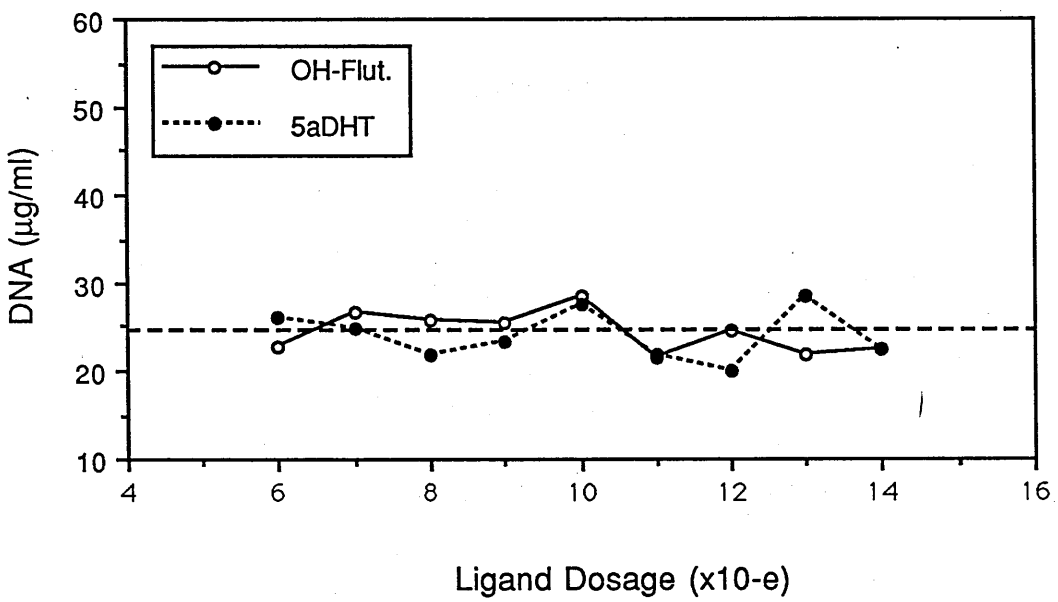


FIGURE 35. **Dose Response Curve for 4-hydroxy-flutamide and 5α–dihydrotestosterone in DU-145 Cells.**

DU-145 cells were exposed to 4-hydroxy-flutamide (OH-Flut) and 5α–dihydrotestosterone (5αDHT) over a range of concentrations from 1×10^{-6}M to 1×10^{-14}M for 48 hours.

Data points represent duplicate wells of cells assayed for DNA in triplicate.

The Effect of Androgen & Antiandrogen on DU-145 Cell Growth.



CONCLUSIONS.

FIGURE 34. shows that LNCaP cell growth responds positively to the influence of the androgen 5αDHT. However, the positive stimulation of LNCaP growth by the antiandrogen OH-Flut. conflicts with the view of the *in vivo* antiandrogenic effect of this drug (POYET & LABRIE, 1985) but is in agreement with a recent report that suggests that this drug does have an androgenic activity (OLEA *et al*, 1990). The possible mechanism behind this anomalous dual activity will be examined elsewhere (see DISCUSSION section 4.)

FIGURE 35. demonstrates equally convincingly that the DU-145 prostate cell line is an androgen-independent cell line in terms of growth regulation; as there was no significant response to either OH-Flut. or 5αDHT.

These two cell lines were exposed to three other ligands in a similar manner to OH-Flut. and 5αDHT. These were flutamide, the non-hydroxylated form of OH-Flut., cyproterone acetate (commercially available as Cyprostat®) and testosterone. Cyproterone acetate is a steroid derivative which has been observed to be an androgenic agent both *in vivo* (POYET & LABRIE, 1985) and *in vitro* (OLEA *et al*, 1990). This activity is said to be independent of its recognised progestational and glucocorticoid action; although in our hands LNCaP cells fail to show any response to cyproterone acetate (data not shown).

There was a similar lack of response to flutamide or testosterone in either cell line (data not shown), possibly because these compounds represent

the systemic, nonactivated forms of OH-Flut and 5αDHT. LNCaP cells may be lacking some activating metabolic process that renders these compound inactive *in vitro*. For example, the LNCaP colony used here may have been deficient in 5α-reductase though time did not permit exploration of this possibility.

CHAPTER 5.

The Effect of Heat and Exogenous Ligands on Prostate Cell Androgen Receptor.

INTRODUCTION.

The collective aim of these experiments was to construct an *in vitro* model for the combined effects of heat and antiandrogens on prostate cancer cells. The preceding two chapters assessed the effects of the individual actions of increased temperature and exogenous ligands on LNCaP and DU-145 prostate cell growth. The experiments described in this chapter were aimed to assess the effects of these two factors on the availability of AR.

METHODS.

LNCaP and DU-145 cells were subcultured as described in METHODOLOGY section 2.2. so that 20% confluent monolayers were formed within 75cm^2 plastic tissue culture flasks. Five flasks were used for each cell line: two of these flasks were used to investigate the effects of heat shock of which one was kept as the control flask and the other was a heat shocked-flask; the other three flasks were used to measure the effects of exogenous ligands and were control, OH-Flut. and 5αDHT respectively. Both cell lines were allowed to "plate-down" for 24 hours in 10% FCS RPMI-1640 culture medium.

Heat shock (HST 1*+2*) was administered in an analogous manner to the experiments described in Part 2. EXPERIMENTAL chapter 2.; however there was only one harvesting stage when all flasks were harvested after the 12 hour recovery period.

The three flasks used to investigate the effects of exogenous ligands were washed free of 10% FCS RPMI-1640 medium, this being replaced by 2.5% HIDCC-dyalised-FCS (METHODOLOGY section 2.2.3.) as described in the preceding chapter. The ligands (OH-Flut. and 5αDHT) were then added to this medium to a final concentration of 1×10^{-9}M, and the cells incubated for 48 hours.

After incubation, the cells were harvested by trypsinisation, washed free of trypsin and 5αDHT or OH-Flut. by resuspension in PBS-A and centrifugation, and prepared for AR ligand binding assay as described in Part 1. EXPERIMENTAL chapter 4. DNA was assayed at this point.

RESULTS.

The results shown are the mean of duplicate experiments each of which involved triplicate assay of AR by the "two-point competition" method (see METHODOLOGY section 3.1.5.).

FIGURE 36. shows the effect of heat shock on the AR binding ability of both LNCaP and DU-145 human prostate cells. FIGURE 37. shows the effects of the androgen 5αDHT and the antiandrogen OH-Flut. on the AR binding ability of these cells. Results are expressed in terms of femtomoles of androgen receptor per mg of DNA.

FIGURE 36. **The Effects of Heat Shock on Prostate Cell AR.**

LNCaP and DU-145 cells were exposed to double heat shock and the effect on cellular AR compared to cells maintained at 37°C.

The double heat shock treatment (HST 1*+2*) consisted of 39°C for 2 hours followed by 37°C for 12 hours, prior to 42°C for 2 hours followed by 37°C for 12 hours.
Controls were maintained at 37°C throughout.

The effects of heat shock was assessed by measuring the amounts of AR using triplicate "two-point" competition ligand binding assay.

Error bars represent the Standard Deviation of the data.

The Effect of Heat Shock on Prostate Cell Androgen Binding Capacity.

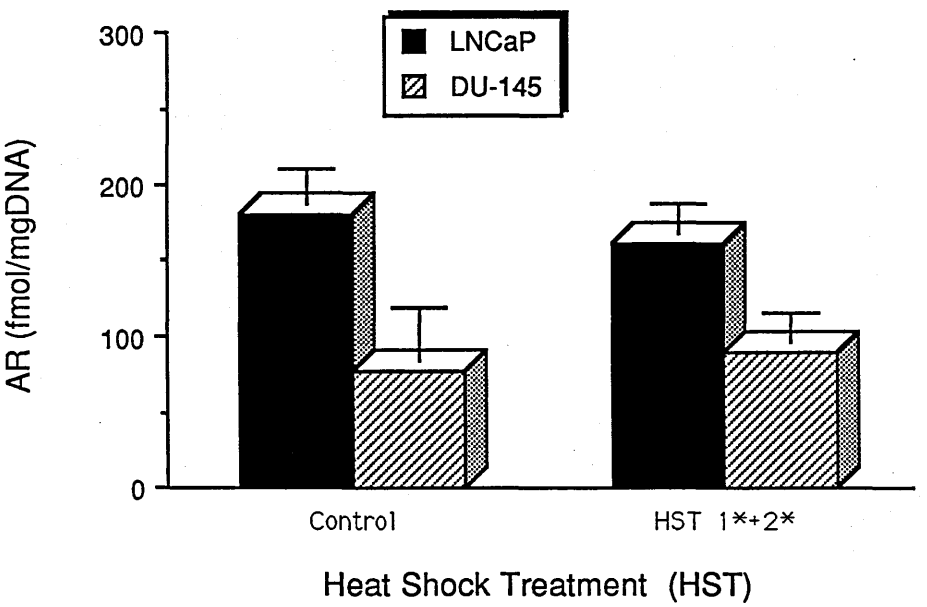


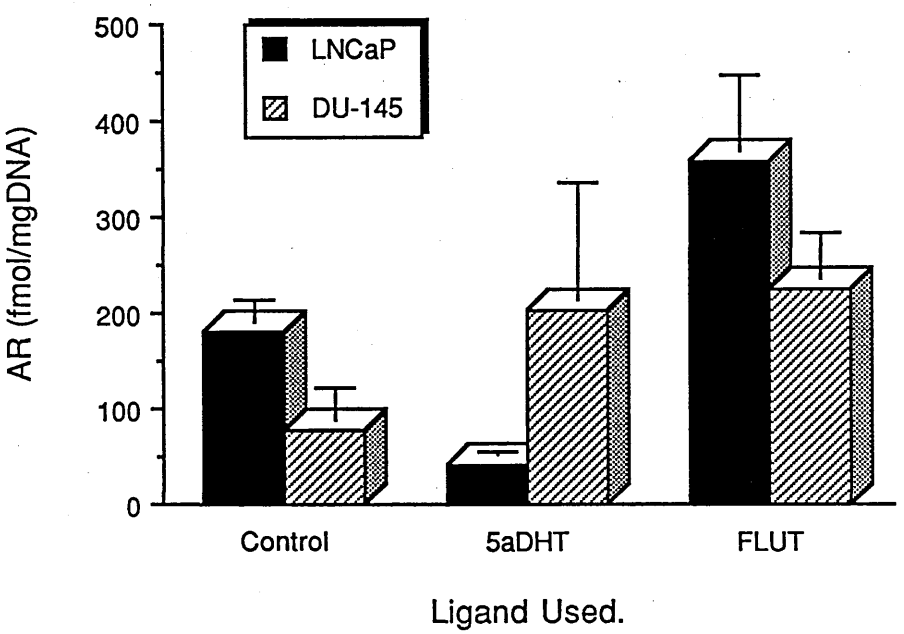
FIGURE 37. **The Effects of Androgen Agonist and Antagonist on Prostate Cell AR.**

LNCaP and DU-145 cells were exposed to 1×10^{-9}M 4-hydroxy-flutamide (OH-Flut) and 1×10^{-9}M 5α–dihydro testosterone (5αDHT) over 48 hours to assess the effects of these ligands on prostate cell AR.

The effects of these exogenous ligands was assessed by measuring the amounts of AR in duplicate experiments using triplicate "two-point" competition ligand binding assay.

Error bars represent the Standard Deviation of the data.

The Effect of Androgen Agonist & Antagonist on Prostate Cell AR.



CONCLUSIONS.

FIGURE 36. demonstrates that heat shock has no effect on the androgen-binding capacity of either LNCaP or DU-145 cells. This result is in contrast to that of the oestrogen-binding capacity of heat shocked MCF-7 cells reported in Part 1. EXPERIMENTAL chapter 4. However this discovery may indicate that there is a fundamental difference in the relationships held between hsp 90 and these two different steroid receptors.

FIGURE 37. confirms the earlier results obtained in Part 2. EXPERIMENTAL chapter 4. showing androgen and antiandrogen sensitivity of LNCaP cells, yet neither ligand was shown to have any effects on DU-145 growth. The LNCaP cell line demonstrates the classic effect of steroid-binding. Androgen-binding was shown to down-regulate its own receptor; reflected in a decrease in available AR levels in response to 5αDHT. OH-Flut., however, resulted in an increase in AR. This supports the hypothesis that the androgenic action of this "antiandrogen" is not by acting through the AR, but instead it acts by relieving some inhibitory function (OLEA *et al*, 1990).

The DU-145 cell line failed to demonstrate any down-regulation of AR in response to either OH-Flut. or 5αDHT. Indeed there was a slight up-regulation of AR though the significance of this, if any, was not examined.

CHAPTER 6.

The Combined Effects of Heat and "Anti-Androgen" on Prostate Cell Growth.

INTRODUCTION.

It was hoped that this cumulative investigation into the combined effects of hyperthermia and antiandrogens on prostate cells *in vitro* could be used to aid the design of suitable models for prostate cancer therapy. In order to simulate the actions of clinical therapeutic methodology the effects of heat together with antiandrogen must be observed. This culminating investigation was completed in two ways:

Firstly, the response of both LNCaP and DU-145 cells to hydroxy-flutamide was measured after growth either at 37°C, or alternatively after heat shock. Conversely, these cell lines were grown in either the presence or absence of hydroxy-flutamide and then any alteration in their ability to survive heat shock was measured.

METHODS.

LNCaP and DU-145 cells were subcultured as described in METHODOLOGY section 2.2. so that 20% confluent monolayers were formed within 75cm^2 plastic tissue culture flasks. Six flasks were used for each cell line: three of these flasks were used to investigate the effects of heat shock on the response to OH-Flut. The other three flasks were used to measure the effects of OH-Flut. on the heat shock response. (See figure legends for the

details of the experimental treatments used).

Prior to the experiment, both cell lines were allowed to "plate-down" for 24 hours in 10% FCS RPMI-1640 culture medium; this was then replaced by 2.5% HIDCC-dyalised-FCS (METHODOLOGY section 2.2.3.).

Heat shock (HST 1*+2*) was administered to both LNCaP and DU-145 cells in an analogous manner to the experiments described in Part 2. EXPERIMENTAL chapter 2. After the final 12 hour recovery period the cell culture medium was brought to a final concentration of 1×10^{-9}M OH-Flut. and the cells were incubated for a further 48 hours before harvesting.

Simultaneously, LNCaP and DU-145 cells were grown in the presence of 1×10^{-9}M OH-Flut. (the dose giving the plateau response in the growth study, see FIGURE 34.) for 48 hours and then subjected to HST 1*+2* heat shock; before being harvested after the final 12 hour recovery period.

Cells were harvested using 0.2% SDS in ETN and then assayed for DNA in triplicate, (previously described in *Part 1.* EXPERIMENTAL chapter 1).

RESULTS.

FIGURES 38a. & 38b. show the effect of prior heat shock on the response of the prostate cell lines LNCaP and DU-145 to the antiandrogen OH-Flut.

Correspondingly, FIGURES 39a. & 39b show the effects of prior OH-Flut. exposure on the subsequent response of these cell lines to heat shock.

These combination experiments were carried out only once, however the values represent triplicate DNA-assay of duplicate flasks of cells.

Statistical analysis of variance (ANOVA) was used to compare treatments.

FIGURE 38. **The Effects of Heat Shock on Antiandrogen action in Prostate Cells.**

FIGURE 38a. shows the LNCaP cell response to antiandrogen after prior double heat shock treatment, assessed in terms of cell growth.

FIGURE 38b. shows the DU-145 cell response to antiandrogen after prior double heat shock treatment, assessd in terms of cell growth.

The histogram categories represent the sequence of the various types of treatment:

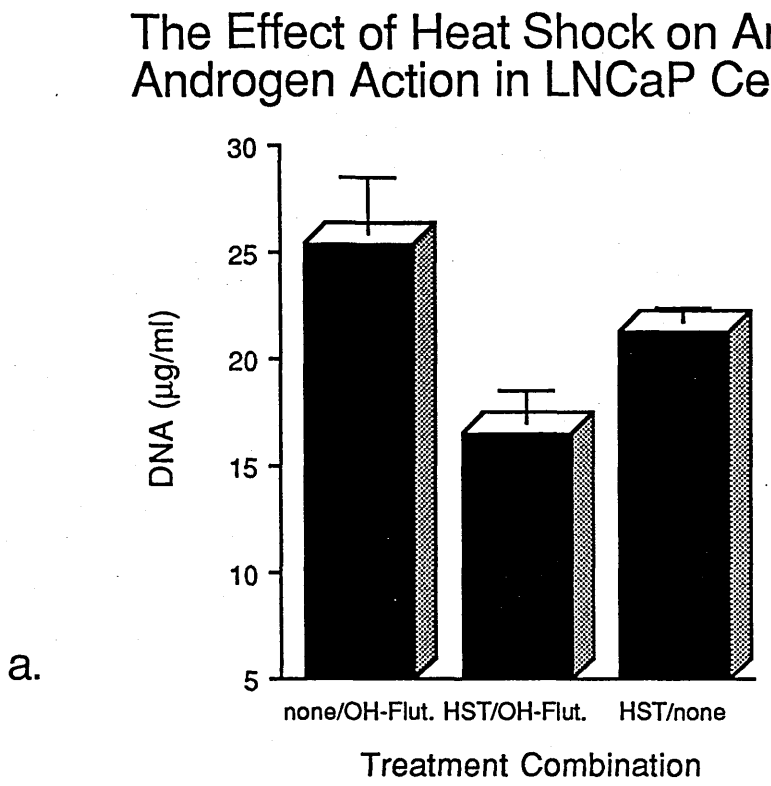
none	37°C/no antiandrogen,
HST	HST 1*+2*,
OH-Flut.	1×10^{-9}M hydroxy-flutamide

The double heat shock treatment (HST 1*+2*) consisted of 39°C for 2 hours followed by 37°C for 12 hours; prior to 42°C for 2 hours followed by 37°C for 12 hours.
Controls were maintained at 37°C throughout.

The cells were exposed to 1×10^{-9}M 4-hydroxy-flutamide (OH-Flut.) for 48 hours, after the second heat shock recovery period.

To assess cell growth, triplicate DNA assays were carried out on duplicate flasks of cells. Error bars represent the Standard Deviation of the data.

a.


The Effect of Heat Shock on Anti-Androgen Action in LNCaP Cells.
DNA (µg/ml)
30
25
20
15
10
5
none/OH-Flut.
HST/OH-Flut.
HST/none
Treatment Combination

b.

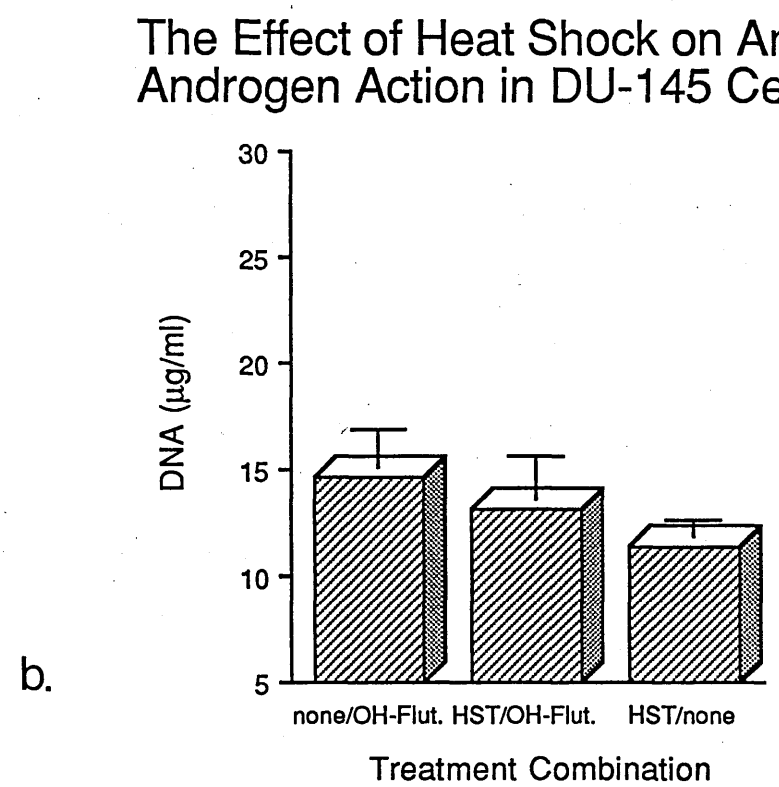

The Effect of Heat Shock on Anti-Androgen Action in DU-145 Cells.
DNA (µg/ml)
30
25
20
15
10
5
none/OH-Flut.
HST/OH-Flut.
HST/none
Treatment Combination

FIGURE 39. **The Effects of Antiandrogen on Prostate Cell Thermotolerance.**

FIGURE 39a. shows the LNCaP cell response to heat shock after prior exposure to antiandrogen, assessed in terms of cell growth.

FIGURE 39b. shows the DU-145 cell response to heat shock after prior exposure to antiandrogen, assessed in terms of cell growth.

The histogram categories represent the sequence of the various types of treatment:

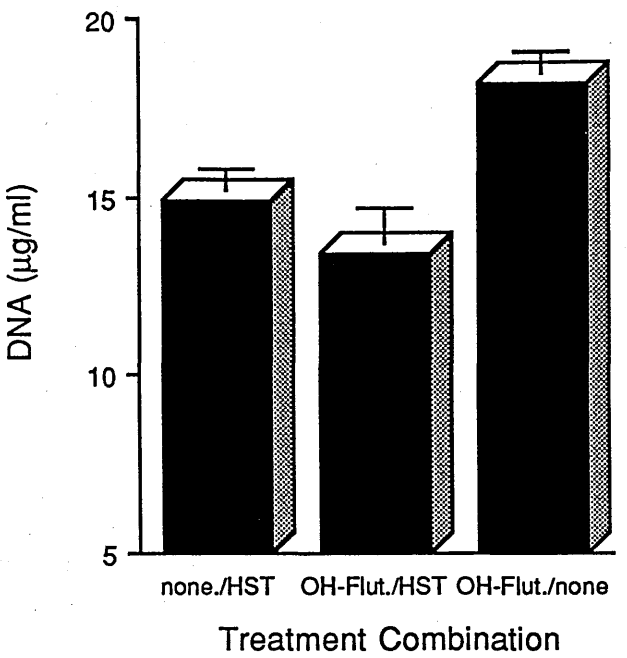
none	37°C/no antiandrogen,
HST	HST 1*+2*,
OH-Flut.	1×10^{-9}M hydroxy-flutamide

The cells were exposed to 1×10^{-9}M 4-hydroxy-flutamide (OH-Flut) for 48 hours, prior to commencing heat shock treatment.

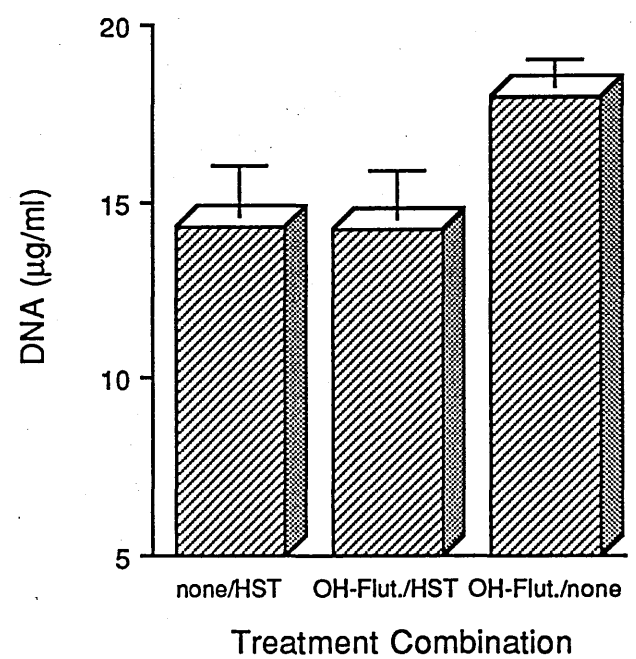
The double heat shock treatment (HST 1*+2*) consisted of 39°C for 2 hours followed by 37°C for 12 hours; prior to 42°C for 2 hours followed by 37°C for 12 hours.
Controls were maintained at 37°C throughout.

To assess cell growth, triplicate DNA assays were carried out on duplicate flasks of cells. Error bars represent the Standard Deviation of the data.

a.


The Effect of Antiandrogen on LNCaP Cell Thermotolerance.
DNA (µg/ml)
20
15
10
5
none./HST
OH-Flut./HST
OH-Flut./none
Treatment Combination

b.


The Effect of Antiandrogen on DU-145 Cell Thermotolerance.
DNA (µg/ml)
20
15
10
5
none/HST
OH-Flut./HST
OH-Flut./none
Treatment Combination

CONCLUSIONS.

These results represented within these figures demonstrate that there exists a complex relationship between the actions of heat shock and the actions of the antiandrogen 4-hydroxy-flutamide. Double heat shock treatment (HST 1*+2*) was shown to induce the thermotolerance response in LNCaP cells (Part 2. EXPERIMENTAL chapter 2.); while resulting in approximately 5-10% loss of overall growth. It is not so surprising then, that cells exposed to a prior heat shock show less growth than cells maintained at 37°C when they are both exposed to OH-Flut. (FIGURE 38a.). However, the combination of heat shock followed by antiandrogen resulted in an approximate 23% loss of growth when compared to heat shock alone ($P<0.001$); suggesting that heat shock may act to inhibit the positive proliferative stimulus of the "antiandrogen" shown for the LNCaP cell line both herein (Part 2. EXPERIMENTAL chapter 4.) and by others (OLEA *et al*, 1990).

The LNCaP cells exposed to antiandrogen prior to HST 1*+2* exhibited similar, though smaller ($P<0.02$), growth inhibition, with a 10% reduction of growth when compared to HST 1*+2* alone (FIGURE 39a.).

As can be seen in FIGURES 38b. & 39b., neither sequence of HST 1*+2* and OH-Flut. had a statistically significant effect on DU-145 cell growth compared with HST 1*+2* alone. Tentatively, it might be concluded that for heat shock to prime the sensitivity of prostate cells to growth inhibition by antiandrogen, the cells must first be growth-regulated by androgen.

In conclusion, it seems that the combination of heat shock and anti-androgen represents a synergistic partnership capable of suppressing prostate cell growth, but, this phenomenon is confined to the androgen/"antiandrogen"-responsive cell line LNCaP; and is most effective if heat shock induction of the thermotolerance reaction precedes antiandrogen exposure. Thus clinical application of this sequential therapy should be restricted to patients whose tumours are known to be growth regulated by androgens.

DISCUSSION.

There has been much recent interest in the application of heat during the management of cancer patients, the treatment of prostatic cancer in particular has greatly benefited from the instigation of thermal-therapy (SERVADIO *et al*, 1987). Treatment involves heating tumour tissue to between 41°C and 45°C; this range includes the temperatures that were shown in *Part 1* to be capable of inducing both hsp 90 and thermotolerance in breast cancer cells. Such development of tumour-thermotolerance *in vivo* has been demonstrated in conjunction with the use of fractionated hyperthermia (KAMURA *et al*, 1982). In addition, this stepwise type of heating has been shown to induce the thermal resistance of tumours, again *in vivo* (MAHLER *et al*, 1981).

Clearly this is a potentially dangerous situation for the physician using hyperthermia, as there exists the possibility of creating a heat-resistant tumour clone by using an inappropriate heating regime.

Thermal-resistance aside, the acquisition of MCF-7 cell thermotolerance was accompanied by an unpredicted effect on breast cell ER binding capacity (*Part 1*). Although this situation represents an artificial *in vitro* manipulation as regards breast cancer; thermal-manipulation of prostate cancer cells is a clinical reality. The implication that there could be an interaction between thermal and endocrine effects on prostate cancer cells *in vivo* remains uninvestigated, yet it is of potentially great importance due to the combination of antiandrogen and thermal-therapies being developed for treatment of prostatic disease.

With these unanswered questions in mind, it was aimed to create an *in vitro* model of prostatic cancer and investigate the effects of heat and

antiandrogens, individually and in combination.

1. LOCALISATION OF ANDROGEN RECEPTOR IN LNCaP & DU-145 CELLS.

Thermal killing of cancer cells is highly selective (GERWECK, 1985); this seems to be attributable to two compounding factors. The less efficient blood supply results in poorer clearance of toxins and, more importantly, local hypoxia. In addition, the altered glycolytic metabolism within neoplastic cells results in both lower energy levels and cellular acidosis. The combination of hypoxia and lowered pH is thought to sensitise malignant cells to the impact of heat shock.

In the case of neoplastic prostate cells there is one other distinction between the tumour cells and the surrounding tissue: this is androgen (and presumably also antiandrogen) sensitivity. (Though not all prostate cancers are hormone sensitive.) For this reasons two prostate cancer cell lines were used that had intrinsically distinct androgen-responsiveness. Both the LNCaP cell line (OLEA *et al*, 1990) and the DU-145 cell line (STONE *et al*, 1978) contain AR, though only LNCaP cells are androgen-responsive (HOROSZWICZ *et al*, 1983; see also FIGURES 34. & 35.). Immunocytochemical staining was used to verify the presence of AR in both these cell lines. The DU-145 AR staining is shown (FIGURES 21. & 22.) as this was the least obvious; even so there is a very definite localisation of AR within the nuclei and cytoplasm of the DU-145 cells. The AR immunocytochemistry results were reflected by the AR ligand-binding

assay of both cell lines (*Part 2.* EXPERIMENTAL chapter 5.), as LNCaP cells maintained a 2 fold higher level of AR when compared to DU-145 cells.

These results were also confirmed when immunofluorecsent detection of AR was used (FIGURES 24. & 25.). FITC-staining was observed in almost all LNCaP cells, predominating in the cell nucleus. Although AR-staining of DU-145 cells was also intranuclear, only a proportion of the cell population was observed to be AR-positive. This result suggests that the lower values of DU-145 cell AR obtained using ligand-binding assay may be due to a mixture of AR-positive and AR-negative cells, rather than merely lower AR levels throughout the whole DU-145 cell population. The reason for this non-homogeneous phenotypic expression of AR is unclear. It is possible that in the DU-145 cell line, AR expression is a function of either the cell cycle or the degree of cellular maturation. Nevertheless, the AR-positive DU-145 component did not show any growth response to either 5αDHT or OH-Flut. (FIGURE 35.). The true interpretation of this anomalous result must await further investigation; and work is currently being carried out by a colleague, Dr. G. Carruba, to answer some of these questions. The data presented in this thesis have only considered sensitivity of cell lines to androgen in terms of growth response. DU-145 may well show metabolic responses to androgen.

2. PROSTATE CELL RESPONSES TO HEAT SHOCK.

In *Part 1*, the MCF-7 breast cancer cell line was shown to be sensitive to temperatures greater than 42°C, unless first primed by an exposure to a lower temperature, in which case this cell line acquired thermotolerance up to 44°C. With these results in mind, it was decided to investigate whether LNCaP and DU-145 cells displayed similar thermal-sensitivities and thermotolerance induction patterns.

The original heat shock treatments used (HST 1, & HST 2) were identical to the temperatures effective in evoking both thermal-lethality and thermotolerance in the MCF-7 cell line. As demonstrated by FIGURES 26. & 27., the first HST of 41°C, was in fact a lethal temperature for both prostate cell lines. This result is surprising, but conclusive; there is absolutely no growth of DU-145 cells after exposure to 41°C. There seems to be a small proportion of LNCaP cells that remain growing after this temperature, though the overall growth levels are significantly less than the 37°C controls. Acquisition of thermotolerance during heat shock has been demonstrated (HERMAN *et al,* 1981) and it is possible that a minority within the LNCaP cell population is able to withstand this temperature due to a spontaneous inducement of thermotolerance.

When the heat shock treatments were both decreased by 2°C (HST 1* & HST 2*; 39°C & 42°C respectively) similar growth profiles were obtained for both prostate cell lines as compared to those previously obtained for the MCF-7 cell line (FIGURES 28. & 29.).

Combining these results allows assignment of a point of thermal-sensitivity for both prostate cell lines. 41°C is the critical temperature at which LNCaP and DU-145 cell growth is inhibited. However, prior exposure to 39°C acts to prime the prostate cells, and endows them with thermotolerance; presumably through hsp induction. Such expression of hsp's, and hsp 90 in particular, has two fold importance:

Firstly, the expression of hsp's may be one possible mechanism for the temperature-mediated suppression of cell growth in the absence of mere thermo-killing. Secondly, the accumulation of excess nuclear hsp 90 could modulate altered androgen and antiandrogen effects and so alter the efficacy of endocrine management of prostate cancer. To assess the relevance of hsp 90 and thermotolerance expression in heat shocked prostate cells immunocytochemistry was used to localise hsp 90 before and after thermotolerance induction.

3. HEAT SHOCK MEDIATES NUCLEAR ACCUMULATION OF PROSTATE CELL hsp 90.

In *Part 1*, heat shock was shown to result in an increase in the overall level of MCF-7 cell hsp 90, and more importantly a nuclear accumulation of hsp 90. When LNCaP and DU-145 cells were subjected to the lesser double heat shock treatments (HST 1*+2*) and the levels of hsp 90 assessed using fluorescent immunocytochemistry, broadly similar results were obtained.

The effect of heat shock is underscored when the immunofluorescent observations are compared with the immunocytochemical staining carried out on both the LNCaP and DU-145 cells and a benign prostate section (*Part 2,* EXPERIMENTAL chapter 1.). Both the DU-145 cells (FIGURE 22b.) and the tissue section (FIGURE 23.) show profoundly cytoplasmic hsp 90 immunocytochemical staining, in broad agreement with the cells maintained at 37°C and stained using the fluorescently-labelled antiserum. In addition, fluorescent immunocytochemistry shows that heat shock results in distinct accumulation of hsp 90 in both the LNCaP and DU-145 cell nuclei. Similar cytoplasmic immunohistochemical staining of hsp 90 in various steroid receptor containing tissues has been noted (PEKKI & TUOHIMAA, 1990), though nuclear accumulation of hsp 90 has not been recorded.

The significance of these observations is two-fold. Firstly, on a general level, this evidence affirms that the results obtained using MCF-7 cells were not peculiar to a breast cell line and that the heat shock-induced nuclear accumulation of hsp 90 is a more general effect. This result also ratifies the validity of the immunocytochemical methodology.

More importantly however, the nuclear accumulation of LNCaP and DU-145 cell nuclear hsp 90 has implications for the responses to steroid receptor-mediated endocrine control mechanisms.

4. LNCaP AND DU-145 CELL RESPONSES TO ANDROGENS AND "ANTIANDROGENS".

The overall aim of these experiments was to create an *in vitro* model to aid understanding of the combined thermal and endocrine therapy of prostate cancer. In order to fully appreciate the responses of prostate cells to these combined therapies, it was first necessary to identify the growth effects of each parameter individually. Having assessed the effects of heat and thermotolerance induction in both LNCaP and DU-145 cells, it was aimed to characterise their responses to both androgen and antiandrogen exposure.

LNCaP cells showed the typical response to the addition of exogenous mitogenic ligands: growth was stimulated in a dose dependent manner. Maximum growth occurred when the androgen 5α-dihydrotestosterone (5αDHT) was used at a concentration of 1×10^{-12}M, although the hormone was significantly mitogenic over a range of 10^{-9} to 10^{-13}M. This value is lower than that found by others (WILDING *et al*, 1989; SCHUURMANS *et al*, 1988) who report optimum concentrations of between 10^{-9}M and 10^{-10}M for 5αDHT mitogenecity. This difference could simply reflect variations between different clones of LNCaP cells, or differences in culture conditions.

What was interesting was the demonstration of the *in vitro* androgenic effect of the "antiandrogen" 4-hydroxy-flutamide (OH-Flut.) on LNCaP cell

growth. 10^{-8}M OH-Flut. was shown to be the optimum concentration for stimulating LNCaP cell growth, although at no point over the range of concentrations used (10^{-6}M - 10^{-14}M) was OH-Flut. found to be in any way inhibitory or antiandrogenic. These data are in entire agreement with the work of others (OLEA *et al*, 1990; WILDING *et al*, 1989) confirming the paradoxical *in vitro* response of this antiandrogen.

The DU-145 cells failed to respond to either 5αDHT or OH-Flut. This androgen ambivalence was the expected resulted due to the characterised non-responsiveness of this cell line to androgen-stimulation (Dr. C. Eaton, *personal communication*).

The confusing observations regarding OH-Flut. action *in vitro* have given rise to speculation as to its mechanism of action *in vivo.* These hypotheses are derived from the theory of *indirect negative* growth control (SOTO & SONNENSCHEIN, 1987).

Direct positive growth control is exemplified by the action of oestrogen or androgen; where ligand-binding results in the direct stimulation of mitogenesis. *Indirect positive* growth control is illustrated in the case of oestrogen-induction of growth factors which are then mitogenic in either a paracrine or autocrine manner. *Indirect negative* growth control can be defined as the binding of the primary ligand (eg. oestrogen or androgen) to an inhibitory factor, thus relieving its inhibition and allowing mitogenesis to occur. Such inhibitory factors have been termed

oestrocolyone and androcolyone respectively, and seem to be present as trypsin sensitive extracellular steroid-binding moieties (SONNENSCHEIN *et al*, 1989).

It is thought that the antiandrogenic action of OH-Flut. is mediated through AR binding, and that the androgenic action of OH-Flut. is mediated through binding of OH-Flut. to androcolyone, relieving androcolyone-inhibition of LNCaP cell proliferation (OLEA *et al*, 1990). This conclusion was formed on the basis of various competition experiments when 5αDHT was given in conjunction with OH-Flut. It was found that the responses of LNCaP cells to 5αDHT were typical steroid receptor-mediated responses, displaying dose dependent proliferation shut-off. However, OH-Flut. failed to display this response, and had similar growth induction kinetics as oestrogens; even though LNCaP cells contain no ER (OLEA *et al*, 1990). These workers stress the possible misappropriateness of using putative antiandrogens for the treatment of androgen-sensitive prostate cancer.

*As previously mentioned (Partl Discussion section 5), tamoxifen has both agonistic and antagonistic effects. This is thought to be due to differential induction of the promoters TAF-1 and TAF-2 with respect to their induction by oestrogen.

The possibility that OH-Flut, or other antiandrogens may modulate androgen-response elements in a similar manner remains uninvestigated, however this type of dual action would result in the paradoxical androgenic activity observed when using OH-Flutamide.

5. THERMAL AND ENDOCRINE REGULATION OF PROSTATE CELL ANDROGEN-BINDING CAPACITY.

In *Part 1*, heat shock accompanied an abolition of the oestrogen-binding capacity of MCF-7 cells. This was hypothesised to be due to an accumulation of nuclear hsp 90, which acted as an inhibitor of ER-binding ability. It was of interest to investigate whether heat shock had a similar effect on prostate cell AR as in breast cell ER. This was not in fact the case. Neither LNCaP or DU-145 cell AR levels, as measured by ligand-binding, altered significantly as a response to heat shock. This disparate result may be attributable to two parameters: either the receptor inhibition could be a phenomenon peculiar to breast cells, or alternatively the blocking of ligand-binding by hsp 90 could be a phenomenon exclusive to ER. In order to answer this question the effect of heat shock on breast cell AR was measured. MCF-7 cells were used as these contain significant levels of AR (40 fmol/mg protein), whereas LNCaP cells are devoid of ER (OLEA *et al*, 1990).

Interestingly, heat shock resulted in a 40% decrease in the AR-binding of heat shocked MCF-7 breast cancer cells (see TABLE 2.). This suggests that the heat shock mediated down-regulation of steroid receptor activity is peculiar to the breast cell line, and absent from the prostate cell lines. However, there is some specific difference in the receptor down-regulation, as the almost total inhibition of ER-binding activity is not completely reproduced by AR. The reason for this inconsistent effect is unclear, but it serves to underline the dangers of extrapolating the

qualities of different steroid receptors within the superfamily in the absence of scientific evidence.

TABLE 2. THE EFFECT OF HEAT SHOCK ON THE ANDROGEN BINDING CAPACITY OF MCF-7 HUMAN BREAST CANCER CELLS.

Treatment	ER (fmol/mg protein)	Std. Devn.	No. of Expts.
HST 0	12.29	2.27	6
HST 1+2	7.32	1.36	6

TABLE 2. shows the effect of heat shock on MCF-7 cell AR as assessed by ligand-binding assay. The experimental methodology was exactly as that previously described for the effect of heat shock on MCF-7 cell ER in *Part 1* EXPERIMENTAL chapter 4., except that AR-assay, as opposed to ER-assay was carried out.

Having shown that heat shock-induced thermotolerance has no effect on ligand-binding by prostate cell AR, it was decided to investigate whether this ineffectiveness is due to an altered AR response, or an altered hsp 90 response. To verify AR functionality, the levels of AR within LNCaP and DU-145 cells were measured in the presence of different endocrine effecters. The results of these experiments showed that LNCaP cell AR is down-regulated when the cells are exposed to androgen, and up-regulated when the cells are exposed to antiandrogen. This pattern of receptor modulation is well documented and is typical of functional steroid receptor.

DU-145 cells, however, failed to show a significant control of AR levels in response to either androgen or antiandrogen. These results confirm the androgen-insensitivity of this cell line.

Taken together, these experiments illustrate that the LNCaP cells contain functional AR, while the DU-145 cells are androgen-insensitive in terms of growth. However, since neither cell line shows a down-regulation of AR accompanying heat shock-induced thermotolerance, it seems unlikely that the functionality of the AR bears any relevance to the heat shock-mediated suppression of steroid-binding ability. There have been recent suggestions that the AR of LNCaP cells is abnormal with respect to steroid specificity and receptor activation (VELDSCHOLTE & MULDER, 1990). This is thought to be due to point mutations within the steroid-binding domain (BRINKMANN *et al,* 1990), and so it may be dangerous to form conclusions based on LNCaP cell AR.

6. COMBINED HEAT AND ANTIANDROGEN TREATMENTS OF PROSTATE CELLS.

The overall aim of these experiments was to create an *in vitro* model representing the clinical treatment of prostate cancer. Having assessed the effects of heat, androgen and antiandrogen on prostate cell growth and AR expression, the various treatments were combined in order to simulate the administration of antiandrogen to patients receiving thermal-therapy.

In practice, administration of antiandrogen in conjunction with thermal-therapy results in more effective treatment of prostate cancer compared with the individual use of either method (SERVADIO *et al*, 1987). The suppression of growth by these therapies seems to be synergistic, and not merely additive. Interestingly, this phenomenon is echoed in the observations in this thesis of the LNCaP cell response to combined heat shock and antiandrogen therapy, while the DU-145 cells remained ambivalent to the inclusion of antiandrogen during heat shock exposure.

DU-145 cell insensitivity to the inclusion of antiandrogen was predicted by the results of previous experiments. Antiandrogen failed to be an effecter of mitogenisis in this cell line, and did not assert any changes in the regulation of AR levels. For these reasons the combination of OH-Flut. with heat was not expected to significantly alter the DU-145 cell response to heat shock alone.

The response of the LNCaP cell-line to combined heat and antiandrogen exposure was especially surprising in view of the positive growth response

displayed by LNCaP cells in response to OH-Flut. reported herein and by others (OLEA *et al*, 1990; WILDING *et al*, 1989). Somehow, heat shock led to an alteration in the LNCaP cell response to OH-Flut.: a conversion of OH-Flut. activity from that of an androgen to that of an antiandrogen. This effect was most obvious when heat was administered prior to OH-Flut. exposure, however when OH-Flut. was present prior to commencing heat shock a smaller, additive inhibition of LNCaP cell growth was still observed.

The mechanism behind the anomalous "conversion" of OH-Flut. activity must be heat-mediated; and may possibly be hsp-mediated. There exists a variety of potential mechanisms capable of reversing the androgen-mitogenicity manifested by LNCaP cells. These hypotheses can be divided into hsp 90-mediated effects or receptor-mediated effects:

6.1. Antiandrogen sensitises androgen-responsive cells to heat shock.

The first hypothesis is that the antiandrogen is able to increase cellular sensitivity to heat shock. Heat shock has been shown both here, and by others (WELCH & SUHAN, 1986; YOST & LINDQUIST, 1986) to result in an over-production of hsp's, including hsp 90 (CATELLI *et al*, 1985). Hsp 90 is also though to play a pivotal role in the regulation of normal protein synthesis during heat shock (ROSE *et al*, 1989); regulating both the translational shut-down, and subsequent recommencement of normal protein synthesis. It is therefore possible that by reducing the cellular pool of hsp 90, the thermotolerant response to heat shock could be debilitated.

How could this reduction in hsp 90 availability occur? Referring to the model of Baulieu discussed in *Part 1* DISCUSSION section 5., the inhibitory action of antisteroids is due to the production of nonactive steroid receptors; created as a result of antisteroid-binding producing a heteromeric receptor incapable of undergoing activation (BAULIEU, 1987a & b.). Hence the steroid-binding subunits are tightly bound to hsp 90; locked together as a consequence of antihormone-binding . It should be reinforced that this model does not entirely agree with all the observations concerning antisteroidal activation of steroid receptors (RICHARD-FOY *et al*, 1987). However, it does raise the possibility that if the action of antisteroids is to promote the steroid receptor-hsp 90 association, then the availability of both components is reduced. The inference then, is that the pool of hsp 90, and nuclear hsp 90 in particular, would be reduced; and as a result, efficient regulation of normal protein synthesis during recovery from heat shock would be debilitated. This concept would explain why antiandrogen evokes thermal sensitivity, selectively within cells that are normally androgen-responsive.

Such a scenario is reflected in the clinical observations of patients undergoing combined endocrine and thermal therapies for prostatic disease (S.N. LLOYD, *personal communication*). Pathalogically AR-positive tumour tissue undergoes significant regression while the surrounding normal tissue, also in contact with the thermal applicator, remains undisturbed. The synergistic action of OH-Flut. may therefore be manifested as a sensitisation agent for heat shock, specific to AR positive tissue.

6.2. Heat shock enhances cellular responses to Antiandrogen.

Heat shock may enhance the putative antiandrogenic qualities demonstrated by OH-Flut. *in vivo,* but not *in vitro.*

As discussed in *Part 1* DISCUSSION section 4., it is thought that part of the androgenic action of OH-Flut. is mediated through a relief of androcolyone-inhibition of LNCaP cell proliferation as a consequence of OH-Flut.-binding to androcolyone (OLEA *et al*, 1990). However, this androgenic effect can be "shutoff" by higher concentrations of either OH-Flut. or 5αDHT, suggesting that a proportion of OH-Flut-androgenicity occurs as a result of AR-binding.

Referring again to the modification of Baulieu's model (*Part 1* DISCUSSION section 5., the inhibitory action of antisteroids is thought to be due to antisteroid-binding producing heteromeric, nonactive steroid receptors (BAULIEU, 1987a & b.). If this supposition was correct, OH-Flut. would promote a large population of AR locked together with inactivating hsp 90. This would negate one pathway of androgen stimulation of proliferation for both OH-Flut. and 5αDHT. Consequently, the inclusion of thermal therapy may not only promote formation of this nonactive AR by virtue of the excess nuclear hsp 90, but may also enhance the "locking" effect of OH-Flut., again by increasing the available nuclear hsp 90. In this manner heat shock is enhancing the LNCaP susceptibility to AR-inactivation by OH-Flut.

Again, this hypothesis is in broad agreement with the clinical observations and the results contained within this thesis. However, much more information

must be obtained on the actual mechanism of OH-Flut. action both *in vivo* and *in vitro*, and the relevance of indirect negative growth regulation by steroid hormones before these theories can be ratified.

On a more general level it is hoped that these results may, at the very least, open a new avenue of thought on the therapeutic mechanism of thermal-therapy. Furthermore, the various observations culled from the use of the cell line models may aid in the optimisation of combined thermal and endocrine therapies during the treatment of prostatic disease.

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