INTRACELLULAR BINDING SITES FOR NONSTEROIDAL ANTIOESTROGENS

by

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SUMMARY

This thesis is concerned with the properties of intracellular binding sites for nonsteroidal antioestrogens. In MCF 7 human mammary carcinoma cells the triphenylethylene antioestrogen and antitumour agent tamoxifen (TAM) was bound to a high affinity (K_D = 0.97 ± 0.15 nM at 4°C), saturable binding site (141,300 ± 20,100 sites/cell) which was distinct from the oestrogen-receptor (ER). The distribution of this antioestrogen binding site (AEBS) between nuclear, mitochondrial, microsomal and cytosol subcellular fractions paralleled that of the endoplasmic reticulum. The interaction between TAM and AEBS was influenced by changes in pH, ionic strength and temperature. The kinetic rate constants k_+ and k_- showed strong temperature dependence, unlike the equilibrium dissociation constant K_D.

The AEBS was also widely distributed in tissues from the rat and was concentrated in the liver (K_D = 0.9 ± 0.1 nM at 0°C, C = 0.78 ± 0.10 nmol/g tissue), where its subcellular distribution and biochemical properties were generally similar to those in MCF 7 cells. Within the rat liver microsomal fraction the distribution of AEBS was intermediate between that of rough and smooth endoplasmic reticulum. The AEBS was found to be an integral membrane protein, apparently requiring liquid for binding activity, and could be solubilized using the detergent sodium cholate, although with reversible loss of binding activity in the solubilized state. Gel filtration and hydrophobic interaction chromatography demonstrated the presence of a truely solubilized, highly hydrophobic AEBS-detergent complex (M_r 440,000 - 490,000).

Ligand specificity studies with a wide range of compounds showed that high affinity binding required a hydrophobic di- or tri-cyclic

aromatic- or heterocyclic-ring structure to which is attached a hydrocarbon side chain terminating in a basic alkyl-substituted secondary or tertiary amino group. Natural or synthetic oestrogens or other steroids had no affinity. Structural changes on several positions influenced ligand affinity, particularly at the terminal amino group. Although ligands for several other cellular binding sites (including calmodulin, cytochrome P-450, and D_1-, H_1- and α_1-adrenergic receptors) showed cross reactivity with the AEBS, structure affinity studies demonstrated that none of these sites had identity with the AEBS.

From the above studies it was concluded that the AEBS was unlikely to be involved in mediating antioestrogenic activity and this was confirmed by in vitro studies of the effects of antioestrogens and structurally related compounds on MCF 7 cell proliferation. Antioestrogenic activity was instead correlated with affinity for ER. However, compounds which bound to AEBS generally displayed cell-cycle specific cytotoxic antitumour activities at high concentrations (5 - 20 μM), which were apparently not ER-mediated. Such effects, however, were not likely to be directly mediated through the AEBS, but rather through cross-reactive binding sites such as calmodulin.

STATEMENT OF ORIGINALITY

The work described in the body of the text was carried out by myself.

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To Sally, Nicky, Emma and David.

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LIST OF ABBREVIATIONS

AEBS;	antioestrogen binding site
B;	bound ligand
BSA;	bovine serum albumin
C;	binding site concentration
DES;	diethylstilboestrol
DMF;	N,N dimethyl formamide
E_1;	oestrone
E_2;	17β-oestradiol
E_3;	oestriol
ER;	oestrogen receptor
FCS;	foetal calf serum
H;	heavy microsomal fraction
IC_n;	concentration required for n % inhibition
IC_{DD};	concentration required to prevent increase in cell number above drugging density
IC_{CX};	concentration required to reduce cell number to 50% that of drugging density
k+;	association rate constant
k-;	dissociation rate constant
K_D;	equilibrium dissociation constant
L;	light microsomal fraction
NSB;	non-specific binding
PEG;	polyethylene glycol
PgR;	progesterone receptor
PMF;	postmitochondrial fraction
PRL	prolactin
RM;	rough membranes
SM;	smooth membranes
TAM;	tamoxifen
U;	unbound ligand

CHAPTER ONE

BIOLOGY AND MECHANISMS OF ACTION OF NONSTEROIDAL ANTIOESTROGENS

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1.1 INTRODUCTION

Synthetic nonsteroidal compounds, in particular triarylalkenes and -alkane derivatives, comprise the most studied group of oestrogen antagonists. Defined in the broadest sense an oestrogen antagonist is any agent whose action results in the reduction of the biological effects of oestrogen. Nonsteroidal antioestrogens are not pure antagonists, however, and are unable to antagonise all oestrogenic effects in all species and all tissues. Such compounds have a variety of effects both *in vivo* and *in vitro* which include antifertility and antiproliferative (e.g. antitumour) actions. There is good evidence that other mechanisms in addition to antioestrogenicity contribute to these effects.

Oestrogen antagonists have been invaluable for defining the mechanisms of oestrogen action at the molecular level. They are also extremely important clinical agents particularly in the treatment of oestrogen-dependent breast cancer and in the control of female fertility. These properties have encouraged research into the mechanisms governing their biological activities particularly with the aim of developing new treatment strategies and more clinically effective compounds.

Although apparently oestrogen-receptor (ER)-mediated, a precise understanding of the molecular events underlying oestrogen antagonism is far from complete. Interactions of antioestrogens with cellular components other than ER have also been described *in vitro*, but to what extent these interactions mediate oestrogen antagonism and other biological activities *in vivo* is largely unknown.

This Chapter reviews the biological properties of nonsteroidal antioestrogenic compounds and the current theories as to their molecular

modes of action.

1.2 HISTORICAL DEVELOPMENT

The earliest known and so called 'classical' oestrogen antagonists were the natural non-oestrogenic steroids, progestins and androgens. Their effects, mediated largely by non-competitive mechanisms and not involving direct interaction with the ER system, modify rather than counteract the actions of oestrogen and have been reviewed elsewhere (Roberts and Szego, 1953). The finding that weak oestrogens such as oestriol (E_3, Figure 1.1) inhibited uterotrophic activity of the more potent oestrogen, oestradiol (E_2, Figure 1.1) in the rat suggested that direct antagonism might occur through the occupancy of specific oestrogen binding sites whose existence at that time had been postulated but not experimentally confirmed (Hisaw et al., 1954).

Several synthetic nonsteroidal oestrogens were identified that also had oestrogen antagonist activity although this was only expressed upon local administration in vivo, and at very high concentrations in vitro. Thus Bárány et al. (1955) and Miquel et al. (1958) demonstrated that di-p-hydroxyphenylalkane and -alkene analogues inhibited proliferation and cornification of vaginal epithelium in rats when administered locally in milligram quantities. Similarly, the oestrogen agonists dimethylstilboestrol and other stilboestrol derivatives (Figure 1.1) (Emmens and Cox, 1958; Emmens et al., 1958; Emmens et al., 1962, 1969) and 3,3',5,5'-tetramethyl α,β-diethylstilboestrol (Clark and McCraken, 1971) inhibited E_2-induced cornification in the ovariectomized mouse vagina. However, continued exposure to such compounds results in a full agonist response (Martin, 1969).

The successful search for a more potent systemically active

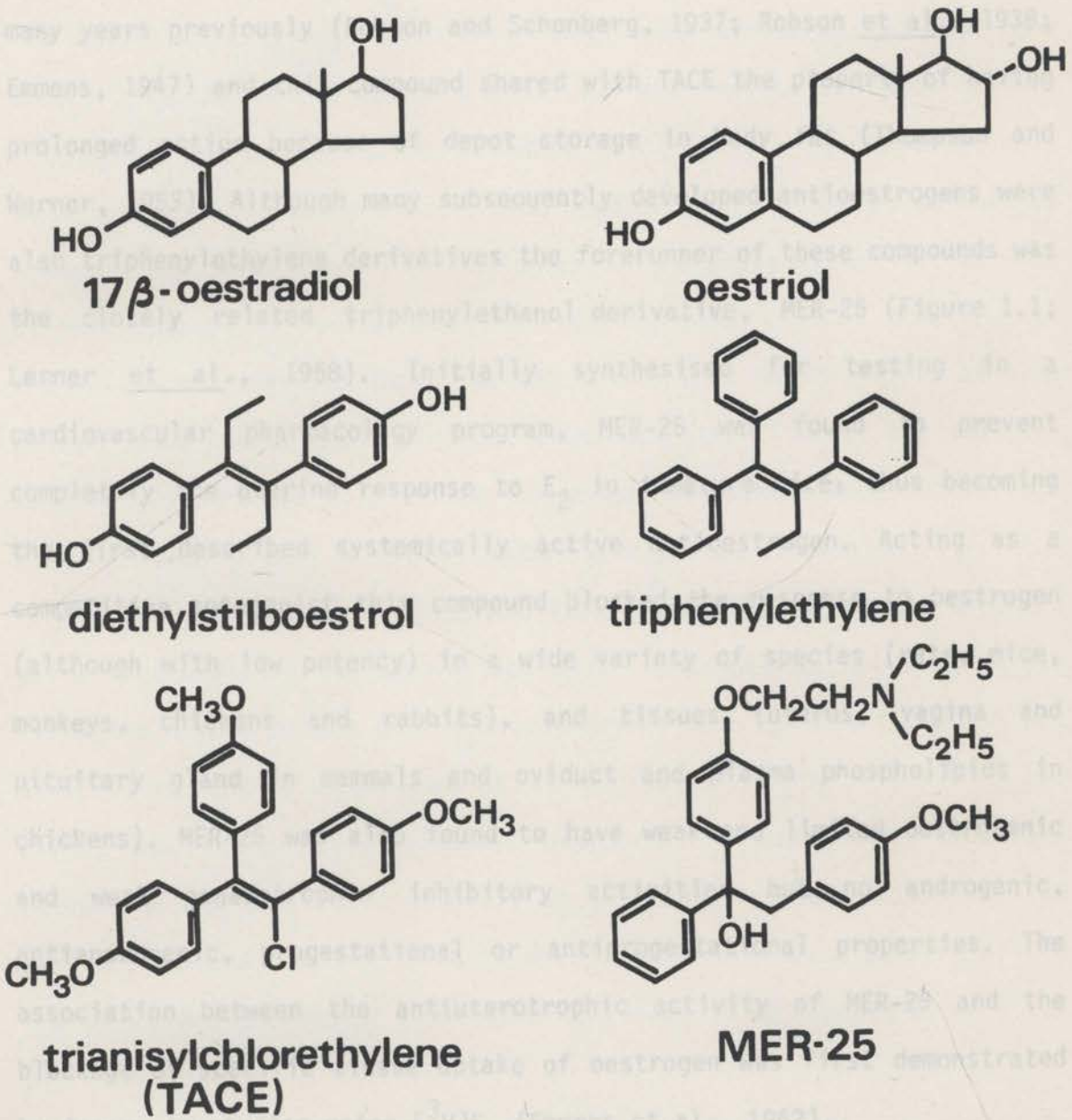


Figure 1.1: Structures of steroidal and nonsteroidal oestrogens and the first nonsteroidal antioestrogen, MER-25.

antioestrogen by Lerner and colleagues at Merrell Laboratories evolved from an existing program to develop synthetic oestrogens. The triphenylethylene derivative, tri-p-anisylchloroethylene (TACE, Figure 1.1), a product of this program, was a clinically useful long acting oestrogen (Thompson and Werner, 1953). The oestrogenic properties of triphenylethylene (Figure 1.1) and its derivatives had been studied for many years previously (Robson and Schonberg, 1937; Robson et al., 1938; Emmens, 1947) and this compound shared with TACE the property of having prolonged action because of depot storage in body fat (Thompson and Werner, 1953). Although many subsequently developed antioestrogens were also triphenylethylene derivatives the forerunner of these compounds was the closely related triphenylethanol derivative, MER-25 (Figure 1.1; Lerner et al., 1958). Initially synthesised for testing in a cardiovascular pharmacology program, MER-25 was found to prevent completely the uterine response to E_2 in immature mice, thus becoming the first described systemically active antioestrogen. Acting as a competitive antagonist this compound blocked the response to oestrogen (although with low potency) in a wide variety of species (rats, mice, monkeys, chickens and rabbits), and tissues (uterus, vagina and pituitary gland in mammals and oviduct and plasma phospholipids in chickens). MER-25 was also found to have weak and limited oestrogenic and weak gonadotrophin inhibitory activities but no androgenic, antiandrogenic, progestational or antiprogestational properties. The association between the antiuterotrophic activity of MER-25 and the blockage of specific tissue uptake of oestrogen was first demonstrated by Jensen in studies using $[^3H]E_2$ (Emmens et al., 1962).

MER-25 proved to have limited clinical application. Kistner and Smith (1959) administered MER-25 to women with chronic cystic mastitis

and breast carcinoma and although the patients experienced some relief from pain, toxic side effects especially in the central nervous system prevented the use of MER-25 in treatment of breast disease. The finding that MER-25 had antifertility properties in the rat (Lerner et al., 1958; Segal and Nelson, 1958; Lerner, 1964) led to an investigation of its use as a potential oral contraceptive agent. Because of low potency and toxic side effects clinical trials were abandoned (Kistner and Smith, 1961; Smith and Kistner, 1963) but the results of these studies stimulated the search for more potent antifertility agents especially among compounds which were basic ethers of triphenylethylene, -ethane or -ethanol and their fused ring analogues. Although it is not the purpose of this chapter to review the antifertility properties of nonsteroidal antioestrogens, many of the compounds synthesised for this purpose also proved to be potent antioestrogens with potential clinical applications in other areas especially in the management of hormone-dependent breast cancer. However, only a limited number were tested clinically.

Clomiphene (chloramiphene, MRL 41, Figure 1.2), a Merrell compound and a triphenylethylene derivative, first described by Holtkamp et al. (1960) as a more potent oestrogen antagonist than MER-25, found use not as a contraceptive agent but as an ovulation inducer. Tyler et al. (1960) had found MER-25 induced ovulation in women with secondary amenorrhea and this led Greenblatt et al. (1961) to use clomiphene for the same purpose finding it to be more potent. Clomid, a mixture of the cis- and trans-isomers of clomiphene continues to be used clinically for this purpose.

Nafoxidine (U 11,100A, The Upjohn Co., Figure 1.2), a diphenyl-dihydronapthalene derivative was found to have antifertility activity in rodents. Duncan et al. (1963) postulated that this was due to its

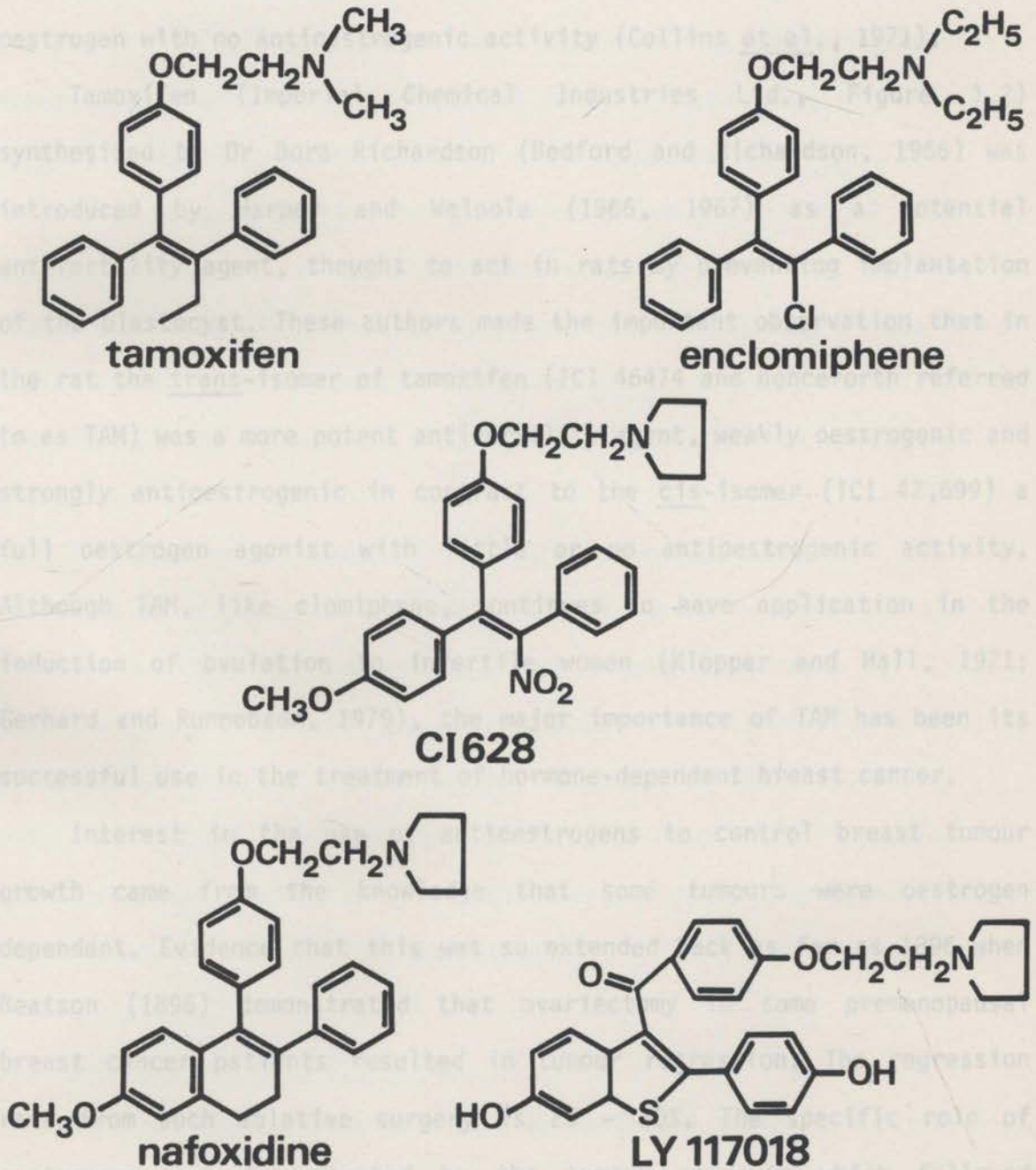


Figure 1.2: Nonsteroidal antioestrogens.

antioestrogenic activity, although subsequently Emmens and Martin (1965) showed that its oestrogenic potency was more likely to be responsible.

CI 628 (nitromifene, CN 55945-27, Parke, Davis and Co., Figure 1.2) was first described by Callantine et al. (1966) and shown to be an antifertility agent with antioestrogenic activity in the rat. In the mouse CI 628 also had weak antifertility activity but was a weak oestrogen with no antioestrogenic activity (Collins et al., 1971).

Tamoxifen (Imperial Chemical Industries Ltd., Figure 1.2) synthesised by Dr Dora Richardson (Bedford and Richardson, 1966) was introduced by Harper and Walpole (1966, 1967) as a potential antifertility agent, thought to act in rats by preventing implantation of the blastocyst. These authors made the important observation that in the rat the trans-isomer of tamoxifen (ICI 46474 and henceforth referred to as TAM) was a more potent antifertility agent, weakly oestrogenic and strongly antioestrogenic in contrast to the cis-isomer (ICI 47,699) a full oestrogen agonist with little or no antioestrogenic activity. Although TAM, like clomiphene, continues to have application in the induction of ovulation in infertile women (Klopper and Hall, 1971; Gerhard and Runnebaum, 1979), the major importance of TAM has been its successful use in the treatment of hormone-dependent breast cancer.

Interest in the use of antioestrogens to control breast tumour growth came from the knowledge that some tumours were oestrogen dependent. Evidence that this was so extended back as far as 1896 when Beatson (1896) demonstrated that ovariectomy in some premenopausal breast cancer patients resulted in tumour regression. The regression rate from such ablative surgery is 25 - 30%. The specific role of oestrogens was demonstrated by the tumour regrowth which followed administration of small doses of oestrogen to ovariectomized patients

(Pearson et al., 1954). Subsequently it was shown that some breast tumours retained [^{3}H]-hexoestrol (Folca et al., 1961) and that the presence of specific oestrogen binding activity in breast tumours was correlated with response to endocrine therapy (Jensen et al., 1967; Jensen et al., 1971).

Studies on the use of TAM (as the clinical agent TAM citrate, 'Nolvadex') as an antitumour agent began at the Christie Hospital, Manchester in 1969 in postmenopausal women with advanced disease (Cole et al., 1971; Ward, 1973). Although clomiphene (Hecker et al., 1974) and nafoxidine (Heuson et al., 1972) were also shown in clinical trials to achieve remissions in advanced breast cancer only TAM remains in widespread clinical use, because of its effectiveness and lower incidence of side effects (Mouridsen et al., 1978; Patterson and Battersby, 1980; Patterson, 1981; Novadex Clinical Trial Organisation, 1985).

The most recent generation of antioestrogens have been developed specifically as potential antitumour agents. Apart from new analogues of existing nonsteroidal compounds these include trioxifene (LY 133314, Jones et al., 1979) and the benzothiophenes LY 117018, (Figure 1.2, Black and Goode, 1980; Scholl et al., 1983) and LY 156758 (Black et al., 1983). While some have been shown to be more potent antioestrogens than TAM (Jordan and Gosden, 1983) a more effective antitumour agent than TAM has not yet reached clinical development.

1.3 METABOLISM OF ANTIOESTROGENS

Antioestrogens show widely varying oestrogen antagonist and agonist properties according to the experimental conditions (e.g. dose range, method of administration), the species, tissue and parameter of

oestrogen action studied. Although the molecular basis for this heterogeneity of response is largely undefined, differences in pharmacodynamics and metabolism could modify the action of a given antioestrogen. However, in general, the antioestrogenic properties of these drugs are not dependent upon metabolic transformation *per se*.

Triphenylethylenes are characterized by long biological half lives (Fromson *et al.*, 1973a,b; Binart *et al.*, 1979; Katzenellenbogen *et al.*, 1979) which can be accounted for by their high lipid solubility (as previously noted in the case of TACE); by their tendency to bind to serum and cellular proteins with relatively high affinities (Section 1.6.3); and because of enterohepatic recirculation of metabolites, the faeces being the main route of excretion in both laboratory animals and humans (Fromson *et al.*, 1973a,b). Hydroxylated compounds such as 4-hydroxyTAM (Jordan and Allen, 1980) and LY 117018 (Jordan and Gosden, 1983), although generally more potent antioestrogens, have short durations of action presumably because of high tissue and extracellular fluid clearance rates.

The triphenylethylenes undergo extensive hepatic biotransformation to yield products with a range of biological activities. Studies *in vitro* and *in vivo* have identified the major metabolites of TAM shown in Figure 1.3 (Fromson *et al.*, 1973a,b; Binart *et al.*, 1979; Adam *et al.*, 1980; Foster *et al.*, 1980; Borgna and Rochefort, 1981; Robertson *et al.*, 1982b; Bain and Jordan, 1983; Jordan *et al.*, 1983; Kemp *et al.*, 1983). Two further metabolites have been identified as 4'-hydroxyTAM and TAM epoxide (Ruenitz *et al.*, 1984). Studies with the cytochrome P-450 inhibitors SKF-525A and metyrapone have shown the involvement of various isoenyzmes in the formation of TAM metabolites with the exception of TAM N-oxide which may be produced by action of the microsomal flavin-

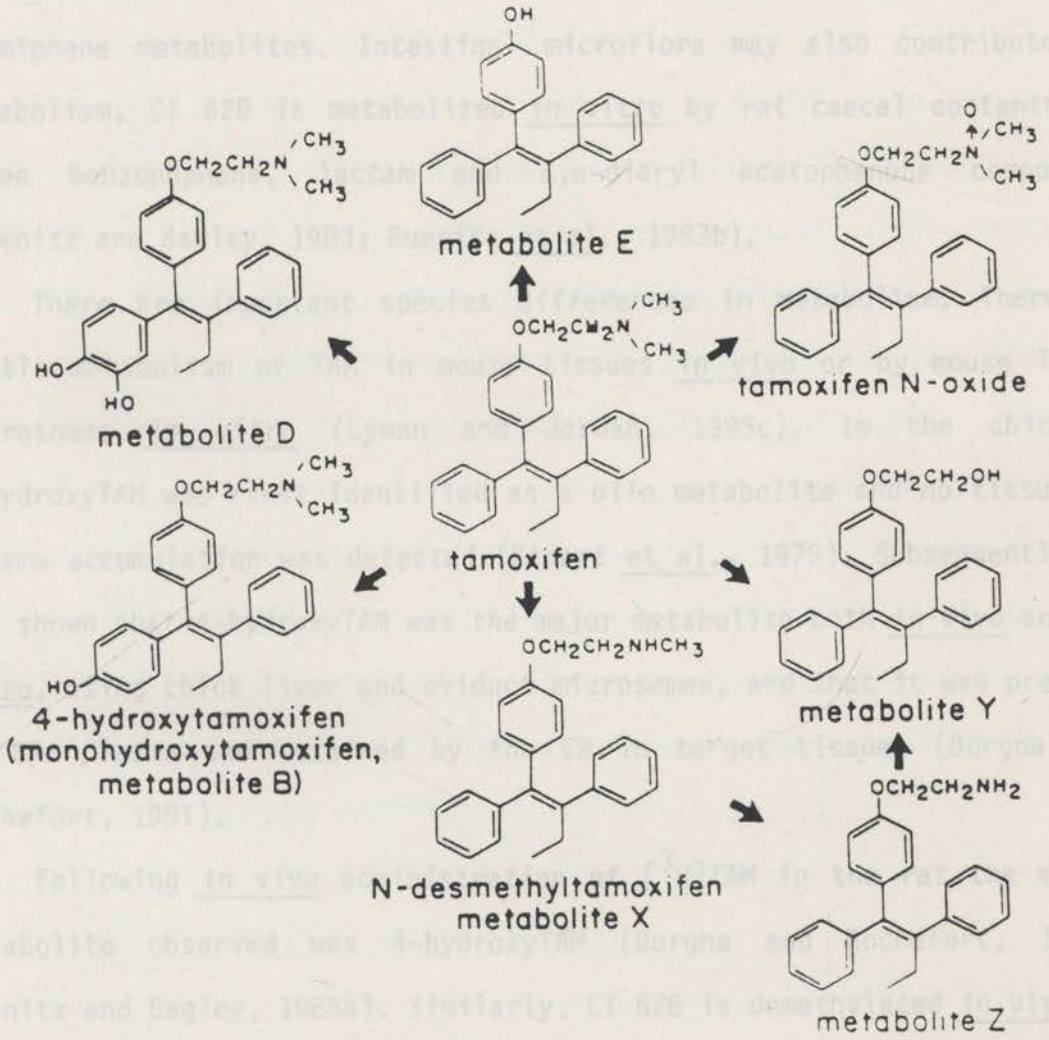


Figure 1.3: Tamoxifen metabolites identified 'in vivo' and 'in vitro' (from Jordan, 1984).

containing mono-oxygenase (Ruenitz et al., 1984).

Like TAM, other triphenylethylenes undergo biotransformation to phenolic metabolites (CI 628, Katzenellenbogen et al., 1978; U 23,469, Tatee et al., 1979; and clomiphene, Ruenitz, 1981; Ruenitz et al., 1983a). The latter studies also identified N-desethyl and N-oxide clomiphene metabolites. Intestinal microflora may also contribute to metabolism. CI 628 is metabolized in vitro by rat caecal contents to three benzophenone, lactam and α,α-diaryl acetophenone compounds (Ruenitz and Bagley, 1983; Ruenitz et al., 1983b).

There are important species differences in metabolism. There is little metabolism of TAM in mouse tissues in vivo or by mouse liver microsomes in vitro (Lyman and Jordan, 1985c). In the chicken, 4-hydroxyTAM was first identified as a bile metabolite and no tissue or plasma accumulation was detected (Binart et al., 1979). Subsequently it was shown that 4-hydroxyTAM was the major metabolite both in vivo and in vitro, using chick liver and oviduct microsomes, and that it was present in the plasma and retained by the ER in target tissues (Borgna and Rochefort, 1981).

Following in vivo administration of [^{3}H]TAM in the rat the major metabolite observed was 4-hydroxyTAM (Borgna and Rochefort, 1980; Ruenitz and Bagley, 1985a). Similarly, CI 628 is demethylated in vivo to the more active phenolic metabolite CI 628M (Katzenellenbogen et al., 1978). Although cis-triphenylethylene derivatives generally have only agonist activity in the rat, cis-4-hydroxyTAM and cis-CI 628 have antagonist activity (Jordan et al., 1981b). This may result from in vivo geometric isomerization of these compounds to the antagonist trans-isomers, preceded in the case of CI 628 by metabolism to CI 628M. Such isomerization of cis- and trans-4-hydroxyTAM has been observed in vitro

(Katzenellenbogen et al., 1985b). Clomiphene, however, is largely unmetabolised in immature female rats (Ruenitz and Bagley, 1985a).

In the human, the major metabolite of TAM is N-desmethylTAM, which accumulates in both plasma and tumours to levels approximately twice those of the parent compound (Daniel et al., 1981). Lesser amounts of Metabolite Y and 4-hydroxyTAM are also formed, at plasma levels 3 - 56% and ≤ 2% those of TAM, respectively (Adam et al., 1980; Bain and Jordan, 1983; Jordan et al., 1983). N-desmethylTAM has similar antioestrogenic activity to TAM both in vitro and in vivo (Coezy et al., 1982; Jordan et al., 1983; Reddel et al., 1983), but 4-hydroxyTAM could be expected to make only a minor contribution to activity in vivo as, despite its high affinity for ER and potent antioestrogenicity in vitro (50 - 100-fold that of TAM), this compound is only 10-fold more potent than TAM in the rat antiuterotrophic assay (Jordan et al., 1977a). This discrepancy may be due to metabolism of TAM or to a shorter half-life of the hydroxylated compound. Metabolite Y has lower antioestrogenic potency than TAM (Jordan et al., 1983) and is probably not a significant contributor to in vivo activity in the human.

1.4 BIOLOGICAL EFFECTS OF ANTIOESTROGENS

As previously stated, there is differential expression of agonist and antagonist activity by nonsteroidal antioestrogens in the oestrogen target tissues of various species. This review concentrates upon the action of these compounds in the mouse, rat and chicken. The spectrum of effects covers those commonly observed in other species as reviewed by Furr et al. (1979) and Sutherland and Jordan (1981).

1.4.1 Effects of Antioestrogens in the Mouse

In the mouse, MER-25 exhibits virtually no agonist activity while inhibiting the uterine weight increase induced by steroidal and nonsteroidal oestrogens (Lerner et al., 1958) and by the nonsteroidal antioestrogens TAM and 4-hydroxyTAM (Lyman and Jordan, 1985c). In fact, MER-25 is unique in this regard as all other compounds related to the triphenylethylenes are generally fully oestrogenic in this species, with weak antagonist responses seen only under certain circumstances. Thus, in immature mice TAM is fully uterotrophic (Terenius, 1971a; Martin and Middleton, 1978) without antioestrogenic activity (Terenius, 1970), and in ovariectomized mice TAM produces full vaginal cornification (Harper and Walpole, 1966). Weak antioestrogenic effects of prolonged high dose TAM administered prior to E_2 have been observed in vaginal smear tests (Emmens, 1971; Jordan, 1975). Presumably acting here as a weak oestrogen, TAM fails to maintain maximal rates of vaginal epithelial proliferation (Martin, 1981). There is no evidence that conversion in vivo to oestrogenic metabolites such as Metabolite E can account for the lack of antioestrogenic activity (Lyman and Jordan, 1985c). Nafoxidine (Emmens and Martin, 1965; Terenius, 1971), enclomiphene (Terenius, 1971) and CI 628 (Emmens, 1973) are also oestrogenic in mice with little or no significant antioestrogenic activity. Although LY 117018 is partially uterotrophic, it also has antioestrogenic properties in the uterine weight test in ovariectomized mice (Black and Goode, 1980).

Although 4-hydroxyTAM is fully oestrogenic for progesterone receptor (PgR) induction in the ovariectomized mouse uterus in vivo, a paradoxical situation exists in primary uterine cell cultures where 4-hydroxyTAM is non-oestrogenic and inhibits E_2 stimulated induction of PgR (Campen et al., 1985). However, the histology of these cultures was

not defined and it may be that 4-hydroxyTAM has differential effects on PgR induction *in vitro* (and *in vivo*), according to the cell type studied. Primary rat uterine cultures established under the same conditions appeared to consist exclusively of myometrial cells.

1.4.2 Effects of Antioestrogens in the Chicken

The chick oviduct and liver have been used extensively as models of both oestrogen and antioestrogen action. Oestrogen responsive parameters of oviduct function have been reviewed by Schimke *et al.* (1975) and include: histology; relative rates of conalbumin and ovalbumin synthesis (Catelli *et al.*, 1980), which have been shown to be proportional to the cellular concentration of the respective mRNA's for these two proteins (Palmiter *et al.*, 1977); and total cellular ER content, DNA polymerase α and cAMP-dependent protein kinase activities (Sutherland *et al.*, 1977b). In contrast to the situation in the mouse, triphenylethylenes and related compounds are full oestrogen antagonists with no oestrogenic activity in the chick oviduct (Sutherland *et al.*, 1977a; Binart *et al.*, 1979; Sutherland, 1981; Sutherland and Foo, 1981; Mester *et al.*, 1981). Even the *cis*-isomers of TAM and clomiphene, which are generally full oestrogen agonists in mammalian species, are devoid of oestrogenic activity and act as weak antagonists in the oviduct model (Sutherland, 1981).

In the chick liver, oestrogens exert control over those metabolic activities involved in vitellogenesis, the synthesis of specific proteins and lipids required for the developing egg yolk. These oestrogenic activities have been reviewed by Lazier *et al.* (1981) and include: effects on secreted proteins (vitellogenin, apo VLDL-B and apo VLDL-II, riboflavin binding protein and transferrin); increases in liver

weight (hypertrophy); changes in microsomal membrane properties; and increases in enzymes associated with transcription and translation. As in the oviduct, triphenylethylenes act only as oestrogen antagonists and potently inhibit all oestrogen-induced protein synthesis and other oestrogenic effects (Gschwendt, 1975; Capony and Williams, 1981; Lazier et al., 1981).

1.4.3 Effects of Antioestrogens in the Rat

The rat uterus has been the most widely studied model of antioestrogen action since this organ is used as the predominant bioassay for oestrogenic and antioestrogenic activity. Many of the current ideas concerning the molecular actions and clinical applications of these compounds arise from studies on rat uteri. For this reason the actions of antioestrogens in this species are reviewed in some detail.

Antioestrogens express partial oestrogen antagonist and/or agonist activities in the rat according to the tissue or biological response studied. In the uterus, antioestrogens administered alone increase uterine weight in immature rats but the response is significantly less than that seen with E_2 alone. The uterine response to E_2 is reduced but not abolished by the presence of the same antagonists. These effects have been observed with: MER-25 (Lerner et al., 1958); CI 628 and nafoxidine (Katzenellenbogen and Ferguson, 1975); TAM (Harper and Walpole, 1967); enclomiphene (Jordan et al., 1981a); and LY 117018 (Black and Goode, 1980), the latter compound being a potent antioestrogen with very weak agonist activity in this model. Typically early (0 - 6 h) uterine responses to antioestrogens are agonistic and similar to those seen with E_2 (Ferguson and Katzenellenbogen, 1977; Martin, 1981), whereas antagonistic responses are seen later (> 24 h;

Harper and Walpole, 1967).

Several studies have attempted to dissect the complex differential responses to oestrogens and antioestrogens in the various cell types which comprise the rat uterus, i.e. the luminal and glandular epithelium, the stroma and the myometrium. In immature and ovariectomized adult rats, antioestrogens produce hypertrophy of glandular and luminal epithelium and inhibit E_2-induced hyperplasia (Martin, 1980, 1981). In the luminal epithelium a small increase in labelling index and cell numbers is observed, but less than that induced by E_2. In contrast, the luminal proliferation induced by uterine dilation is augmented by TAM (Martin, 1981), which in this experimental situation appears to be converted from a partial to a full oestrogen agonist. In immature rats, both E_2 and TAM induce synthetic and secretory activity in luminal and glandular epithelium with development of the Golgi system, the rough endoplasmic reticulum and the formation of secretory granules (Holinka et al., 1980).

In the glandular epithelium of ovariectomized adult rats, antioestrogens have cytotoxic activity (Martin, 1981). At first TAM induces a small but transient increase in the labelling index, less than that induced by E_2 but there is a time-related decrease in gland cell numbers with high dose TAM. This results from a sustained increase of cell death rate above control levels. The decrease in gland cell numbers (6-fold over two weeks) is not reversed by E_2. E_2 alone increases both labelling and dead cell indices but in the long term there is little increase in cell numbers.

In neonatal rats, TAM and E_2 disrupt uterine development (Branham et al., 1985). TAM was found to inhibit gland genesis in neonatal animals (day 1 - 5) at doses which did not increase uterine weight, and

in infantile animals (day 10 - 14) at uterotrophic doses. E_2 reduced gland number but much less so than TAM. The action of TAM is irreversible and uteri failed to achieve full weight after subsequent exposure to E_2. This decreased responsiveness was not the result of decreased ER levels which remained unchanged. TAM appears to act independently of ER, blocking cell division in the luminal epithelium that is required for gland differentiation.

In the myometrium, neither E_2 nor antioestrogens increase myometrial proliferation. High dose TAM causes an initial hypertrophy and an increase in the number of stromal cells labelled with thymidine over that seen with E_2 but long term treatment results in atrophy of the stromal cell population (Martin, 1981).

Antioestrogens act in the uterus as antagonists of oestrogen-induced uterine peroxidase activity (DeSombre and Lyttle, 1978) but as full oestrogen agonists without antagonist activity in the induction of 'Induced Protein' (IP; Mairesse and Galand, 1979) and PgR (Dix and Jordan, 1980; Kirchhoff et al., 1983). However, in primary cultures of rat uterine myometrial cells, TAM, 4-hydroxyTAM and LY 117018 inhibit the E_2-stimulated induction of PgR (Campen et al., 1985).

In rat liver, as in the uterus, both oestrogenic and anti-oestrogenic effects of antioestrogens have been observed. Both TAM and nafoxidine act as agonists in the stimulation of renin substrate (Bichon and Bayard, 1979; Kneifel and Katzenellenbogen, 1981). In contrast, TAM blocks the oestrogen-induced decrease in hepatic 3β-hydroxy steroid dehydrogenase (Lax et al., 1980); and nafoxidine antagonises E_2-induced stimulation of hepatic tryptophan oxygenase and tyrosine amino-transferase (Hamburger, 1981).

Prolactin (PRL) synthesis by primary cultures of rat pituitary

cells is a useful model for the *in vitro* study of antioestrogen action. Oestrogen is a direct regulator of prolactin (PRL) gene transcription (Maurer, 1982; Shull and Gorski, 1984), and antioestrogens can reversibly antagonise the oestrogen-stimulated increase in PRL synthesis (Lieberman *et al.*, 1983a,b). This system avoids the complications encountered *in vivo* of bioavailability, pharmacokinetics and metabolism, making it particularly suitable for structure-activity studies. Biological potencies of both oestrogens and antioestrogens are found to be directly related to their relative binding affinities for ER (Lieberman *et al.*, 1983b) and these findings have led to the development of a hypothetical model for ligand binding to the ER (Section 1.6.2).

1.4.4 Effects of Antioestrogens on Human Breast Cancer Cells

Many models of hormone-dependent breast, endometrial, prostatic and renal cancers have been used to investigate the effects of nonsteroidal antioestrogens as antitumour agents. Of the *in vivo* breast cancer models, tumours established in rodents have been most widely studied, as reviewed by Sutherland and Jordan (1981) and Jordan (1982). A major deficiency of such tumour models is that they are not generally directly dependent upon oestrogens for growth.

Direct effects of oestrogens and antioestrogens on tumour cells are best studied *in vitro* and a number of hormone responsive breast carcinoma cell lines are available for this purpose as reviewed by Engel and Young (1978). The MCF 7 cell line has been the most widely studied. It contains ER (Brooks *et al.*, 1973) and oestrogens stimulate cell proliferation at low concentrations (Lippman *et al.*, 1976a, 1977). Nonsteroidal antioestrogens completely inhibit E_2 binding to the MCF 7 ER (Brooks *et al.*, 1973; Lippman *et al.*, 1976b) and are potent (but

oestrogen reversible) inhibitors of cell proliferation (Lippman and Bolan, 1975; Lippman et al., 1976a) except at high concentrations where their effects become oestrogen-irreversible (Sutherland et al., 1983b). Other factors influence the oestrogen-responsiveness of these cells including insulin (Butler et al., 1981) PRL (Shafie and Brooks, 1977) and other serum factors (Reddel et al., 1984). Receptors for progesterone, androgens, glucocorticoids (Horwitz et al., 1975), 1,25-dihydroxy vitamin D_3 (Eisman et al., 1980), PRL (Shiu, 1979), insulin (Osborne et al., 1978), epidermal growth factor (Imai et al., 1982), calcitonin (Martin et al., 1980) and growth hormone (Murphy et al., 1984a) have also been identified.

MCF 7 and other ER-positive cell lines are excellent models for investigating structure-activity relationships among nonsteroidal antioestrogens. A particular advantage of this system is that the cells appear not to metabolize these drugs (Horwitz et al., 1978). Detailed studies in this laboratory have documented three effects of antioestrogens in the MCF 7 cell line summarized in Figure 1.4 (Sutherland et al., 1983a,b, 1984; Reddel et al., 1983; Reddel and Sutherland, 1984; Taylor et al., 1983; Murphy and Sutherland, 1985). At concentrations < 0.1 - 5 μM TAM causes an oestrogen-reversible growth inhibition which can be accounted for by a dose-dependent decrease in cell proliferation rate. Between approximately 5 and 7.5 μM, more marked growth inhibition is observed but this is only partially reversed by the simultaneous administration of a dose of E_2 sufficient to completely displace TAM from the ER. No evidence of cytotoxicity, as assessed by clonogenic survival, is apparent over this dose range following 24 h exposure to the drug. At concentrations > 10 μM, TAM has a cytotoxic effect on MCF 7 (Sutherland et al., 1983a,b) and other breast cancer

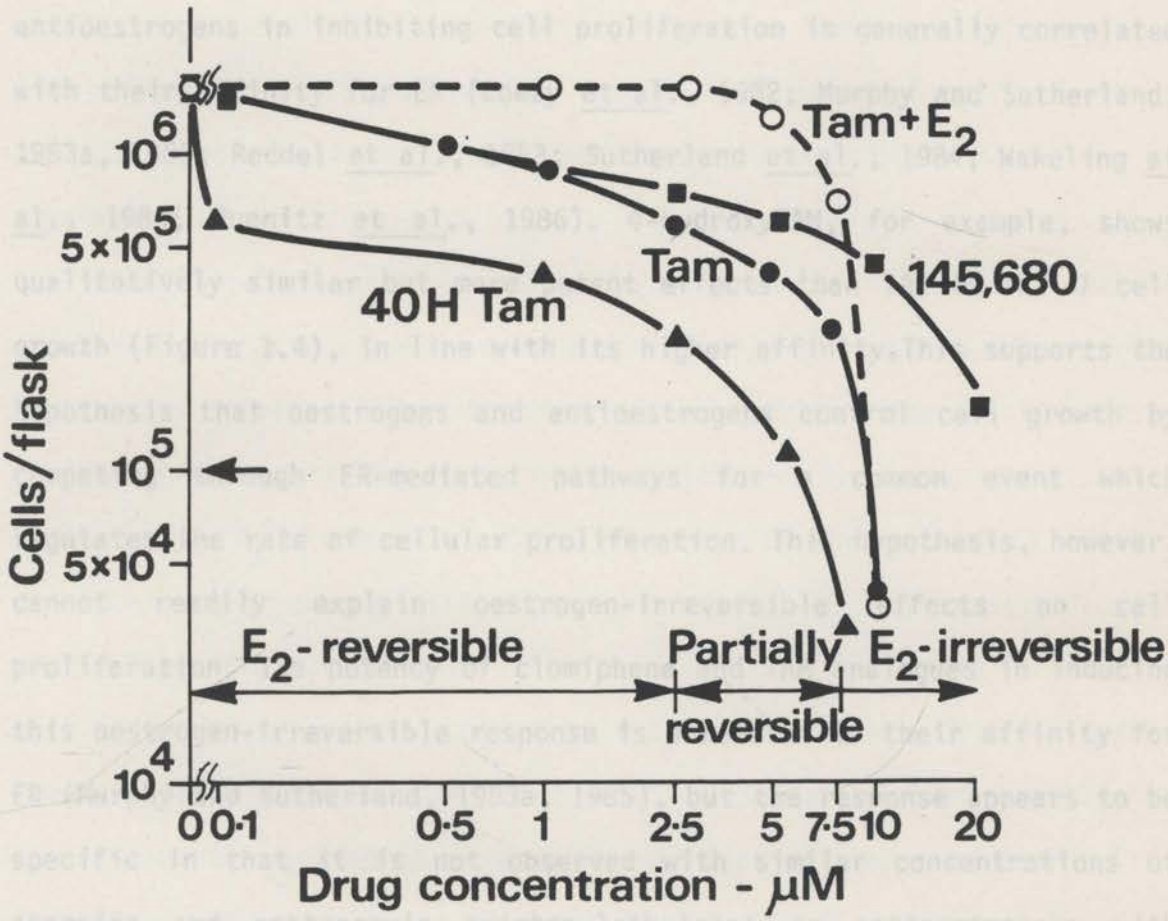


Figure 1.4: Antiproliferative actions of antioestrogens on the MCF 7 human breast cancer cell line. 10^5 cells were plated into 25 cm^2 flasks and allowed to grow in the presence of 4-hydroxyTAM, ICI 145680 or TAM (with or without the simultaneous presence of E_2, at a 10-fold lower concentration). Flasks were harvested after approximately four population doublings and viable cells counted.

cell lines (Reddel et al., 1985). This effect is not reversed by simultaneous administration of E_2 and in some circumstances a synergistic cytotoxic effect is seen.

At low oestrogen-reversible concentrations the potency of antioestrogens in inhibiting cell proliferation is generally correlated with their affinity for ER (Coezy et al., 1982; Murphy and Sutherland, 1983a, 1985; Reddel et al., 1983; Sutherland et al., 1984; Wakeling et al., 1984; Ruenitz et al., 1986). 4-hydroxyTAM, for example, shows qualitatively similar but more potent effects than TAM on MCF 7 cell growth (Figure 1.4), in line with its higher affinity.This supports the hypothesis that oestrogens and antioestrogens control cell growth by competing through ER-mediated pathways for a common event which regulates the rate of cellular proliferation. This hypothesis, however, cannot readily explain oestrogen-irreversible effects on cell proliferation. The potency of clomiphene and TAM analogues in inducing this oestrogen-irreversible response is unrelated to their affinity for ER (Murphy and Sutherland, 1983a, 1985), but the response appears to be specific in that it is not observed with similar concentrations of steroids and oestrogenic triphenylethylenes or antioestrogens with non-basic side chains. Thus ICI 145680, a TAM analogue with a non-basic side chain (Table 6.8), but having an equal affinity for ER, is a significantly less potent inhibitor of growth in the E_2-irreversible concentration range, and is not cytotoxic, even at a concentration of 50 μM (Figure 1.4).

Decreased growth rate induced by antioestrogens is accompanied by a concentration-dependent decrease in the proportion of cells in the DNA synthetic portion of the cell cycle (S phase), with a corresponding increase in the proportion of G_0/G_1 phase cells. At TAM concentrations

< 5 μM the presence of E_2 reverses these effects, but only partial reversal is achieved at higher concentrations. These effects result from a reduction in the rate of progression through G_1 phase, antioestrogens having much less effect on progression of cells through subsequent phases of the cell cycle (Sutherland et al., 1983b; Taylor et al., 1983). Drug sensitivity is maximal in a 2 - 4 h interval in mid-G_1 phase but the biochemical basis of the antioestrogen-induced G_1 block is unknown.

Oestrogen-irreversible inhibition of cell proliferation involves similar cell cycle changes to those in the oestrogen-reversible range (Sutherland et al., 1983b). Oestrogen-irreversible cytotoxicity observed at high TAM concentrations is also accompanied by an accumulation of cells in the G_0/G_1 phase except at very high concentrations (> 20 μM), where cell death occurs without changes in the cell-cycle distribution (Sutherland et al., 1983a).

In addition to its effects on cell proliferation, TAM influences several oestrogen-responsive biochemical parameters in MCF 7 cells, in particular, the synthesis of several oestrogen-induced proteins. PgR is potently induced by low concentrations of TAM (Horwitz and McGuire, 1978a; Horwitz et al., 1978), which appears to act as an oestrogen agonist in this regard. However, at higher concentrations (1 μM) PgR levels are suppressed below control levels. The oestrogen-induced synthesis of several other intracellular proteins is antagonised by antioestrogens in MCF 7 cells. Included among these are the 'Induced Protein' (Mairesse et al., 1980), subsequently identified as the brain-type isoenzyme of creatinine kinase (Reiss and Kaye, 1981); thymidine kinase (Bronzert et al., 1981); DNA polymerase α (Edwards et al., 1980) and lactate dehydrogenase (Burke et al., 1978). The

extracellular secretion of several glycoproteins is also antagonised by antioestrogens: plasminogen activator (Butler et al., 1983; Katzenellenbogen et al., 1984); and a 52K glycoprotein (Westley and Rochefort, 1980) of unknown function but hypothesised to be the product of an as yet uncharacterized mammary oncogene having autocrine growth factor activity (Rochefort et al., 1984).

Effects of antioestrogens similar to those observed in the MCF 7 cell line have been reported for several other breast cancer cell lines. These include T-47D (Reddel et al., 1984); ZR-75 (Allegra and Lippman, 1978, 1980; Darbre et al., 1984), and a variety of other ER-positive and -negative lines (Goldenberg and Froese, 1982; Reddel et al., 1985).

1.5 OESTROGEN ACTION AND THE OESTROGEN RECEPTOR SYSTEM

In outline, oestrogen action involves the entry of oestrogen into target cells followed by high affinity binding to receptor protein, resulting in the conversion of the steroid hormone-receptor complex to a form which binds with higher affinity to one or more nuclear acceptor sites, in turn activating a number of oestrogen-responsive genes. DNA, RNA and protein synthesis are altered with consequent effects on cell proliferation, differentiation and function.

Entry of oestrogen into cells is thought to occur through passive diffusion (Müller and Wotiz, 1979). Although low levels of ER have been detected in the plasma membrane which could be postulated to play a role in membrane transport (reviewed by Szego and Pietras, 1984), these findings have been questioned (Müller et al., 1979), and recent immunohistochemical studies with monoclonal antibodies to ER have failed to confirm the presence of membrane-bound ER (see below).

Thus the first identifiable event in oestrogen action is the

binding of the steroid ligand to its specific receptor. Although such a receptor had previously been postulated, the first direct evidence for the ER was the observation that $[^3H]E_2$ localized in oestrogen target tissues in the immature rat (Jensen and Jacobsen, 1962), and in ovariectomized mice (Stone, 1963; Stone et al., 1963). The specific binding of $[^3H]E_2$ to protein was localized in the cytosol and nuclear subcellular fractions (Noteboom and Gorski, 1965; Toft and Gorski, 1966), and a cytosolic receptor protein sedimenting at 8 - 9.5S on sucrose gradients was identified (Toft and Gorski, 1966; Jensen et al., 1967), whereas the 0.4 M KCl-extracted nuclear receptor protein sedimented at 5S on gradients containing KCl (Erdos, 1968). The 8S cytosolic receptor was found to dissociate into 4S subunits in the presence of 0.4 M KCl (Jensen et al., 1967).

Following the binding of oestrogen, the ER undergoes a time- and temperature-dependent process (activation) which increases the binding of the ER-ligand complex to nuclei and polyanions. A conformational change of the 4S subunit is involved, resulting in the exposure of a nuclear binding domain (Milgrom, 1981; Müller et al., 1983). In addition, conformational changes in the oestrogen-binding domain of each receptor subunit (Müller et al., 1985a) result in an ER complex with a slower rate of ligand dissociation, i.e. with higher affinity for oestrogen (Notides and Nielsen, 1974; Weichman and Notides, 1977). Another receptor domain mediates a third receptor event, transformation, i.e. conversion of the cytosolic into the nuclear binding form (Jensen and DeSombre, 1973; Notides and Nielsen, 1974; Notides et al., 1975). Transformation involves the association of 4S monomers into 5S dimers and can also be considered equivalent to subunit disaggregation from the oligomeric untransformed native 8 - 10S receptor (Skipper et al., 1985).

This process is inhibited by molybdate (Sherman and Stevens, 1984), which stabilises the oligomeric receptor and also prevents the formation of variably large aggregates of native receptor. It has been suggested that these may be the primary actions of molybdate rather than the apparent protection of the receptor from degradation by proteases, phosphatases and nucleases (Skipper et al., 1985).

Evidence that the 5S receptor is in fact a homodimer comes from several studies. Using dense amino acid labelling techniques, Scholl and Lippman (1984) found the nuclear 5S receptor was a dimer of two identical or closely related subunits. Miller et al. (1985), using techniques involving SDS-gel electrophoresis of 5S nuclear ER complexes in which the subunits were chemically cross-linked and labelled covalently with [^{3}H]TAM-aziridine, have shown that the subunits are two identical M_r 65,000 monomers. Other in vitro observations which have shown that E_2 binding to ER displays positive cooperativity support this model (Notides et al., 1981). However in uterine cell suspensions only non-cooperative E_2 binding is observed (Müller et al., 1985b), presumably because in undisrupted cells receptor monomers are not able to freely interact due to immobilization within the nucleus. Receptors immobilized on hydroxyapatite also do not exhibit cooperative oestrogen binding (Sakai and Gorski, 1984).

In view of the above findings, the conclusions of some earlier studies which indicated that 5S receptor consisted of a 4S receptor associated with another distinct protein, must be questioned (Yamamoto, 1974; Thampan and Clark, 1981). However, stronger evidence that additional components may under some circumstances be associated with ER comes from studies in which a monoclonal antibody (D5) against soluble ER complexes from human myometrium was found to react with cytoplasmic

ER from several ER-positive human tissues but not human nuclear ER or cytoplasmic ER from other species. The D5 antibody was shown to bind to a cytosolic M_r 29,000 non-hormone binding component of unknown function which appeared, under activating conditions, to complex with cytosolic ER (Coffer et al., 1985). In epithelial tissue and in human breast tumours there was a highly significant correlation between D5 staining using an immunoperoxidase technique and ER measured by E_2 binding assay (King et al., 1985a). Baulieu's group has also identified, through the use of a monoclonal antibody raised against partially-purified molybdate-stabilised chick oviduct PgR, a 90K non-hormone binding protein apparently common to 8S non-transformed chick progesterone, oestrogen, androgen and glucocorticoid receptors (Joab et al., 1984). Whether this protein is involved in receptor function or is merely an artifact of tissue homogenization (Birnbaumer et al., 1984) is unknown.

Traditionally, the early events in oestrogen action (activation, conversion to a high affinity conformation and transformation) were thought to occur in the cytoplasm prior to the translocation of the transformed ER to the nucleus where binding to nuclear acceptor sites occurred (Gorski et al., 1968; Jensen et al., 1968). Evidence for this view came from observations that in tissues from immature or ovariectomized animals unoccupied ER was almost entirely confined to the cytosol and not the nuclear fraction (Toft et al., 1967; Shyamala and Gorski, 1969). Oestrogen administration caused redistribution of ER to the nuclear fraction (Gorski et al., 1968; Jensen et al., 1968). Other observations, however, are in conflict with this model. Nuclear as opposed to cytosol transformation has been observed (Linkie and Siiteri, 1978), while Zava and McGuire (1977), Sheridan et al. (1979) and Carlson and Gorski (1980) have demonstrated the presence of unoccupied ER in the

nuclear fraction of various tissues.

In rat uterus, following administration of high affinity ligands, nuclear ER levels increase at the expense of cytosol levels, whereas cytosol and nuclear levels remain constant when low affinity ligands are present (Jordan et al., 1985). However both classes of ligands stimulate growth and induce PgR induction, requiring ER to be located in the nucleus. These apparently conflicting data may result from differential extraction of loosely bound, unoccupied nuclear ER, arising in the case of low affinity ligands as the result of ligand dissociation following cell disruption.

The use of enucleation and immunohistochemical techniques strongly supports the conclusion that ER is in fact confined to the nucleus in intact cells and that the presence of receptors in cytosolic fractions is artifactual and arises as the result of extraction of loosely bound, i.e. untransformed, receptors during cell fractionation procedures (Gorski et al., 1984). Welshons et al. (1984) showed that, following cytochalasin B enucleation of rat pituitary GH_3 cells, unoccupied receptor was located within the nucleoplast fraction. However, if the cells were instead homogenized, high cytosolic concentrations of unoccupied receptors were observed. Immunocytochemical localization of several distinct monoclonal antibodies to the ER (King and Greene, 1984; McClennan et al., 1984; King et al., 1985b) has shown that specific staining for ER is confined to the nuclei of intact target cells, and is localized in the chromatin (Press et al., 1985).

The precise nuclear locations of unoccupied and occupied receptors are unknown. Specific binding (acceptor) sites for ER are believed to exist with direct evidence coming from studies such as those in which chick oviduct and rat uterine chromatin have been fractionated following

digestion with micrococcal nuclease (Senior and Frankel, 1978; Lebeau et al., 1981, 1982). Proposed locations of acceptor sites for steroid hormone receptors have included specific DNA sequences (Mulvihill et al., 1982; Payvar et al., 1983; Cato et al., 1984; Jost et al., 1985); nuclear matrix (Clark et al., 1980; Barrack and Coffey, 1983; Barrack, 1984), and acidic nonhistone protein DNA complexes (Spelsburg et al., 1983).

DNA sequences which represent specific binding sites for receptors have been identified both upstream from the start of transcription of hormone-responsive genes and within the genes themselves. Thus ER binds 1 - 2Kb from the start site of the PRL gene (Chambon et al., 1984a; Maurer, 1985; von der Ahe et al., 1985). Other hormone receptor binding regions have been mapped 150 - 200 bp upstream from the start of transcription in mouse mammary tumour virus DNA for the glucocorticoid-receptor complex (Hynes et al., 1983) and at upstream sites from the chicken lysozyme gene (Renkowitz et al., 1983) and ovalbumin gene (Dean et al., 1983) for PgR. Oestrogen- and progesterone-receptor complexes have also been shown to induce the ovalbumin gene by relieving the effect of a negative regulatory element (Chambon et al., 1984b), which is upstream from the promoter element identified by Dean et al. Progesterone-receptor complexes also bind selectively to DNA sequences from within the ovalbumin gene (Mulvihill et al., 1982; Compton et al., 1983), although whether such sites are involved in modulating transcription rate is unknown (Parker and Page, 1984).

There is limited knowledge regarding the DNA binding site of the ER. Enzymatic cleavage of purified ER has shown that the DNA- and steroid-binding regions are separable (Greene et al., 1984). From amino acid sequences predicted from nucleotide sequences of cDNA for ER, a

domain which may be involved in DNA recognition has been located 300 - 500 amino acids from the carboxy terminus (Greene et al., 1986). The region is rich in cysteine, lysine and arginine and has significant homology with a similarly placed region in the glucocorticoid receptor (Weinberger et al., 1985), and with a region in the v-erb-A oncogene protein (Debuire et al., 1984; Green et al., 1986). The ER steroid binding site has also been tentatively identified as a relatively hydrophobic region at the carboxy terminus.

The transcription of oestrogen-responsive genes is accompanied by increased activity of RNA polymerases and hence RNA synthesis (Gorski, 1964; Hamilton, 1968). In response to oestrogen a transient rise occurs in RNA polymerase II activity (a nucleoplasmic enzyme synthesising heterogeneous nuclear RNA, the precursor of messenger RNA), followed by a sustained rise in the same enzyme and RNA polymerase I (a nucleolar enzyme responsible for ribosomal RNA synthesis). Changes in RNA polymerase III activity (a nucleoplasmic enzyme synthesising 5S ribosomal RNA and transfer RNA) paralleled those for RNA polymerase I (Bouton et al. 1977; Nicholson et al., 1981).

Although it has been shown that the synthesis of specific mRNA is probably involved in oestrogen-induced DNA synthesis, the corresponding oestrogen-responsive genes have not been identified. Dihydrofolate reductase (Cowan et al., 1982) and thymidine kinase (Bronzert et al., 1981) are oestrogen-responsive proteins potentially involved in this process but stimulation of thymidine and uridine incorporation in MCF 7 cells does not occur in parallel with the induction of mRNA for either of these enzymes (Aitken et al., 1985), suggesting the involvement of other genes in oestrogen-induced DNA synthesis.

Similarly the identity of oestrogen responsive genes which control

cell proliferation and differentiation and the biochemical events underlying these processes are unknown. Possible post-transcriptional effects of oestrogens are largely unexplored.

The ultimate fate of nuclear ER is unknown. Following a single oestrogen exposure, and consequent receptor transformation and apparent translocation of receptor from cytosol to nucleus, there is a replenishment of untransformed cytosolic receptor (Gorski et al., 1968; Jensen et al., 1968; Sutherland and Baulieu, 1976). Continuous oestrogen exposure causes nuclear and total ER levels to undergo an apparent rapid decline. This receptor destruction or 'processing' (Horwitz and McGuire, 1978a,b) has been considered to be necessary for oestrogen action and is correlated with PgR synthesis. It has been suggested to involve receptor destruction per se, alteration in binding affinity, ability to exchange hormone, and/or the binding of receptor to non-exchangeable sites. Nuclear ER synthesis and turnover studies using dense amino acid labelling techniques (Eckert et al., 1984; Scholl and Lippman, 1984), and covalent labelling with [^{3}H]TAM-aziridine (Monsma et al., 1984; Miller et al., 1985), have shown that in MCF 7 cells occupied and unoccupied nuclear receptors have similar turnover rates ($t_{\frac{1}{2}}$ = 3 - 4 h). This turnover time is only a fraction of that necessary for continuous exposure of E_2 to produce the majority of its effects in MCF 7 cells (Strobl et al., 1980), indicating that multiple interactions of receptor with nuclear sites are necessary to produce these responses. A ratchet-like model has been proposed by Stack and Gorski (1985a) to explain this aspect of oestrogen action in rat uterus.

Auricchio and colleagues (Auricchio et al. 1984a) have proposed a receptor model based on evidence that the ER appears to require phosphorylation by a cytosolic receptor kinase before it can bind E_2. In

the nucleus, however, the E_2-ER complex is dephosphorylated by a nuclear receptor phosphatase, resulting in inactivation of the hormone binding activity of receptor which is then returned to the cytoplasmic form perhaps to be degraded or rephosphorylated and recycled. The receptor is phosphorylated exclusively on tyrosine, a process which is stimulated by calmodulin (Migliaccio et al., 1984).

A second class of ER (Type II), of unknown function, has been described in rat uterine cytosol and nuclei (Eriksson et al., 1978; Clark et al., 1980). Oestrogens do not cause translocation of cytoplasmic Type II sites to the nucleus (Clark et al., 1978), although nuclear levels are elevated during oestrogen stimulated uterine growth (Markaverich et al. 1981a,b). The affinity of these sites for E_2 is lower than that of the classical Type I receptor (K_D for Type I and II 0.1 and 30 nM respectively). Evidence for a natural ligand for the Type II receptor has been presented (Markaverich et al., 1983).

Different types of ER also exist in chick oviduct, classified as Type X (K_D = 0.1 nM) and Type Y (K_D = 1 nM) (Raymoure et al., 1985). Type X stimulates RNA polymerase II activity directly whereas Type Y requires ATP or ADP in the presence of Mg^{2+} to be converted from a nonsteroid-binding form to an active receptor. Type Y in the non-binding form may represent a recycling pool of receptor.

Although apparently direct effects of E_2 on DNA synthesis can be demonstrated in isolated rat uterine nuclei (Stack and Gorski, 1984), and on breast cancer cells grown in chemically defined medium, these experiments do not rule out the possibility of autocrine secretion of growth regulatory substances. It has been proposed that the proliferative effects of oestrogen may be mediated indirectly via the secretion of oestrogen-like growth factors (estromedins; Sirbasku and

Benson, 1979; Soto and Sonnenschein, 1980; Ikeda and Sirbasku, 1984; Ikeda et al., 1984). In fact breast cancer cells have been shown to produce many such activities including insulin-like growth factor 1 (somatomedin C), EGF- receptor binding activity, α transforming growth factor and possibly the 52K protein (Baxter et al., 1983; Vignon et al., 1983; Lippman et al., 1984; Rochefort et al., 1984; Salomon et al., 1984; Dickson et al., 1986).

There is also evidence that intracellular polyamines may mediate some of the proliferative effects of oestrogens. Oestrogens stimulate ornithine decarboxylase (ODC), a rate-limiting enzyme in polyamine biosynthesis, and this is associated with increased proliferation in T-47D and ZR-75-1 breast cancer cell lines (Lima and Shiu, 1985). Difluoromethylornithine (DFMO) blocks ODC activity and E_2-induced cell proliferation in these cell lines and in N-nitrosomethylurea-induced mouse mammary tumours in vitro (Manni and Wright, 1983, 1984) and in vivo (Manni et al., 1985). Polyamines, which have no mitogenic effects themselves, reversed the effects of DFMO and TAM. Other unknown serum factors were evidently required for these actions (Lima and Shiu, 1985).

The understanding of the molecular mechanisms underlying oestrogen action is obviously far from complete and only the initial ligand binding to ER and subsequent events leading to high affinity nuclear interaction have been described in any detail. Of post nuclear-binding events and the mechanisms leading to the expression of biological activities we are comparatively ignorant. Further study of the ER system is essential to an understanding of the organization and control of the eukaryotic genome.

1.6 MOLECULAR MECHANISMS OF ANTIOESTROGEN ACTION

The mechanisms by which nonsteroidal antioestrogens exert their biological effects have been intensively investigated for more than 30 years. Recent reviews of this area of research include those of Nicholson and Griffith (1980), Sutherland and Jordan (1981), Jordan (1982, 1984, 1986) and Katzenellenbogen et al. (1983a).

1.6.1 Molecular Basis of ER-Mediated Antioestrogen Action

Antioestrogens, by acting as competitive inhibitors of oestrogen binding to the ER (Skidmore et al., 1972), with consequent formation of antioestrogen-receptor complexes, clearly interfere with proper receptor function. Which particular properties of these complexes are responsible for the expression of antioestrogenic activity, and the details of the molecular processes involved are poorly understood.

Numerous studies have established a correlation between ER affinity determined in vitro and antioestrogenicity as manifested, for example, by antiuterotrophic potencies in the rat (Sutherland and Jordan, 1981), or by oestrogen-reversible inhibition of breast cancer cell proliferation in vitro (Coezy et al., 1982; Murphy and Sutherland, 1983a, 1985; Sutherland et al., 1984; Reddel et al., 1985; Ruenitz et al., 1986). Initially it was believed that oestrogen antagonism arose from low affinity interaction with ER with consequent premature dissociation of ligand resulting in failure to express agonist activity (Emmens et al., 1962; Martin, 1969; Korenman, 1970). It was also suggested that the rapid dissociation of antioestrogens failed to protect the ER against thermal degradation (Capony and Rochefort, 1978). Such mechanisms may adequately explain the antioestrogenic properties of compounds such as E_3 which produce inadequate nuclear accumulation

and retention of ER (Clark et al., 1978) but which produce a full oestrogenic response with continuous administration (Miller, 1969; Müller et al., 1985b). However, nonsteroidal antioestrogens must act through other mechanisms. This was clearly demonstrated by the recognition that potent hydroxylated antioestrogens generally had ER affinities at least as high as that of E_2 (Borgna and Rochefort, 1980; Hayes et al., 1981; Black and Goode, 1980) and that low affinity agonists as well as antagonists also failed to protect ER against thermal degradation (Pavlick and Katzenellenbogen, 1980). In fact no kinetic differences between agonist and antagonist binding to the non-activated ER have been found that could explain antioestrogenic activity (Sutherland and Murphy, 1982).

Following antioestrogen binding, defects in ER activation or transformation could potentially account for antioestrogenic activity. Although in vitro CI 628 apparently failed to activate receptor to the 5S form (DeBoer et al., 1981), subsequent studies with high affinity ligands (4-hydroxyTAM, Rochefort and Borgna, 1981; CI 628M, Katzenellenbogen et al., 1981) showed that antioestrogen-induced ER activation could occur. Differences, however, have been noted in the physical properties and dissociation kinetics of the ligand-receptor complexes. Rochefort and Borgna (1981) demonstrated that following heat activation of ER at 25°C, the dissociation rate of E_2 but not that of 4-hydroxyTAM was decreased. However, Katzenellenbogen et al. (1981) observed no such differences in the case of CI 628M. Fishman and Fishman (1985) have identified apparently non-dissociable E_2-ER complexes in rat uterine cytosols following heat treatment at 37°C, whereas TAM-ER complexes remain dissociable. Whether these in vitro observations at physiological temperatures reflect the true in vivo situation has yet to

be determined. Interestingly such non-dissociable complexes have also been observed in rat uterine cytosol and nuclear fractions after in vivo E_2 administration (Jakesz et al., 1983).

Differences in ER conformation that are believed to occur following antioestrogenic or oestrogenic ligand binding may account for the observed effects on dissociation kinetics. Several lines of evidence support the notion that such conformational differences do exist. A polyclonal antibody to calf uterine ER (Tate et al., 1984) decreased affinity of unlabelled ER for E_2 but not 4-hydroxyTAM (except at very high concentrations), suggesting that the antibody prevents a conformational change which occurs upon E_2 (but not 4-hydroxyTAM) binding, 'locking' the ligand into the ligand binding site. Binding properties were unaffected if antibody was added to the ligand-receptor complexes. On sucrose gradients the antioestrogen-ER-antibody complexes sedimented as higher molecular weight species than the corresponding oestrogen complexes, reinforcing the conclusion that different forms of ligand-ER complex occur.

Differences in the physical properties of nuclear salt-extracted ligand receptor complexes from MCF 7 cells have also been noted (Eckert and Katzenellenbogen, 1982; Miller et al., 1985; Sheen et al., 1985a). High affinity antioestrogens (e.g. CI 628M, H1285 and 4-hydroxyTAM) always favour maintenance of a 5.5S receptor complex whereas E_2-receptor complexes sediment at 4S - 5S depending upon sucrose gradient buffer composition. Similarly, H1285-receptor complexes from rat uterine nuclei show a predominance of 5S over 4S forms in contrast to a reverse distribution in the case of E_2 (Ruh and Ruh, 1983). However, only 5S oestrogen and antioestrogen complexes could be identified in GH_3 cells (Tate and Jordan, 1984). These apparent differences in the stability of

the 5S receptor complex with regard to dissociation or perhaps degradation may reflect differences in receptor conformation, producing, for example, differential sensitivity to proteases (Attardi, 1983). It has also been noted that unlike E_2-ER complexes, antioestrogen-receptor complexes are not inactivated by a nuclear receptor phosphatase (Auricchio et al., 1984a)

The reduced ability of antioestrogen-complexes to bind to DNA-cellulose (Katzenellenbogen et al., 1981) and calf thymus DNA (Evans et al., 1982) is further evidence of conformational differences in ligand-ER complexes. It should be noted, however, that other studies using DNA- and synthetic polynucleotide-celluloses have found no such differences (Borgna and Rochefort, 1980, 1981; Murphy and Sutherland, 1983b).

Many early studies (reviewed by Sutherland and Murphy, 1982) showed failure of antioestrogens to stimulate cytoplasmic ER replenishment subsequent to nuclear translocation. Clark et al. (1974) proposed that oestrogen antagonism was therefore due to the inhibition of ER resynthesis. Subsequent observations argue against this hypothesis. In the chick, E_2 can reverse the effects of TAM and induce an oestrogenic response even if administered several hours later when cytoplasmic receptor levels are at their lowest (Mester et al.,1977). Cytoplasmic depletion is also a phenomenon observed with high dose oestrogen agonists (Katzenellenbogen et al., 1977), suggesting that inhibition of cytoplasmic ER replenishment may only be apparent, and result instead from continued ER translocation, due to the long biological half-lives of antioestrogens in plasma and tissue. A similar explanation holds for the observations that, in general, antioestrogens cause prolonged elevation of nuclear ER (Clark et al., 1974; Jordan et al., 1977b; Katzenellenbogen et al., 1977; Ruh and Baudendistel, 1977; Sutherland et

al., 1977a). A study using dense amino acid labelling techniques to follow synthesis and degradation of ER in MCF 7 cells (Eckert et al., 1984) confirms that antioestrogens do not prevent ER synthesis and neither do they accelerate nor block receptor degradation.

Differences in the nuclear processing of antioestrogen and ER complexes have been described in MCF 7 cells in vitro (Koseki et al., 1977; Horwitz and McGuire, 1978b; Tate and Jordan, 1984). TAM-ER complexes are partially resistant and nafoxidine-ER complexes completely resistant to processing, paralleling the respective effects of these compounds on PgR induction. Other data, however, showing that processing does not occur in vivo in the rat uterus (Jakesz et al., 1983), brings into question the relevance of ER processing to antioestrogen action.

Antioestrogen-receptor complexes are more readily extracted from the nucleus by KCl, spermine, actinomycin D and ethidium bromide (Ruh and Baudendistel, 1977). Although this was thought to indicate that agonist and antagonist receptor complexes bind to different chromatin acceptor sites, the observation that the oestrogenic triphenylethylene derivative, zuclomiphene, does not form a salt-resistant nuclear receptor (Ruh and Baudendistel, 1977) suggests such phenomena may not be relevant to antioestrogen action and may instead be explained by the dissociation of low affinity ligands with subsequent differential extraction of unoccupied nuclear receptors.

Digestion of chick oviduct chromatin with micrococcal nuclease has shown that E_2, but not TAM or 4-hydroxyTAM, induces a specific 13 - 14S binding peak on sucrose gradients (Lebeau et al., 1981, 1982). Thus, by this criterion, nuclear binding of antioestrogen-receptor complexes to chromatin acceptor sites appears qualitatively different to that of oestrogen-receptor complexes. However, 13 - 14S binding sites appear if

TAM and progesterone are given simultaneously, although under these conditions TAM has an amplifying effect on gene transcription, in contrast to a suppressive effect when administered together with or after oestrogen (see below).

Although there is limited knowledge of the precise differences between agonist and antagonist receptor complexes, the evidence available shows these differences mediate biological activity primarily through effects on rates of gene transcription rather than on post-transcriptional effects on mRNA stability or protein synthesis. The effects of TAM on RNA polymerases have been summarized by Nicholson et al. (1981). In rat uterus and DMBA-induced rat mammary tumours TAM produces effects on RNA polymerase II qualitatively similar to the effects of E_2, although TAM is unable to maintain the secondary stimulation of activity seen with E_2. TAM is a poor inducer of RNA polymerase I activity compared with E_2, and also has little ability to promote large alterations in RNA polymerase III activity. In mouse uterus, where it has agonist activity, TAM produces identical responses to E_2 on RNA polymerase I and II activities. In the rat when administered simultaneously, TAM antagonises the effects of E_2 on RNA polymerase I and III but is without effect on RNA polymerase II activity.

Little is known about the variety and quantity of mRNA transcripts produced by RNA polymerase II when activated by the TAM-receptor complex. Palmiter et al. (1977) showed TAM decreased the concentration of ovalbumin mRNA after TAM administration to oestrogen-stimulated chickens. Simultaneous administration of TAM and diethylstilboestrol (DES) stopped or inhibited the induced transcription of ovalbumin and conalbumin genes as determined by their respective mRNA levels

(Schweizer et al., 1985); mRNA levels decreased markedly 3 hours after administration. The effect was greater if TAM was injected 6 hours after oestrogen, presumably because the extracellular levels of DES had fallen by this time, allowing more efficient competition for receptor binding by TAM. A good correlation was found between levels of gene transcription and rates of conalbumin and ovalbumin protein synthesis. In contrast TAM amplified the mRNA synthesis induced by dexamethasone and progesterone. The results from this study indicate that these actions of TAM involve effects at the transcriptional level, although TAM alone had no effect on gene transcription, in agreement with previous findings on conalbumin and ovalbumin protein synthesis (Catelli et al., 1980). However, other studies (Binart et al., 1982; Le Bouc et al., 1985) have shown slight increases in conalbumin but not ovalbumin synthesis, perhaps indicating post-transcriptional effects of TAM under these experimental conditions.

Despite many years of intensive research only a limited understanding of the mechanisms of antioestrogen action has been achieved. Major questions remain unresolved especially with regard to the differential expression of antagonist and agonist activities and species/tissue differences. In addition, almost nothing is known of the biochemical basis for the apparently non-ER mediated biological activities of antioestrogenic compounds (Section 1.6.3).

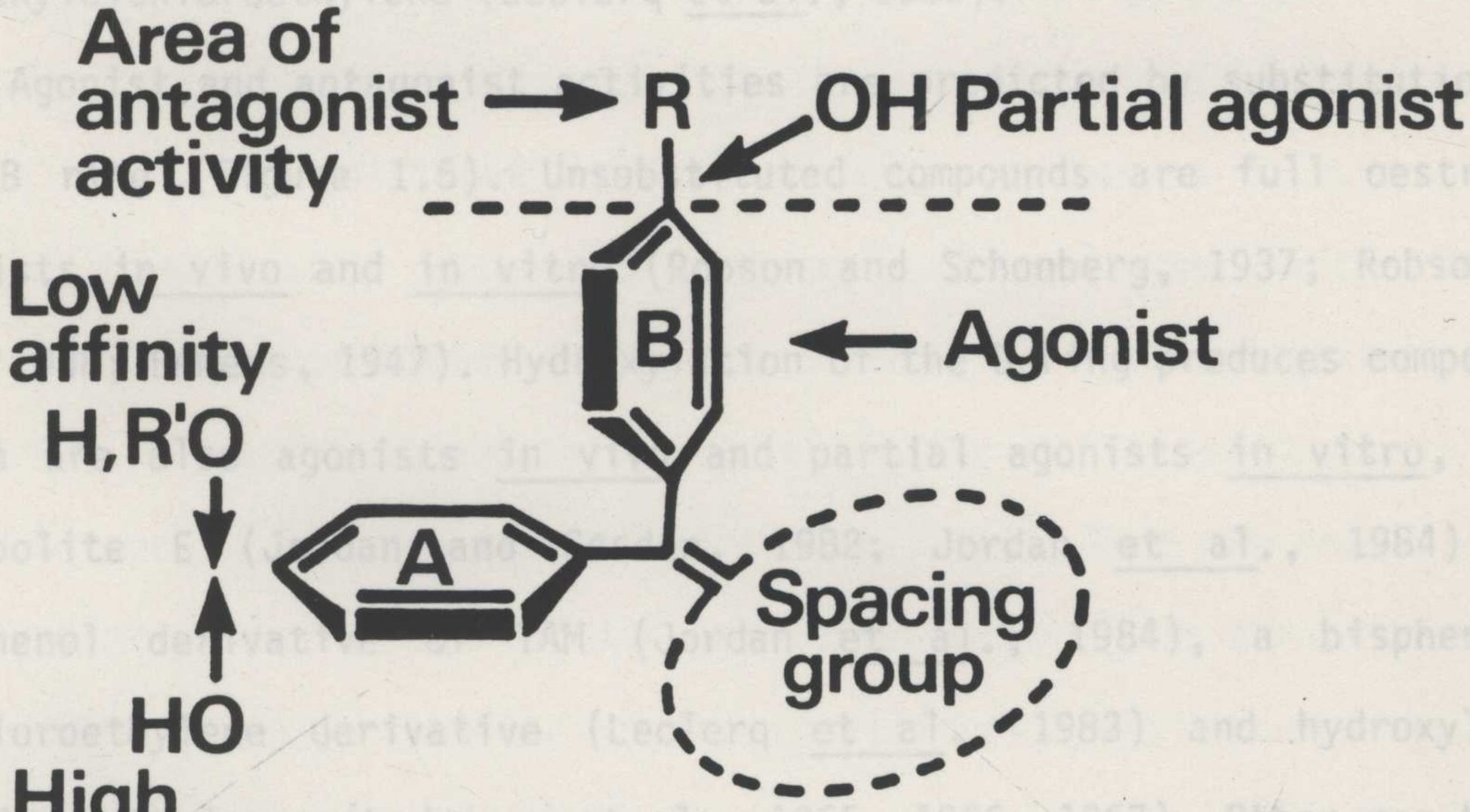
1.6.2 The Antioestrogenic Ligand and a Model for Antioestrogen Interaction with ER

Structure-activity studies have defined those structural features of the antioestrogenic ligand responsible for the expression of oestrogen antagonist and agonist activities and those which are

responsible for potency.

Structural requirements for agonist binding to ER have been reviewed elsewhere (Duax and Weeks, 1980). In respect of nonsteroidal antioestrogens and their structurally related agonist analogues, a general ligand model has been proposed (Figure 1.5) describing the structural requirements for biological activity (Campen et al., 1985). In this model, binding affinity (i.e. potency) is largely determined by substitution on the A ring. Thus a phenolic hydroxyl group at the 4 position, equivalent to the C_3 phenol of E_2, results in compounds with high affinity for ER (Korenman, 1969; Jordan et al., 1977a; Black and Goode, 1980; Hayes et al., 1981; Ruenitz et al., 1982; Keene et al., 1984; Jordan et al. 1984). The structural requirements for high affinity binding are quite specific: a hydroxyl group at position 3 rather than 4 on the A ring of TAM markedly reduces affinity and hence potency (Ruenitz et al., 1982; Jordan et al., 1984). 3,4-hydroxylation of TAM produces a catechol compound with high affinity but low biological activity because of oxidation to a quinone (Jordan et al., 1984). Alkyl ether (e.g. methoxy) substitutions on the A ring result in decreased affinity for receptor but an increased duration of action in vivo (Jordan, 1984).

A variety of 'spacing' groups (Figure 1.5) can occupy the ligand binding site. Triphenylethylenes and related compounds such as the triphenylethanols with phenyl spacing groups have a planar trans-stilbene structure similar to the conformation of E_2 and are thus endowed with relatively high affinity. Substitution of this phenyl group with -OH or $-OCH_3$ groups does not significantly affect activity or potency (Jordan, 1984). Among hydroxylated triphenylethylene derivatives, the non-phenolic vinyl substituent influences binding



R	Agonist Activity		Antagonist Activity	
	'in vivo'	'in vitro'	'in vivo'	'in vitro'
H	full	full	-	-
OH	full	partial	-	partial
glyceryl	partial	-	partial	full
alkylaminoalkoxy	partial	-	partial	full
acetoxy	full	-	-	full

Figure 1.5: Generalized structure of the antioestrogen ligand. The Table shows the effect of side chain structure on the expression of agonist and antagonist activities in the rat. Similar results are obtained in vitro in the MCF 7 model. (-) indicates no activity. (Adapted from Jordan, 1984; and Campen et al., 1985).

affinity (Ruenitz et al., 1983b, 1986), perhaps because resonance effects transmitted through the vinyl double bond can influence hydroxyl group acidity. Smaller spacing groups are permissible such as the cyclohexane ring system of cyclofenil (Leclerq et al., 1983), the chlorine-substituted cyclopropane group of a series of stilbene analogues (Pento et al., 1981) and the chlorine substituents of diphenyldichloroethylene (Leclerq et al., 1983).

Agonist and antagonist activities are predicted by substitution on the B ring (Figure 1.5). Unsubstituted compounds are full oestrogen agonists in vivo and in vitro (Robson and Schonberg, 1937; Robson et al., 1938; Emmens, 1947). Hydroxylation of the B ring produces compounds which are also agonists in vivo and partial agonists in vitro, e.g. Metabolite E (Jordan and Gosden, 1982; Jordan et al., 1984) the bisphenol derivative of TAM (Jordan et al., 1984), a bisphenolic dichloroethylene derivative (Leclerq et al., 1983) and hydroxylated dihydronapthalenes (Lednicer et al., 1965, 1966, 1967). Other non-basic substituents result in varying degrees of expression of antioestrogenicity. Acetoxy-substituted compounds can be either agonists in vivo (cyclofenil), or antagonists both in vivo and in vitro (Schneider et al., 1982; 1985; Jordan, 1984; Schneider and Ball, 1986). An ethyl substituent on the B ring of hydroxylated bromotriphenylethylene (Borgna et al., 1982) confers antioestrogenic properties whereas an allyoxy-substituted fixed-ring hydroxylated triphenylethylene is an agonist both in vivo (Acton et al., 1983) and in vitro (Jordan et al., 1984). Glyceryl side chains on the B ring of a fixed ring triphenylethylene (Jordan et al., 1984) and dihydronapthalene (U 23469, Lednicer et al., 1969) produce antioestrogenic derivatives.

Compounds with basic alkylamino alkoxy side chains which extend

away from the binding site of the A ring 4-hydroxy substituent are almost invariably antioestrogens, and several structure-activity studies have investigated the importance of side chain structure on antioestogenic properties. Lednicer proposed from a study of the antiuterotrophic activities of 3,4-dihydronapthalene derivatives (Lednicer et al., 1966, 1967) that an effective antioestrogen required the presence of basic side chain groups 'at a given position in space', for although the ether oxygen of the side chain could be replaced by carbon without significant loss of potency, reduction in side chain length dramatically decreased potency. Observations with MER-25 confirmed that restriction of the positions that the aminoethoxy side chain could adopt by dimethylation ortho to the side chain reduced antioestrogenic activity (Clark and Jordan, 1976). That the correct side chain orientation is required for antagonist activity is demonstrated by the absence of such activity in the cis-triphenylethylene isomers such as zuclomiphene and ICI 47699 (Harper and Walpole, 1966; Clark and Guthrie, 1981). Robertson et al. (1982a) have challenged the view that a basic group is a necessary side chain component by demonstrating a lack of correlation between the side chain basicity of TAM analogues and binding affinity for ER or antiuterotrophic activity. Hydrogen bonding rather than ionic interactions was suggested to be important for optimal antioestrogenic activity as evidenced by the antioestrogenicity of compounds substituted with non-basic hydroxyethoxy or glyceryl groups. However, despite the fact that these and other studies (Lednicer et al., 1965; Murphy and Sutherland, 1983a, 1985) have shown that side chain structure in general, and terminal amino group structure in particular, influence antioestrogenic activity, the molecular basis of these observations is obscure. It may be that these structures influence

both ligand binding affinity and the expression of antioestrogenic activity by interaction with a putative receptor domain that determines such activity.

A hypothetical molecular model to account for the interaction of ligands with the ER and subsequent expression of antagonist or agonist activity, specifically on prolactin secretion by rat pituitary cells *in vitro*, has been proposed (Lieberman *et al.* 1983b; Jordan, 1984; Jordan *et al.*, 1984). In this model (Figure 1.6), E_2 and other agonist ligands such as DES bind with high affinity because of interaction between the ligand phenolic hydroxy group and a hypothetical 'phenolic' receptor domain. The conformational change induced in the receptor decreases the dissociation rate of the ligand. Hydroxylated antioestrogenic ligands are also able to bind with high affinity to this phenolic site, but the alkylamino alkoxy side chain, perhaps interacting with a specific antioestrogen domain, prevents the conformational changes necessary for the formation of a ligand-receptor complex which is fully functional in subsequent interactions with chromatin. Because of the lack of a phenolic hydroxy group, the A ring of TAM and similar ligands interacts with low affinity with the phenolic site and thus these compounds are less potent antagonists than the corresponding hydroxylated compounds. The oestrogenic *cis*-isomers are suggested to form the preferred *trans*-stilbene-like structure with the ether oxygen of the side chain interacting weakly with the phenolic domain, allowing a conformational change that allows expression of agonist activity. The agonist, Metabolite E, might bind in such a manner that the B-ring phenolic hydroxy group interacts with the phenolic receptor domain. The phenyl spacing group would, however, produce steric inhibition in this model which would account for the reduced receptor affinity observed. Finally,

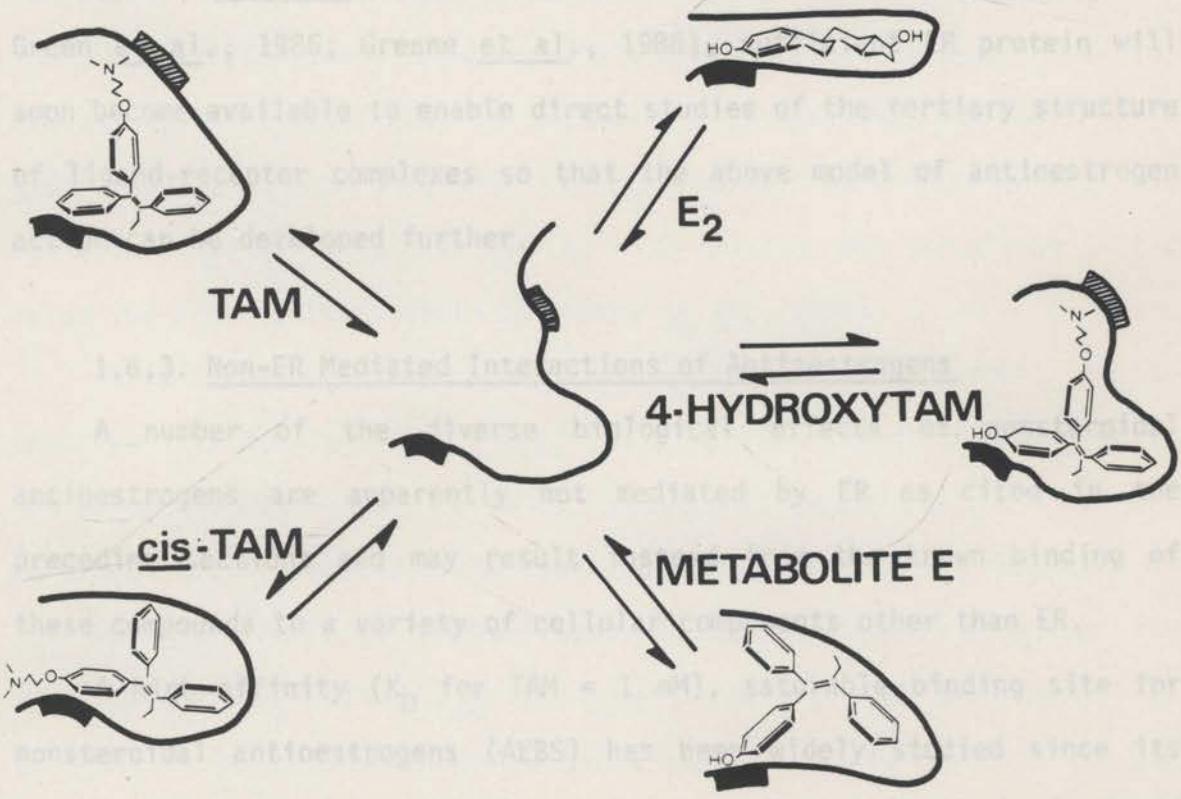


Figure 1.6: Hypothetical model of ER binding of agonist and antagonist ligands. The solid and hatched areas represent putative 'phenolic' and 'anticestrogenic' receptor domains, respectively. (Adapted from Lieberman et al., 1983b).

this model proposes that a partial agonist such as the bisphenol derivative of TAM might produce an equilibrium mixture of agonist and antagonist complexes.

This proposed model of antioestrogen action at the receptor level is of necessity an over-simplification, and fails, for example, to provide any explanation for species, tissue and *in vivo*/*in vitro* differences in pharmacological activity. Hopefully, with the recent cloning and *in vitro* expression of the ER gene (Walter *et al.*, 1985; Green *et al.*, 1986; Greene *et al.*, 1986), sufficient ER protein will soon become available to enable direct studies of the tertiary structure of ligand-receptor complexes so that the above model of antioestrogen action can be developed further.

1.6.3. Non-ER Mediated Interactions of Antioestrogens

A number of the diverse biological effects of nonsteroidal antioestrogens are apparently not mediated by ER as cited in the preceding sections and may result instead from the known binding of these compounds to a variety of cellular components other than ER.

A high affinity (K_D for TAM = 1 nM), saturable binding site for nonsteroidal antioestrogens (AEBS) has been widely studied since its discovery in this laboratory (Sutherland and Foo, 1979; Sutherland and Murphy, 1980; Sutherland *et al.*, 1980). These initial studies in oestrogen-target tissue cytosols showed an excess of binding sites for [^{3}H]TAM and [^{3}H]CI 628 over the number of ER binding sites labelled with [^{3}H]E_2. Subsequent investigations, described in detail in the following Chapters, have shown the AEBS to be a membrane-bound microsomal protein, binding not only nonsteroidal antioestrogens substituted with side chains terminating in basic alkyl amino groups, but also a wide

variety of structurally related compounds with a range of biological activities. The AEBS has no affinity for E_2 or other natural or synthetic steroids. Putative natural ligands for this site have been partially characterized (Clark et al., 1983; Murphy et al., 1985). There is considerable interest in the function of the AEBS especially as a potential mediator of antioestrogenic activity.

Two other specific antioestrogen binding proteins have been described. The benzothiophene antioestrogen LY 117018, conjugated to epoxy-activated agarose, specifically binds a protein present in rat and rabbit uterine cytosol that is neither the ER nor AEBS (Van Oosbree et al., 1984). A binding site with similar binding specificity to the AEBS but with lower affinity for TAM (K_D = 28 nM) has been described on rat serum low density lipoprotein (Winneker et al., 1983).

Antioestrogens have inhibitory effects on smooth muscle contractility (Callantine et al., 1966; Martin, 1981). However, it is not clear whether these effects, presumably occurring in the cell membrane, are non-specific and caused by solution of these compounds in the membrane itself or whether specific membrane-bound receptors are involved. Both E_2 and the cis- and trans-isomers of TAM have been shown to inhibit rat myometrial contractions in response to oxytocin (Lipton et al., 1984) while TAM and 4-hydroxyTAM specifically block histamine-induced contractions of canine tracheal smooth muscle, partially reverse the effect of serotonin and potassium but fail to inhibit acetylcholine-induced contractions (Kroeger and Brandes, 1985). These observations are interesting in light of the finding that TAM has low but detectable affinity for the histamine H_1 receptor as determined by competition with [^{3}H]pyrilamine (Brandes et al., 1985). A further study, however, brings into question the possibility of a receptor-mediated action. Morris

(1985) has shown that although TAM and clomiphene inhibit the contractions of guinea pig ileum produced by a wide range of spasmogens, the effects are non-specific and non-competitive. The actions of acetylcholine, histamine, bradykinin and the muscarinic agonist β-methylcholine were all reversed. It is suggested that these effects are due to changes in membrane ion-permeability.

Triphenylethylene-related antioestrogens (but not E_2, DES, cyclofenil or hexoestrol) are able to increase the sensitivity of doxorubicin-resistant P-388 murine leukaemia cells to doxorubicin treatment (Ramu et al., 1984). This phenomenon may be due to cell membrane interactions resulting in decreased packing density facilitating an increased diffusion rate of doxorubicin into the cell.

The binding of clomiphene to rat brain muscarinic receptors was demonstrated by Ben-Baruch et al. (1982) and it was hypothesised that antioestrogens might thus antagonize the effects of acetylcholine in the autonomic nervous system producing some of the side effects i.e. vasomotor instability, gastrointestinal discomfort, nausea and visual disturbances seen, albeit rarely, during antioestrogen therapy. TAM also binds to brain dopamine D_2 receptors as shown by the competitive inhibition of [^{3}H]spiperone binding (Hiemke and Ghraf, 1984) and in vivo has inhibitory effects on dopamine synthesis and increases dopamine receptor levels (Baksi et al., 1985).

Antioestrogens interact directly with cytochrome P-450 and undergo cytochrome P-450-mediated drug metabolism (Section 1.3). In rats treated for 3 days with TAM, desmethylTAM or 4-hydroxyTAM, potent inhibitory effects on hepatic microsomal oxidations are observed (Meltzer et al. 1984). It is unknown whether the metabolism of co-administered pharmacological agents or endogenous substrates is affected during long-

term TAM administration.

Lam (1984) has shown that TAM is a potent competitive inhibitor of calmodulin in the activation of cAMP phosphodiesterase (IC_{50} = 2 μM). The relevance of calmodulin antagonism to the biological properties and in particular the antitumour activity of TAM is unknown. TAM and several other calmodulin antagonists have been shown to perturb platelet polyphosphoinositide metabolism, resulting in increased levels of ^{32}P-phosphatidylinositol-4-phosphate and ^{32}P-phosphatidylinositol 4,5-bisphosphate (Tallant and Wallace, 1985). Calmodulin inhibition might result in the effects of antioestrogens on muscle contractility previously described, perhaps by altering Ca^{2+} flux across the plasma membrane.

O'Brian et al. (1985) have reported that TAM is an inhibitor of rat brain Ca^{2+} and phospholipid-dependent protein kinase C (IC_{50} 100 μM) and also inhibits the high affinity binding of [^{3}H]phorbol dibutyrate to mouse fibroblast C3H10T½ cells (IC_{50} 5 μM). TAM did not interact with the active site of the enzyme and possibly is inhibitory because of competition with phospholipids.

Antioestrogens have been shown to have direct effects on oestrogen biosynthesis (Watson and Howson, 1977) and on prostaglandin biosynthesis (Fenwick et al., 1977; Sharma and Pugh, 1977; Ritchie, 1978), to alter the overall hormonal environment by changes in the levels of hormones and their plasma binding proteins (Sherman et al., 1979; Habib et al., 1979; Hammond et al., 1980; Manni et al., 1981; Baksi et al., 1985), and to have direct effects on enzymes of carbohydrate metabolism (Deshpande et al., 1985).

The role of non-ER interactions, if any, in directly or indirectly mediating the biological activities of antioestrogens has generally yet

to be established. These aspects of antioestrogen action are dealt with in greater detail in the following Chapters.

1.7 CONCLUSION AND AIMS OF THIS THESIS

The preceding sections have outlined the present state of knowledge regarding antioestrogenic action, and the molecular mechanisms thereof. Clearly there are many deficiencies in this knowledge. We lack precise details regarding the changes that occur to ER upon antioestrogen binding, and the subsequent defects in nuclear interactions and gene transcription. We cannot yet explain the basis for differences in biological activity between species, tissues and the in vitro/in vivo situation, nor many of the effects of antioestrogenic compounds which are not oestrogen reversible and apparently not mediated through ER.

This thesis is concerned with the action of nonsteroidal antioestrogens as antitumour agents; in particular, the intracellular binding of these compounds and their effects on breast cancer cell proliferation. The study had its origins in an existing program in Dr Robert Sutherland's laboratory which had identified and partially characterized the properties of the AEBS (Section 1.6.3), a cytoplasmic binding site for antioestrogens which has properties distinct from those of the oestrogen-binding site of the ER. The major impetus to this work was the possibility that the AEBS could play a role in mediating antioestrogenic or other biological activity either directly by unknown mechanisms or indirectly by influencing ligand distribution or availability. If such a role was established it would lead to increased understanding of many unexplained features of antioestrogen action. Thus a full biochemical characterisation of the AEBS was considered essential, including investigations into tissue and subcellular

distribution, binding properties, with emphasis on ligand specificity, and factors influencing stability and binding activity. Purification procedures were also explored. From these studies arose investigation into the possible identity of the AEBS with other cellular receptors and binding sites.

The biology of human breast cancer cell lines especially with regard to antioestrogen action is one of the major areas of interest in this laboratory. Therefore the MCF 7 cell line was chosen as an in vitro model to test the hypothesis that the AEBS might be involved in mediating antioestrogenic or antitumour activity. By determining the growth inhibitory activities and cell cycle effects of various classes of compounds with known affinities for ER and AEBS it was hoped to clarify the relationship between biological effects and interaction with AEBS.

CHAPTER TWO

GENERAL METHODOLOGY

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2.1 MATERIALS

2.1.1 Chemicals

Unless otherwise noted general laboratory chemicals were supplied by BDH Chemicals, Port Fairy, Victoria, Australia, or by Ajax Chemicals, Sydney, Australia and all other chemicals and biochemicals were supplied by Sigma Chemical Co., St. Louis, MO., U.S.A.

2.1.2. Radiochemicals

Trans-[N-methyl-^{3}H]tamoxifen ([^{3}H]TAM, 71-89 Ci/mmol) was purchased from Amersham Australia, Sydney, Australia, or from New England Nuclear, Boston, MA., U.S.A. An ethanol stock solution of 4 μM [^{3}H]TAM was prepared and stored at -20°C for < 6 months. Aqueous solutions were prepared as described in Section 2.1.3. [^{3}H]adenosine 5'-monophosphate ([^{3}H]AMP), 19.3 Ci/mmol; [^{3}H]oestradiol ([^{3}H]E_2), 85-110 Ci/mmol; [^{3}H]pyrilamine, 26 Ci/mmol; and [^{3}H]spiperone, 77 Ci/mmol, were from Amersham Australia. [^{3}H]flupenthixol, 10.2 Ci/mmol, and [^{3}H]fluphenazine, 30.8 Ci/mmol, were from New England Nuclear.

2.1.3 Triphenylethylene Analogues and Related Compounds

Tamoxifen (TAM; Nolvadex; ICI 46474; trans-1-(4-β-dimethylaminoethoxyphenyl)-1,2 diphenylbut-1-ene) and its analogues were supplied by I.C.I. Pharmaceuticals Division, Macclesfield, Cheshire, England through the courtesy of Drs B. Tait and A. Wakeling. TAM N-oxide was synthesized as reported by Foster et al. (1980). The identity of the compound was confirmed in this laboratory by Dr Roger Reddel using a modification of the HPLC method described by the same authors.

Enclomiphene (trans-1-(p-β-diethylaminoethoxyphenyl)-1,2-diphenyl-2 chloroethylene) and its analogues were supplied by Merrell-Dow

Pharmaceuticals, Cincinnati, OH., U.S.A. through the courtesy of Dr W.L. Albrecht. The PR series of hydroxylated enclomiphene derivatives was synthesized by Drs Peter Ruenitz and Jerome Bagley, University of Georgia, Athens, GA., U.S.A. (Ruenitz et al., 1986). Under the storage conditions used, the PR compounds were present as 50:50 mixtures of the cis- and trans-isomers.

CI 628 (nitromifene, α-4-pyrrolidinoethoxy)phenyl-4-methoxy-α-nitrostilbene) and its analogues (as 50:50 mixtures of the cis- and trans-isomers) were from Dr E. Elslager of the Warner-Lambert Park Davis Company, Ann Arbor, MI., U.S.A.

Bibenzyl and stilbene derivatives (the H series of compounds) were synthesized by Professor C.W. Emmens, University of Sydney, Sydney, Australia. Cyclofenil and related diphenylethylene derivatives were supplied by Dr Guy Leclercq, Institut Jules Bordet, Brussels, Belgium. SKF-525A and its analogues were supplied by Smith, Kline and French Laboratories, Philadelphia, PA., U.S.A. Fluphenazine hydrochloride was supplied through the courtesy of Mr P.F. Levvey, E.R. Squibb and Sons Pty. Ltd., Noble Park, Victoria, Australia. The structures of all these compounds are shown in Tables 6.1 - 6.10.

Stock solutions (10^{-6} - 5 x 10^{-2} M) of these and other compounds, used in competitive binding studies and in studies of the effects on breast cancer cell growth, were prepared in ethanol or in N,N dimethyl-formamide (DMF) when compounds were insoluble in ethanol. Stock solutions were stored at -20°C in glass vials.

Prior to preparation of aqueous stock solutions of these and the radiolabelled compounds, ethanol or DMF was removed under a stream of dry nitrogen. Aqueous solutions, unless otherwise stated, were in the following buffers: 10 mM Tris(tris(hydroxymethyl)aminomethane)-HCl, 0.25

M sucrose, 25 mM KCl, pH 7.4 at 22°C (TSK); or 10 mM Tris-HCl, 1 mM sodium ethylenediaminetetraacetic acid (EDTA), pH 7.4 at 22°C (TE); containing 0.2% (w/v) bovine serum albumin (BSA; Calbiochem-Behring Corp., La Jolla, CA., U.S.A.). The presence of BSA in these buffers (i.e. TSKB and TEB respectively) was necessary to maintain the solubility of these generally hydrophobic compounds and to prevent adsorption to contacting surfaces. Aqueous solutions were stored in polypropylene tubes and all assays were performed in tubes of the same material to minimize adsorption of ligands. In this regard, polypropylene was superior to glass or polystyrene. Exposure to light was minimized at all times and solutions were stored at -20°C for up to 2 months.

2.2. PREPARATION OF SUBCELLULAR FRACTIONS

2.2.1 Cell Culture and Harvesting

MCF 7 human mammary carcinoma cells in their 299th passage were supplied by Dr Charles McGrath, Meyer L. Prentis Cancer Center, Detroit, MI., U.S.A. and maintained as described elsewhere (Sutherland et al., 1983b). Stock cells were passaged weekly with an inoculation density of 2×10^5 cells/150 cm^2 flask into 50 ml RPMI 1640 medium supplemented with 20 mM Hepes buffer, 14 mM sodium bicarbonate, 6 mM L-glutamine, 20 µg/ml gentamicin (Essex Laboratories, Sydney, Australia), 10 µg/ml porcine insulin (CSL-Novo, Parramatta, Australia), 0.06% phenol red and 10% (v/v) foetal calf serum (FCS). All materials were from Flow Laboratories, Sydney, Australia, unless otherwise indicated.

Cells used for preparation of subcellular fractions were obtained by plating 2×10^6 cells in exponential growth phase into 150 cm^2 flasks in 50 ml of medium, and harvesting 6 - 7 days later when cell numbers

were 3 - 5 x 10^7 per flask. Cells were washed once with 1 mM EDTA in Dulbecco phosphate buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.3), and then harvested with 3 ml of the same solution. Cells were collected by centrifugation at 800 x g for 5 min and the cell pellet was washed once with PBS and once with TE containing 0.25 M sucrose (TES) at 4°C.

2.2.2 Subcellular Fractionation of MCF 7 Cells

2.2.2.1 Homogenization: All procedures were at 0° - 4°C. Cells were suspended in TE or TES (3 x 10^7 cells/ml). When hypotonic TE was used cells were allowed to swell for 10 min which enabled easier homogenization. Cells were homogenized in a Teflon-glass Potter-S homogenizer (10 - 20 strokes at 800 r.p.m., or until > 90% of cells were ruptured as assessed by phase-contrast microscopy).

2.2.2.2 Post-mitochondrial fraction: A post-mitochondrial fraction, consisting of the microsomal fraction and cytosol, was prepared by centrifuging the homogenate at 10,000 x g for 20 min. The supernatant was diluted with TE or TES (generally 1:5 - 1:10) and is subsequently referred to as PMF. This preparation was used in all binding studies unless otherwise indicated.

2.2.2.3 Analytical subcellular fractionation: MCF 7 cells were homogenized in 10 mM Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid) buffer, pH 7.4 containing 0.25 M sucrose (HS) (2 - 3 x 10^7 cells/ml, 8 strokes of the homogenizer at 1000 r.p.m.). The homogenate was centrifuged at 800 x g for 10 min to obtain the crude nuclear pellet, which was washed twice with HS. The combined post-nuclear supernatants were centrifuged at 10,000 x g for 20 min to obtain the mitochondrial pellet which was washed twice with the same

buffer. The microsomal fraction was obtained by centrifuging the combined post-mitochondrial supernatants at 130,000 x g_{av} for 1 h in a Beckman 50 Ti rotor. The supernatant was the cytosol fraction (C). The microsomal pellet was washed with HS containing 0.15 M KCl to remove loosely adsorbed protein. The nuclear, mitochondrial and microsomal pellets were resuspended in HS (N, M and Mc fractions, respectively) and assayed for AEBS, ER and various enzyme activities as described below.

2.2.2.4 Nuclear fractions: Crude nuclei were prepared from the crude nuclear pellet by resuspending it in TES containing 2 mM $MgCl_2$ (TESM) and 1% (w/v) Triton X-100, followed by an immediate centrifugation at 800 x g for 5 min. The pellet was washed three times in approximately 10 volumes of TESM.

Purified nuclei were prepared according to the method Chauveau et al. (1956). The pellet was resuspended in 9 volumes of 10 mM Tris-HCl, 2.4 M sucrose, 3 mM $MgCl_2$, pH 7.4 and centrifuged at 76,000 x g_{av} for 60 min in a Beckman SW 27 rotor. Pure nuclei were washed twice with TESM. Nuclear extracts were prepared by glass-glass homogenization of crude nuclei in TE containing 1 M NaCl (approx. 3 x 10^7 nuclei/ml). After extraction for 30 min at 0°C and dilution with an equal volume of TE (final NaCl concentration 0.5 M) the preparation was centrifuged at 140,000 x g_{av} for 30 min in a 50 Ti rotor. The resulting supernatant contained the 'soluble' nuclear receptor fraction. Any unbound oestrogen which was present in this fraction was removed by a 30 min incubation with 0.5% charcoal/0.05% dextran (Section 2.4.1) and centrifugation at 1,500 x g for 10 min.

2.2.2.5 Microsomal subfractions: Microsomes were subfractionated on a discontinuous sucrose gradient containing Cs^+ according to the method of Dallner (1978) for the separation of rat liver rough (ribosome-

bearing) and smooth microsomes. 5 ml of PMF prepared from a homogenate of MCF 7 cells in 0.44 M sucrose was underlayed with 1 ml of 0.6 M sucrose - 15 mM CsCl and 4 ml of 1.3 M sucrose - 15 mM CsCl. The gradients were centrifuged at 130,000 x g_{av} for 1 h in a 50 Ti rotor. The material banding in the 0.6 M sucrose layer (designated the light microsomes, L) and in the pellet (heavy microsomes, H) was collected and washed with TSK and resuspended in TES. The position of these fractions in the gradient corresponds to rat liver smooth- and rough-microsomal subfractions respectively.

2.2.3 Subcellular Fractionation of Rat Liver

2.2.3.1 Preparation of KCl-washed microsomes: Livers were removed from 18 h starved female Sprague-Dawley or Fischer rats (200 - 250 gms) and rapidly cooled on ice. The tissue was minced, washed in ice cold TSK and homogenized (10% or 20% w/v in TSK) in a Teflon-glass Potter S homogenizer (6 strokes at 1,000 r.p.m.). A PMF was prepared by centrifugation at 10,000 x g for 20 min and from the supernatant a microsomal pellet was prepared by centrifugation at 180,000 x g_{av} for 1 h in a 50 Ti rotor. The microsomes were washed free of adsorbed proteins using TSK containing 150 mM KCl followed by recentrifugation. The microsomal fraction (Mc) was resuspended by homogenization in TSK to the original volume of the homogenate and stored at -20°C or in liquid N_2 for up to 3 months without significant loss of activity. The Mc fraction was used for all binding and biochemical studies unless otherwise stated.

2.2.3.2 Analytical subcellular fraction: A 20% (w/v) rat liver homogenate, prepared as described above, was filtered through 100 μm nylon mesh. Centrifugation at 800 x g for 10 min gave the crude nuclear

pellet (N) which was washed twice with TSK. The combined supernatants were centrifuged at 10,000 x g for 20 min to produce the mitochondrial pellet (M) which was washed twice with TSK. The combined supernatants from this fraction were further centrifuged at 130,000 x g_{av} for 1 h to produce the pelleted microsomal fraction (Mc), which was washed with TSK containing 150 mM KCl, and the supernatant containing the soluble cellular proteins. The combined supernatant and microsomal wash constituted the cytosol (C). All pellets were resuspended in TSK. Rough (RM) and smooth microsomes (SM) were prepared by the method of Dallner (1978) as described in Section 2.2.2.5 from a 1:5 (w/v) homogenate of rat liver in 0.44 M sucrose, using either a 50 Ti rotor or, in a separate series of experiments using a slightly modified technique with a swinging bucket rotor (Beckman SW 50.1). In the latter experiments 2 ml of PMF was underlayed with 1 ml of 0.6 M sucrose containing 15 mM CsCl, and 2 ml of 1.3 M sucrose also containing 15 mM CsCl and centrifuged for 3 h at 234,000 x g_{av} at 4°C. 5M were collected as a band at the 0.6 M/1.3 M interface and RM as the pelleted material. All microsomal subfractions were washed free of cytosolic and adsorbed proteins with TSK containing 0.15 M KCl.

2.2.4 Biochemical Analysis of Subcellular Fractions

Analytical subcellular fractionation of MCF 7 cells and rat liver was accompanied by a biochemical analysis of specific marker enzymes for various organelles within each subcellular fraction. The marker enzymes selected for MCF 7 cells were the same as those used for rat liver, in view of the fact that at the time of these studies there were no published data on the isolation and properties of MCF 7 subcellular organelles.

5'-nucleotidase (E.C 3.1.3.5) was the enzyme marker for the cell surface (plasma) membrane, and was assayed by the method of Newby et al. (1975) which measures the release of [^{3}H]adenosine from [^{3}H]AMP. A stock solution of 200 µM AMP was prepared in 50 mM Tris-HCl pH 8.0. [^{3}H]AMP was added to a concentration of 40 - 60 x 10^3 cpm/ml. 500 µl of this solution and 50 µl of the various subcellular fractions were incubated at 37°C for 30 min and the reaction stopped with 100 µl of 0.15 M $ZnSO_4$, followed by 100 µl of 0.15 M $Ba(OH)_2$. After 5 min centrifugation in an Eppendorf Microfuge 0.5 ml of the supernatant was counted. After subtraction of the control value obtained using 50 µl of buffer in place of the subcellular fractions, radioactivity was directly proportional to enzyme activity.

Cytochrome c oxidase (E.C 1.9.3.1) was the enzyme marker for mitochondria. The assay was based on that of Yonetani and Ray (1965) and measures the oxidation of reduced cytochrome c by following the decrease in absorbance at 550 nm. Reduced cytochrome c was prepared by reducing a 2 mM solution of cytochrome c (Type VI, Sigma Chemical Co.) in 0.067 M sodium phosphate buffer pH 7.4 with excess ascorbic acid (approximately 4 mg). The solution was applied to a PD-10 Sephadex column (Pharmacia, Uppsala, Sweden) and eluted with 2.5 ml of phosphate buffer. The reduced cytochrome c in the eluate was diluted to approximately 25 µM with sodium phosphate buffer containing 1 mM EDTA and 0.05% Triton X-100. The reaction mixture consisted of 2.8 ml of this solution and 200 µl of the various subcellular fractions. The absorbance at 550 nm was monitored for up to 5 min at 25°C. The reaction obeys first order kinetics and the slope of the plot of $\ln[A_t - A_\infty]$ vs time gave the pseudo-first order rate constant, which is directly proportional to oxidase concentration, where A_t is the absorbance at time t and A_∞ is determined by adding a

drop of $K_3Fe(CN)_6$ to the cuvette to fully oxidise the remaining reduced cytochrome c.

NADPH cytochrome c reductase (E.C. 1.6.2.4) was the enzyme marker for the endoplasmic reticulum and was measured by a modification of the technique described by Beaufay et al. (1974). The reaction mixture consisted of 2.7 ml of cytochrome c (0.05 mM in 50 mM Hepes buffer, pH 7.5 containing 0.3 mM NaCN) and 0.2 ml of the various subcellular fractions. 0.1 ml of 2.25 mM NADPH was then added and the absorbance monitored at 550 nm at 25°C. The slope of the plot of absorbance vs time is directly proportional to activity.

β-glucuronidase (E.C. 3.2.1.31) was the enzyme marker for lysosomes and was assayed as described by Beaufay et al. (1974). The assay measures the rate of release of phenolphthalein from the sodium salt of phenolphthalein mono-β-glucuronidic acid (Sigma Chemical Co.). 0.25 ml of sodium acetate buffer (0.2 M sodium acetate, pH 5.0, containing 1 M sucrose and 0.4% Triton X-100), 0.25 ml of phenolphthalein glucuronide (4 mM in water), and 0.5 ml of the various subcellular fractions were incubated at 37°C for 60 min. The reaction was stopped with 3 ml of a solution containing 0.133 M glycerol, 0.067 M NaCl and 0.083 M Na_2CO_3, and the mixture was centrifuged for 15 min at 1,500 x g. The absorbance was read at 470,552 and 610 nm and the increase in absorbance at 552 nm (which is directly proportional to enzyme activity) was measured using a three-wavelength method on a Shimadzu UV-240 spectrophotometer.

Lactate dehydrogenase (E.C. 1.1.1.27) was the marker enzyme for the soluble protein (cytosol) fraction and was assayed by the method of Schwartz and Bodansky (1966). Pyruvate is reduced to lactate with the concomitant oxidation of NADH to NAD^+ which is monitored at 340 nm. The reaction mixture consisted of 2.3 ml of 0.067 M sodium phosphate buffer

(pH 7.4), 0.1 ml NADH (3 mM) and 0.5 ml of the various subcellular fractions. After incubation for 20 min at 37°C, 0.1 ml of sodium pyruvate (0.01 M, pH 7.4) was added and the reaction followed at 340 nm at 37°C to obtain the reaction velocity in the zero order part of the curve, which is directly proportional to the enzyme activity.

Cytochrome P-450 was assayed as described by Estabrook and Werringloer (1978). Microsomal fractions were diluted in 50 mM Tris-HCl pH 8.0 to a protein concentration of approximately 1.5 mg/ml and divided equally into two cuvettes. The contents of the sample cuvette were gassed with carbon monoxide, and sodium dithionite (1 mg) was added to both cuvettes. The difference spectrum of the CO complex of reduced cytochrome P-450 minus the spectral contribution of reduced cytochrome P-450 was recorded on a Shimadzu UV-240 spectrophotometer. The change in absorbance at 450 nm relative to 490 nm was converted to a cytochrome P-450 concentration using the difference extinction coefficient of 91 cm^{-1} mM^{-1}.

RNA was the marker for rough (ribosome-bearing) endoplasmic reticulum and was assayed by a modified Schmidt-Thanhauser method as described by Munro and Fleck (1966). To a 5 ml sample in a conical polypropylene tube 2.5 ml of ice cold 0.6 M $HClO_4$ was added, and after 10 min at 0°C the mixture was centrifuged at 1,000 x g for 10 min. The pellet was washed twice with ice-cold 0.2 M $HClO_4$, drained and solubilized with 0.3 M KOH at 37°C for 1 h. After cooling on ice 2.5 ml of 1.2 M $HClO_4$ was added to precipitate protein and DNA. After standing a further 10 min at 0°C the mixture was centrifuged at 1,000 x g for 10 min. The absorbance of the supernatant was read at 260 nm. Standard curves were prepared under the same conditions using calf liver RNA (Type IV, Sigma Chemical Co.).

Protein, AEBS and ER were assayed as described elsewhere (Sections 2.3, 2.4, 2.5).

Results of the subcellular fractionation studies are presented according to de Duve et al. (1955), where the relative specific activity (RSA) of a given component in the various subcellular fractions is plotted against the fractional protein content of the subcellular fractions expressed as a percentage. RSA is calculated as the fractional amount of total recovered activity divided by the fractional amount of total recovered protein. RSA is also the ratio of the absolute specific activity of the fraction to the absolute specific activity of the starting material, corrected for recovery. The area of one block on the graphical representation, RSA x percent protein, gives the portion of total activity found in that fraction. The total area of the diagram is equal to 100%.

2.3 PROTEIN DETERMINATION

Protein concentration was determined by the method of Lowry et al. (1951), using BSA as a standard. In PMF, microsomal protein concentration was determined in the microsomal pellet produced by the addition of 10 mM $CaCl_2$ to PMF followed by centrifugation at 9,000 x g for 5 min (Schenkman and Cinti, 1978). In the presence of Hepes-containing buffers, protein was determined by the method of Bradford (1976) using the Bio-Rad Protein Assay with human γ-globulin as standard.

2.4 DETERMINATION OF BINDING PARAMETERS OF THE AEBS

2.4.1 Standard Assay Conditions

Measurements of bound (B) and unbound (U) TAM were made by incubating in 2.5 ml polypropylene tubes 100 µl microsomes, PMF or other subcellular fraction (diluted in TSK or TE and containing 1 µM E_2, sufficient to saturate any ER present and thus prevent any interaction of [^{3}H]TAM with ER) with 50 µl [^{3}H]TAM in TSKB or TEB, and either 50 µl TSKB (or TEB) or 50 µl unlabelled TAM (400 nM or 4 µM) in TSKB or TEB giving a total reaction volume of 200 µl. After 16 - 20 h at 0° - 4°C or 1 - 2 h at 22°C, by which time equilibrium had been reached, B and U were separated by incubation at 0°C with 500 µl of 0.5% (w/v) activated charcoal (BDH Chemicals) - 0.05% (w/v) Dextran-T70 (Pharmacia) suspension followed by centrifugation at 1,500 x g for 5 - 10 min at 4°C. By this means unbound ligand was adsorbed by the charcoal and pelleted. The charcoal separation technique also results in reduction of the high levels of non-specific binding by partial removal of [^{3}H]TAM from low affinity (non-receptor) binding sites. A 500 µl aliquot of the supernatant containing bound [^{3}H]TAM was removed, mixed with 5 - 10 ml of scintillant (ACS; Amersham Australia) and counted in a LKB 1217 Rackbeta or Beckman 2800 Liquid Scintillation counter, using external standardization. Duplicate or triplicate incubations were routinely used.

To test the optimal conditions for separation of protein-bound from unbound TAM, PMF binding sites from MCF 7 cells were labelled with 4 nM [^{3}H]TAM in two parallel incubations for 16 h at 0°C, one in the absence (total binding) and the other in the presence of 1 µM unlabelled TAM (non-specific binding). After addition of charcoal/dextran (Figure 2.1) specific binding of [^{3}H]TAM remained constant between 20 min and 90 min,

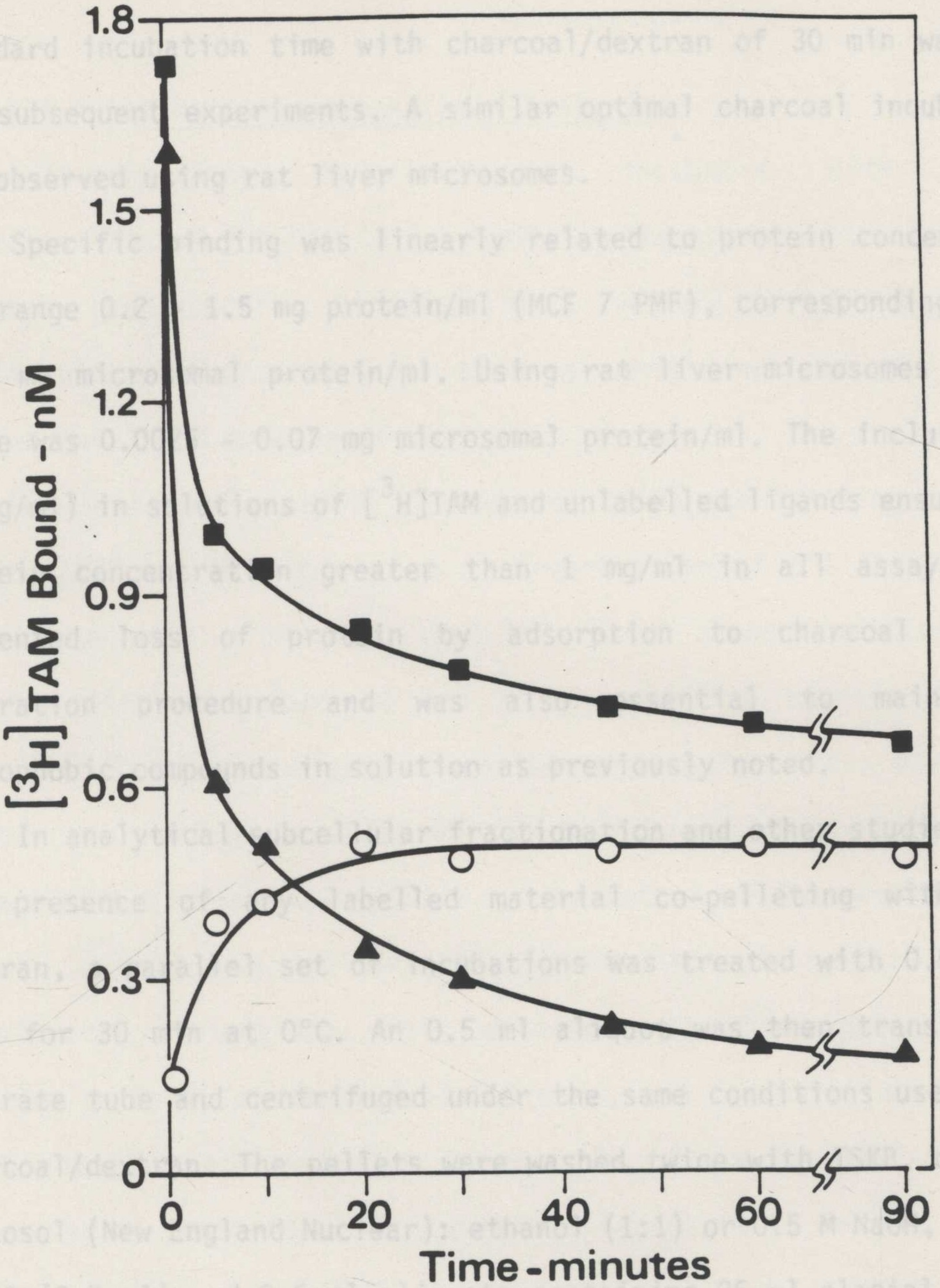

Figure 2.1: Effect of time of exposure to charcoal/dextran on the binding of [^{3}H]TAM to the AEBS. PMF from MCF 7 cells was incubated at 0°C for 16 h with 5 nM [^{3}H]TAM in the absence (total binding) or presence (non-specific binding) of 1 µM unlabelled TAM. Five volumes of 0.5% charcoal/0.05% dextran were added to 2 volumes of the labelled complexes and at the times indicated 700 µl aliquots were removed, centrifuged at 1,500 x g for 10 min at 4°C and 500 µl of the supernatant counted. The concentration of specifically bound [^{3}H]TAM (○) was the difference between total binding (■) and non-specific binding (▲).

while non-specific binding declined slowly during this period. A standard incubation time with charcoal/dextran of 30 min was selected for subsequent experiments. A similar optimal charcoal incubation time was observed using rat liver microsomes.

Specific binding was linearly related to protein concentration in the range 0.2 - 1.5 mg protein/ml (MCF 7 PMF), corresponding to 0.04 - 0.45 mg microsomal protein/ml. Using rat liver microsomes the linear range was 0.0025 - 0.07 mg microsomal protein/ml. The inclusion of BSA (2 mg/ml) in solutions of [^{3}H]TAM and unlabelled ligands ensured a final protein concentration greater than 1 mg/ml in all assays and this prevented loss of protein by adsorption to charcoal during the separation procedure and was also essential to maintain these hydrophobic compounds in solution as previously noted.

In analytical subcellular fractionation and other studies to detect the presence of any labelled material co-pelleting with charcoal-dextran, a parallel set of incubations was treated with 0.5 ml HSB or TSKB for 30 min at 0°C. An 0.5 ml aliquot was then transferred to a separate tube and centrifuged under the same conditions used to pellet charcoal/dextran. The pellets were washed twice with TSKB, dissolved in Protosol (New England Nuclear): ethanol (1:1) or 0.5 M NaOH, 0.1% Triton X-100 (0.7 ml) and 0.5 ml aliquots containing 25 µl glacial acetic acid or 50 µl concentrated HCl (to eliminate chemiluminescence) were counted. The sum of the binding in the charcoaled supernatant and in the pelleted material was thus equal to the total binding within a particular incubation. If an aliquot was not transferred to a separate tube prior to centrifugation, as described above, [^{3}H]TAM adsorbed to the assay tube during the initial incubation was solubilized, resulting in high levels of non-specific binding, masking any low levels of specific

binding present.

2.4.2 Saturation and Scatchard Analysis

AEBS-containing material was incubated with increasing concentrations of [^{3}H]TAM (0.5 - 6 nM) in the absence (total binding) or presence (non-specific binding) of 1 μM unlabelled TAM. Saturable (specific) components of the binding were given by the difference between total and non-specific binding.

For Scatchard analysis (Scatchard, 1949) the limiting value of B/U ($\lim_{B\to\infty}$[B]/[U]) was determined at a concentration of 2 - 3 nM [^{3}H]TAM by competition with 1 μM unlabelled TAM. Under these conditions the high affinity AEBS is saturated and as non-specific binding is directly proportional to free ligand concentration the B/U ratio asymptotically reaches a limiting value on a Scatchard plot. Correction for non-specific binding was applied by the method of Chamness and McGuire, (1976), using programs written for Hewlett Packard 41CV and 97 calculators. In outline, data entered into these programs are the specific activity of the radiolabel used, and for each (averaged) assay point total radioactivity added (as d.p.m.) and total bound radioactivity measured after incubation (as d.p.m.). Unbound (i.e. free) ligand concentration [U] is calculated and raw data points (including the limiting ratio above) for a Scatchard plot are printed i.e. [B]/[U] and [B], where [B] is the concentration of total bound ligand. The plot is generally curvilinear, being a composite curve of specific and non-specific components which are separated by calculation when the limiting ratio is entered. For each raw data point the concentration of non-specific binding ([NSB]) and specific binding ($[B]_{specific}$) are calculated and data for the linearized Scatchard plot are printed i.e.

$[B]_{specific}/[U]$ and $[B]_{specific}$ where:

$$[B]_{specific} = [B] - [NSB]$$

and

$$[NSB] = [U] \lim_{B \to \infty} \frac{[B]}{[U]}.$$

The binding capacity (C) and apparent equilibrium dissociation constant (K_D) of the specific, saturable binding sites are calculated after least squares linear regression analysis of the plot of $[B]_{specific}/[U]$ vs $[B]_{specific}$.

2.4.3 Single Saturating Dose Assay

Specific binding capacity was alternatively measured as the difference in the amount of TAM bound after incubation with a concentration of [^{3}H]TAM (5 nM) sufficient to saturate the AEBS in the absence or presence of 1 μM unlabelled TAM. Bound and unbound ligand were separated by charcoal/dextran adsorption or by the pelleting technique (Section 2.4.1).

2.4.4 Kinetic Studies

2.4.4.1 Association rate: PMF (MCF 7) or microsomes (rat liver) were incubated at various temperatures with 5 nM [^{3}H]TAM in the absence (total binding) or presence (non-specific binding) of 1 μM unlabelled TAM. Bound TAM was determined at various times points by halting the reaction with 0.5% charcoal/0.05% dextran at 0°C followed by incubation for 30 min. Specifically bound TAM was calculated as the difference between total and non-specific binding. The kinetic association rate constant (k_{+1}) was calculated assuming second-order kinetics for the

formation of the TAM-AEBS complex. Plots of $\log[(R_o/L_o)(L_o-RL)/(R_o-RL)]$ against time, where L_o and R_o were the concentrations of unbound TAM and uncomplexed AEBS at zero time and RL was the concentration of the TAM-AEBS complex at time t, produced straight lines the slopes of which were related to the second order rate constant such that $k_{+1} = [2.303/(L_o-R_o)] \times$ slope. The association rate may be underestimated by this method as the concentration of unbound ligand (i.e. L_o-RL) is not corrected for non-specific binding.

2.4.4.2 Dissociation rate: An isotopic dilution technique was used to estimate the rate of dissociation of the TAM-AEBS complex. PMF or microsomes were pre-labelled with 5 nM [^{3}H]TAM for 16 h at 0°C or for 1 h at 22°C in the absence or presence of 1 μM unlabelled TAM. Unbound ligand was removed by addition of 5% charcoal/0.5 dextran (to a final charcoal concentration of 0.5%) followed by incubation at 0°C for 30 min and centrifugation at 1,500 x g for 10 min. Unlabelled TAM (10^{-4} M in ethanol) was added to a final concentration of 1 μM and at various times thereafter specifically bound [^{3}H]TAM was determined at various temperatures by charcoal/dextran adsorption at 0°C. Plots of log $[RL/RL_0]$ against time, where RL_0 and RL were the concentrations of the TAM-AEBS complex at time 0 and t respectively, gave straight lines the slopes of which were related to the first order kinetic dissociation rate constant (k_{-1}) such that $k_{-1} = 2.303 \times$ slope.

The enthalpy changes ($\Delta H°$) for k_{+1} and k_{-1} were calculated from the plots of log k against 1/T(°K) which yielded straight lines of slope $-H°/2.303R$ where R is the gas constant.

2.4.5 Competitive Binding Studies

To determine the ability of various ligands to compete with TAM for

binding to the AEBS, [^{3}H]TAM at a final concentration of 4 - 5 nM was incubated for 16 h at 0°C or for 1 - 2 h at 22°C with increasing concentrations of unlabelled ligand over the range 2.5 nM - 10 μM. The incubation mixture consisted of 50 μl of 16 - 20 nM [^{3}H]TAM in TEB or TKB, 50 μl of the unlabelled competing ligand up to 40 μM in TEB or TSKB, and 100 μl of MCF 7 PMF or rat liver microsomal suspension containing 1 μM E_2, sufficient to saturate any ER present. Bound and free [^{3}H]TAM were separated by charcoal/dextran adsorption (Section 2.4.1).

In some experiments the unlabelled competing ligands were added as ethanol stock solutions in which case instead of 50 μl of labelled ligand in TSKB, 40 μl of TKB and 10 μl of ethanol stock solution were added. This concentration of ethanol had no significant effect on the determination of relative binding affinity (RBA). The use of ethanol stocks allowed high concentrations of ligands with limited solubility in aqueous solutions to be used. Stock solutions prepared in DMF could also be used in the same way.

Data were plotted as percent total or specific [^{3}H]TAM bound versus log ligand concentration. RBA was calculated by the method of Korenman (1970), where RBA = (IC_{50} of TAM/IC_{50} of the test compound) x 100%. The RBA of TAM for AEBS is defined as 100%.

2.4.6 Thermal Stability of AEBS and Effects of pH, Ionic Strength and Enzyme Treatment

To test the thermal stability of the AEBS, MCF 7 PMF or rat liver microsomes were incubated at various temperatures and the level of AEBS activity remaining was determined by the single saturating dose technique (Section 2.4.3).

To test the effects of pH on [^{3}H]TAM binding to MCF 7 AEBS, Scatchard analyses were performed (0 - 4°C, overnight incubation) on PMF prepared in a universal buffer system (citric acid, monopotassium phosphate, boric acid, barbital, all 0.03 M) pH-adjusted with 0.2 M NaOH and to constant ionic strength with 0.2 M NaCl (Dawson et al., 1969). pH was measured in the final assay incubation mixture at 4°C.

To test the effects of pH on rat liver AEBS, TSK buffer was pH-adjusted with HCl or NaOH (which had minimal effect on ionic strength) and then used to prepare dilute microsomal suspensions for assay. The pH of the final assay incubation mixture was measured at 22°C and [^{3}H]TAM binding was assessed by Scatchard analysis after 1 h incubation at 22°C.

The sensitivity of [^{3}H]TAM binding to ionic strength was assessed by adjusting MCF 7 PMF or rat liver microsomal suspensions to various ionic strengths with KCl. [^{3}H]TAM binding was measured by Scatchard analysis.

To test the sensitivity of the AEBS to enzymes MCF 7 PMF was incubated at 37°C for 1 h with trypsin (Type I), protease (Type VII), lipase (Type XI), phospholipase C (Type I), phospholipase A_2, or RNase (Type IA) from Sigma Chemical Co. In other experiments, MCF 7 PMF was incubated for 30 min with lipase followed by the addition of trypsin and a further 30 min incubation. Rat liver microsomes were incubated at 30°C for 1 h with trypsin (in the presence of 2 mM Ca^{2+}), lipase or RNase (in the presence of 0.5 mM Mg^{2+}). After dilution AEBS concentration was measured by the single saturating dose technique (Section 2.4.3).

2.5 MEASUREMENT OF OESTROGEN RECEPTOR BINDING PROPERTIES

In MCF 7 subcellular fractions ER concentrations were measured by the single saturating dose technique (Section 2.4.3) using 5 nM [^{3}H]E_2.

Incubation was at 0°C for 16 h in the absence or presence of 100 nM unlabelled E_2. Bound and unbound $[^3H]E_2$ were separated by charcoal/dextran adsorption or by the alternative pelleting technique as described in Section 2.4.1.

Competitive binding studies of the binding of ligands to ER were carried out as described in Section 2.4.5 using 0.5 nM $[^3H]E_2$ (final concentration) in TEB or TSKB containing 1 M NaCl and nuclear extracts from MCF 7 cells as a source of ER. Nuclear extracts were prepared from crude nuclei (Section 2.2.2.4). RBA values were calculated as for AEBS (Section 2.4.5). The RBA of E_2 for ER is defined as 100%.

2.6 MEASUREMENT OF MCF 7 CELL GROWTH INHIBITION IN VITRO

MCF 7 cells (5 x 10^4/flask) in exponential growth phase were plated into 25 cm^2 flasks in 5 ml medium described above (Section 2.2.1) except that the concentration of FCS was reduced to 5% (v/v). No attempt was made to remove endogenous steroids from the FCS. The same batch of FCS was used throughout this study.

Twenty-four hours after plating the medium was changed and the drugs added from ethanolic or DMF stock solutions such that the final solvent concentration was 0.1% or 0.2% in all flasks, a concentration without effect on cell growth. After approximately four population doublings of the control cultures (4 - 5 days for MCF 7 cells) the cells were harvested with 0.05% trypsin, 0.02% EDTA in PBS. Viable cell counts were made under phase contrast on a haemocytometer and the cells stained for flow cytometry.

2.7 FLOW CYTOMETRY

Approximately 10^6 cells were stained for DNA flow cytometry with ethidium bromide-mithramycin as previously described (Taylor, 1980). Analysis was performed on an ICP22 pulse cytometer (Ortho Instruments, Westwood, MA.) with excitation at 360 - 460 nm and fluorescence detection at greater than 550 nm. Estimates of the cell cycle kinetic parameters, i.e. the proportion of cells in the G_0/G_1, S and G_2 + M phases of the cell cycle, were calculated from the resulting DNA histograms using a planimetric method of analysis (Milthorpe, 1980). Figure 2.2 shows a representative DNA histogram of MCF 7 cells in exponential growth phase.

2.8 STATISTICS

Statistical analyses were carried out using either a Hewlett-Packard HP-41C calculator equipped with Stat-Pac or the Minitab program run on a PDP-11/73 computer. Where applicable results are presented as mean ± S.E.M.

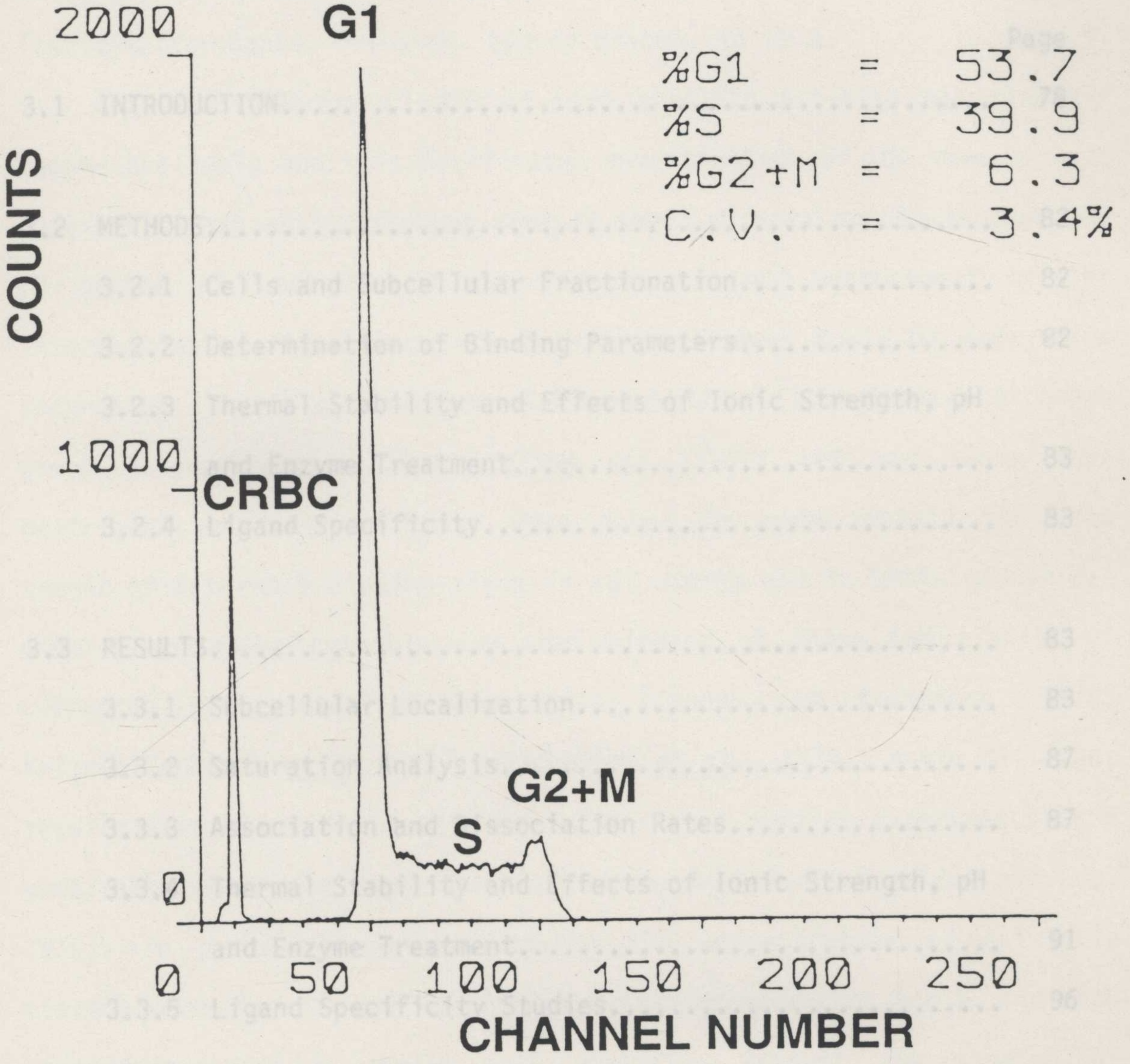

Figure 2.2: Histogram of cellular DNA content of exponentially growing MCF 7 cells as determined by flow cytometry. Channel number (arbitrary units) is linearly related to intensity of fluorescence emitted by DNA fluorochromes. CRBC, chicken red blood cells; C.V., coefficient of variation of G_1 peak.

CHAPTER THREE

ANTIOESTROGEN BINDING SITES IN MCF 7 HUMAN MAMMARY CARCINOMA CELLS

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3.1 INTRODUCTION

Studies on the molecular mechanisms of action of TAM and other nonsteroidal oestrogen antagonists, with emphasis on their antitumour activity, commenced in Dr R.L. Sutherland's laboratory at the Ludwig Institute for Cancer Research, Sydney Branch, in 1978.

Tritiated TAM and CI 628 of high specific activity had recently become available and this facilitated investigation of the then current hypothesis that antioestrogens mediate their effects solely through the ER system of oestrogen target tissues. Direct measurement of the interactions of these compounds in various tissues could be made using saturation analysis and competitive binding techniques. Although previous experiments using [^{3}H]TAM and [^{3}H]CI 628 had shown that oestrogens and antioestrogens were bound to approximately the same number of saturable binding sites in rat uterus and in DMBA-induced rat mammary carcinoma cytosols and that binding of these two classes of compounds were mutually competitive (Capony and Rochefort, 1978; Katzenellenbogen et al., 1978; Nicholson et al., 1979), quite different results were obtained in this laboratory when similar experiments were undertaken with oestrogen-withdrawn chick oviduct (Sutherland and Foo, 1979). In cytosol from this tissue the concentration of saturable binding sites for TAM and CI 628 was 3-fold greater than for E_2 and high concentration of E_2 could only partially inhibit the binding of tritiated antioestrogens to their saturable binding sites. These data indicated, for the first time, the presence of high affinity (K_D approximately 2 - 10 nM), saturable antioestrogen binding sites (AEBS) distinct from the oestrogen binding site of the ER. The possibility that AEBS might mediate the antagonistic effects of antioestrogens, either directly, or indirectly by participating in intracellular binding and

thereby regulating the amount of antioestrogens available for interaction with ER stimulated further investigation of the AEBS by this and other laboratories.

An examination of eight rat, chick and human oestrogen target tissue cytosols (Sutherland et al., 1980) showed that all tissues except rat uterus contained significantly more antioestrogen binding sites than could be accounted for by the concentration of ER. E_2 could only partially inhibit specific [^{3}H]TAM and [^{3}H]CI 628 binding. No high affinity sites were detected in chick skeletal muscle cytosol or in rat plasma. This study also showed that antioestrogen specific binding was present in MCF 7 human mammary carcinoma cell cytosol, and that the antioestrogens TAM, CI 628, nafoxidine and 4-hydroxyTAM and the oestrogenic cis-isomer of TAM (ICI 47699) had comparable affinity for these sites whereas N-desmethylTAM had reduced affinity. In contrast, natural and synthetic oestrogens, androgens and progestins had no affinity. Binding activity was shown to be destroyed by trypsin but not by lipase, RNase or DNase.

A possible role for the AEBS in the antitumour activity of TAM was suggested by a study of the binding of [^{3}H]TAM to a small series of cytosols from human mammary tumours (Sutherland and Murphy, 1980). AEBS was present in ER-positive tumours at an average 8.6-fold higher level than ER, and no AEBS was detected in ER-negative tumours.

In early studies, several other groups were able to confirm the existence of the AEBS. Gulino and Pasqualini (1980) found that [^{3}H]TAM bound to a class of saturable binding sites distinct from ER in foetal guinea pig uterine cytosol with an apparent K_D of 0.39 ± 0.01 nM, and Jozan et al. (1981) were able to confirm the presence of AEBS in MCF 7 cells. This latter study also reported the interesting observation that

AEBS concentrations were substantially reduced in a TAM-resistant MCF 7 cell line. Faye et al. (1980) examined the properties of AEBS in rat uterine cytosol after thermal denaturation of the ER. [^{3}H]TAM bound to these sites with a K_D of 3.3 nM and was competed for by CI 628 and nafoxidine. Uterine AEBS content was found to vary as a function of maturity, phase of the oestrous cycle and following castration.

The presence of AEBS in rat uterine cytosol was demonstrated in this laboratory after depletion of cytosolic ER by prior treatment of animals with E_2 (Murphy and Sutherland, 1981a). The AEBS did not undergo nuclear translocation, demonstrating that the AEBS is unlikely to be an antioestrogen binding domain on the ER. The AEBS was present at levels only 5% that of ER, and this was confirmed in experiments where cytosolic ER was saturated in vitro with E_2, a technique which eliminates interaction between tritiated antioestrogens and ER and simplifies quantitation of the AEBS. K_D values of 1.0 - 1.6 nM were determined for the interaction of CI 628 and TAM with rat uterine AEBS.

The structural specificity of the AEBS was defined in greater detail in a study of the binding of clomiphene analogues to AEBS in MCF 7 cytosol (Murphy and Sutherland, 1981b). It was concluded that the aminoether side chain, which is essential for antioestrogenic activity, is a major structural determinant of the binding of synthetic triphenylethylene antioestrogens to the AEBS.

Gulino and Pasqualini (1982) extended their studies on AEBS in foetal guinea pig tissues, confirming findings from this laboratory on binding specificity, sensitivity to proteolytic enzymes and failure to undergo nuclear translocation (although a similar binding site was identified in the nuclear fraction presumably due to microsomal contamination, see below). This study also showed the AEBS to be

relatively heat stable, present at only low concentrations in non-oestrogen target tissues, and that levels decreased with maturity.

Thus, at the beginning of the present study the presence of binding sites apparently specific for triphenylethylene antioestrogens but distinct from ER had been reported in a variety of tissues from several species. Although there was some preliminary characterization of biochemical properties, the reports were fragmentary and there was insufficient detail to allow an assessment of the extent to which these sites varied between tissues and species and of what their role might be in the normal physiology of the cell or in mediating the actions of antioestrogens as pharmacological agents. Major questions remained as to the biochemical nature of the AEBS, its binding properties, ligand specificity, tissue distribution, subcellular localization and hormonal regulation. The possibility of a natural ligand for the AEBS had also been suggested (Sutherland et al., 1980).

Therefore, in parallel with studies being undertaken by others in this laboratory on the effects of antioestrogens on the proliferation kinetics of MCF 7 human mammary carcinoma cells, an extensive biochemical characterization of the AEBS in this cell line was undertaken. Although the MCF 7 AEBS was initially described in high speed cytosol (Sutherland et al., 1980; Jozan et al., 1981; Murphy and Sutherland, 1981b) prepared using techniques designed for the study of ER, it became apparent from preliminary investigations to the present study that the AEBS might not in fact be cytoplasmic in origin. Thus, gel filtration chromatography of MCF 7 cytosol on Sephacryl S-300 showed that specific [^{3}H]TAM binding in the presence of E_2 was confined to the column void volume (exclusion M_r approximately 1.5×10^6), suggesting either that the AEBS was highly aggregated or that it could be

associated with a subcellular organelle (or fragment of the same) which had failed to sediment during cytosol preparation. Furthermore, specific [^{3}H]TAM binding activity of suspensions of the high speed pellet formed during cytosol preparation was many fold greater than that of the cytosol itself. Therefore, an important aspect of this study was an investigation into the subcellular distribution of the AEBS. In addition, the kinetic properties of [^{3}H]TAM binding to the AEBS were investigated, and a determination of the structural requirements for ligand binding was made using a series of structural analogues of TAM. Further aims of the study were: to examine whether the AEBS was truly a distinct entity from the cystosolic ER; to investigate factors affecting the stability or activity of the AEBS, in particular those which might be encountered in possible purification procedures; and to investigate the reasons for the failure of several workers to successfully detect the AEBS in cytosol preparations (Katzenellenbogen et al., 1981; Eckert and Katzenellenbogen 1982; Coezy et al., 1982; Borgna and Rochefort, 1980).

3.2 METHODS

3.2.1 Cells and Subcellular Fractionation

MCF 7 cells were grown, harvested and subcellular fractions prepared and analysed as described in Section 2.2.

3.2.2 Determination of Binding Parameters

The binding properties of [^{3}H]TAM to MCF 7 AEBS were studied as outlined in Section 2.4. Kinetic rate constants were determined at 4°, 20° and 37°C and all other binding studies were carried out at 0° - 4°C for 16 - 20 h.

3.2.3 Thermal Stability and Effects of Ionic Strength, pH and Enzyme Treatment

The influence of the above factors on the binding of [^{3}H]TAM to the AEBS was determined as described in Section 2.4.6.

3.2.4 Ligand Specificity

Structural requirements for the binding of ligands to the MCF 7 AEBS were determined from competition studies as described in Section 2.4.5 using a wide range of nonsteroidal antioestrogens and structurally related compounds. The results of these studies are more fully presented in Chapter 6, but the general principles of ligand binding are illustrated in this Chapter by the relative binding affinity data for a number of TAM analogues. These results are compared to the relative binding affinities of the same compounds for the MCF 7 ER, determined as described in Section 2.5.

3.3 RESULTS

3.3.1 Subcellular Localization

Figure 3.1 shows the distribution of the AEBS, ER, and the enzyme markers for mitochondria (cytochrome c oxidase), lysosomes (β-glucuronidase), plasma membranes (5'-nucleotidase), endoplasmic reticulum (NADPH cytochrome c reductase) and cytosol (lactate dehydrogenase) between the cytosol, mitochondrial, microsomal and nuclear fractions of MCF 7 cells.

The AEBS was concentrated in the microsomal fraction and throughout the fractions tended to parallel the distribution of the endoplasmic reticulum marker enzyme. Purified nuclei showed an 80% reduction in specific binding activity of TAM in the presence of E_2, and this

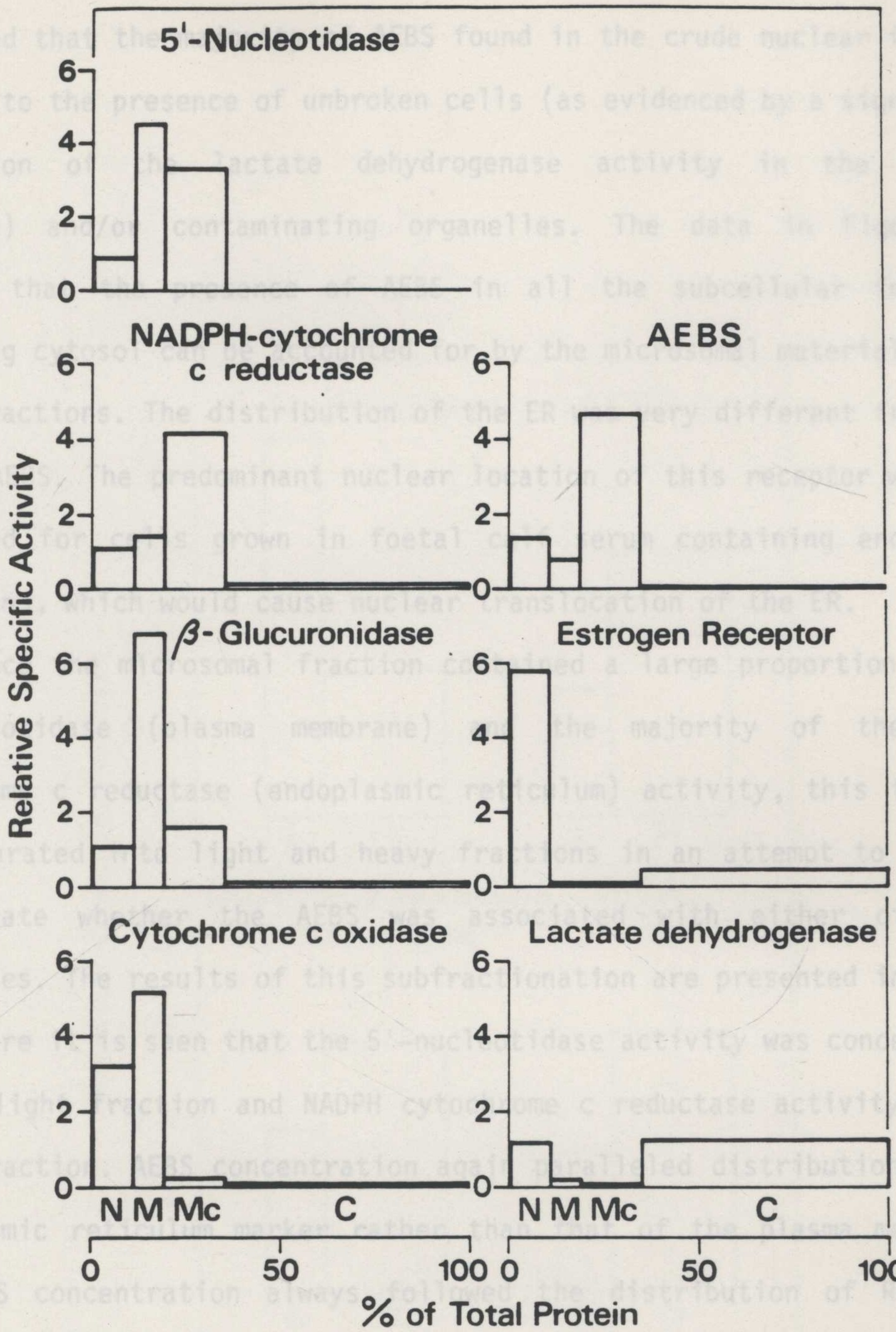

Figure 3.1: Subcellular localization of AEBS in MCF 7 cells. MCF 7 cell homogenates were separated by differential centrifugation into nuclear (N), mitochondrial (M), microsomal (Mc) and cytosol (C) fractions. The distribution of marker enzymes for plasma membranes (5'-nucleotidase), endoplasmic reticulum (NADPH cytochrome c reductase), lysosomes (β-glucuronidase), mitochondria (cytochrome c oxidase) and cytosol (lactate dehydrogenase), and the concentration of AEBS and ER in each fraction are shown.

suggested that the majority of AEBS found in the crude nuclear fraction was due to the presence of unbroken cells (as evidenced by a significant proportion of the lactate dehydrogenase activity in the nuclear fraction) and/or contaminating organelles. The data in Figure 3.1 suggest that the presence of AEBS in all the subcellular fractions including cytosol can be accounted for by the microsomal material within these fractions. The distribution of the ER was very different from that of the AEBS. The predominant nuclear location of this receptor would be predicted for cells grown in foetal calf serum containing endogenous oestrogens, which would cause nuclear translocation of the ER.

Since the microsomal fraction contained a large proportion of the 5'-nucleotidase (plasma membrane) and the majority of the NADPH cytochrome c reductase (endoplasmic reticulum) activity, this fraction was separated into light and heavy fractions in an attempt to further investigate whether the AEBS was associated with either of these organelles. The results of this subfractionation are presented in Figure 3.2, where it is seen that the 5'-nucleotidase activity was concentrated in the light fraction and NADPH cytochrome c reductase activity in the heavy fraction. AEBS concentration again paralleled distribution of the endoplasmic reticulum marker rather than that of the plasma membrane. The AEBS concentration always followed the distribution of RNA more closely than that of NADPH cytochrome c reductase activity, suggestive of an association of the AEBS with rough endoplasmic reticulum.

Because the [^{3}H]TAM binding properties of pelleted and resuspended microsomes were not demonstrably different to those of PMF (microsomal fraction plus cytosol), the latter preparation was used, for convenience, in subsequent studies of MCF 7 AEBS.

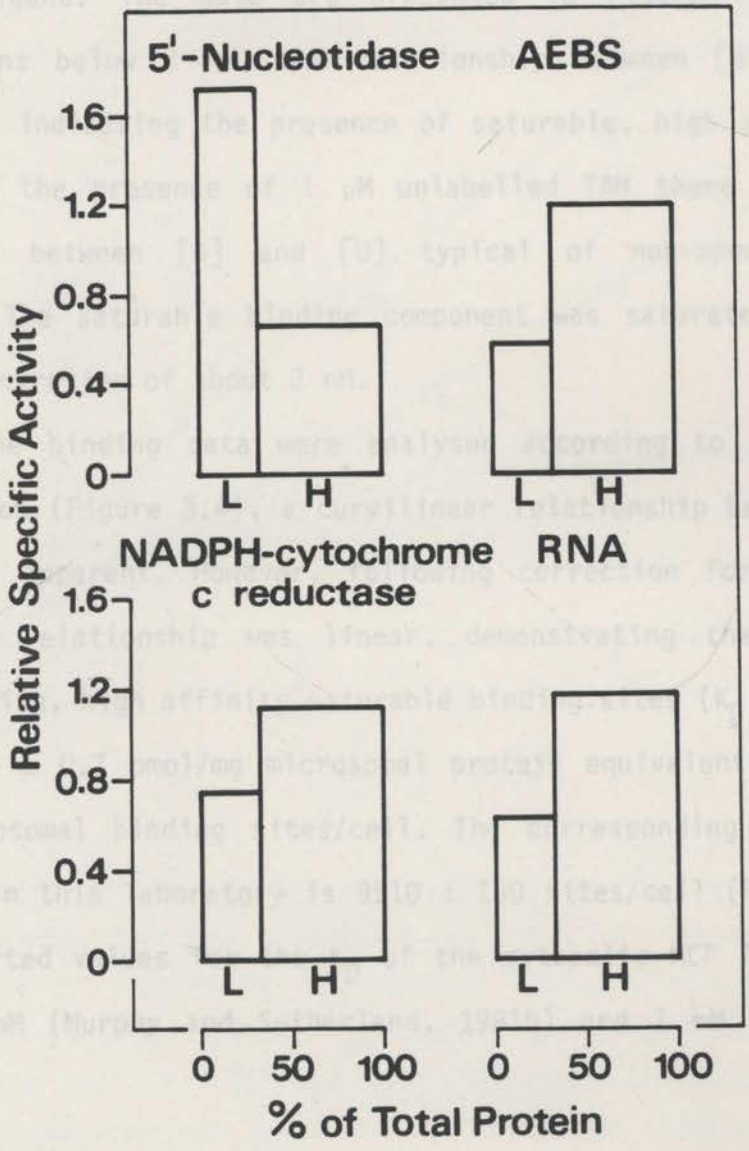

Figure 3.2: Subfractionation of the MCF 7 microsomal fraction. Microsomes were separated on a discontinuous sucrose gradient containing CsCl into heavy (H) and light (L) fractions. The distribution of marker enzymes for plasma membranes and endoplasmic reticulum and the concentrations of AEBS and RNA are shown.

3.3.2 Saturation Analysis

Since time course experiments at 4°C showed that association of [^{3}H]TAM with the AEBS was maximal by 6 h and remained unchanged for at least another 18 h, increasing concentrations of [^{3}H]TAM were incubated with PMF for 16 h and [B] and [U] determined at different concentrations of total ligand. The data are presented in Figure 3.3 where, at concentrations below 3 nM, the relationship between [B] and [U] is curvilinear, indicating the presence of saturable, high affinity sites for TAM. In the presence of 1 μM unlabelled TAM there was a linear relationship between [B] and [U] typical of non-specific binding components. The saturable binding component was saturated at a total ligand concentration of about 3 nM.

When the binding data were analysed according to the Scatchard transformation (Figure 3.4), a curvilinear relationship between [B]/[U] and [B] was apparent. However, following correction for non-specific binding the relationship was linear, demonstrating the presence of non-interacting, high affinity saturable binding sites (K_D = 0.97 ± 0.15 nM, C = 4.5 ± 0.7 pmol/mg microsomal protein equivalent to 141,300 ± 20,100 microsomal binding sites/cell. The corresponding value for ER determined in this laboratory is 9510 ± 150 sites/cell (Reddel *et al.*, 1985). Reported values for the K_D of the cytosolic MCF 7 AEBS include 2.7 ± 0.5 nM (Murphy and Sutherland, 1981b) and 1 nM (Faye *et al.*, 1983).

3.3.3 Association and Dissociation Rates

A representative time course of association of [^{3}H]TAM with PMF binding sites at 4°C is shown in Figure 3.5. The mean association rate constant (k_{+1}), was calculated from the linear transformation of the

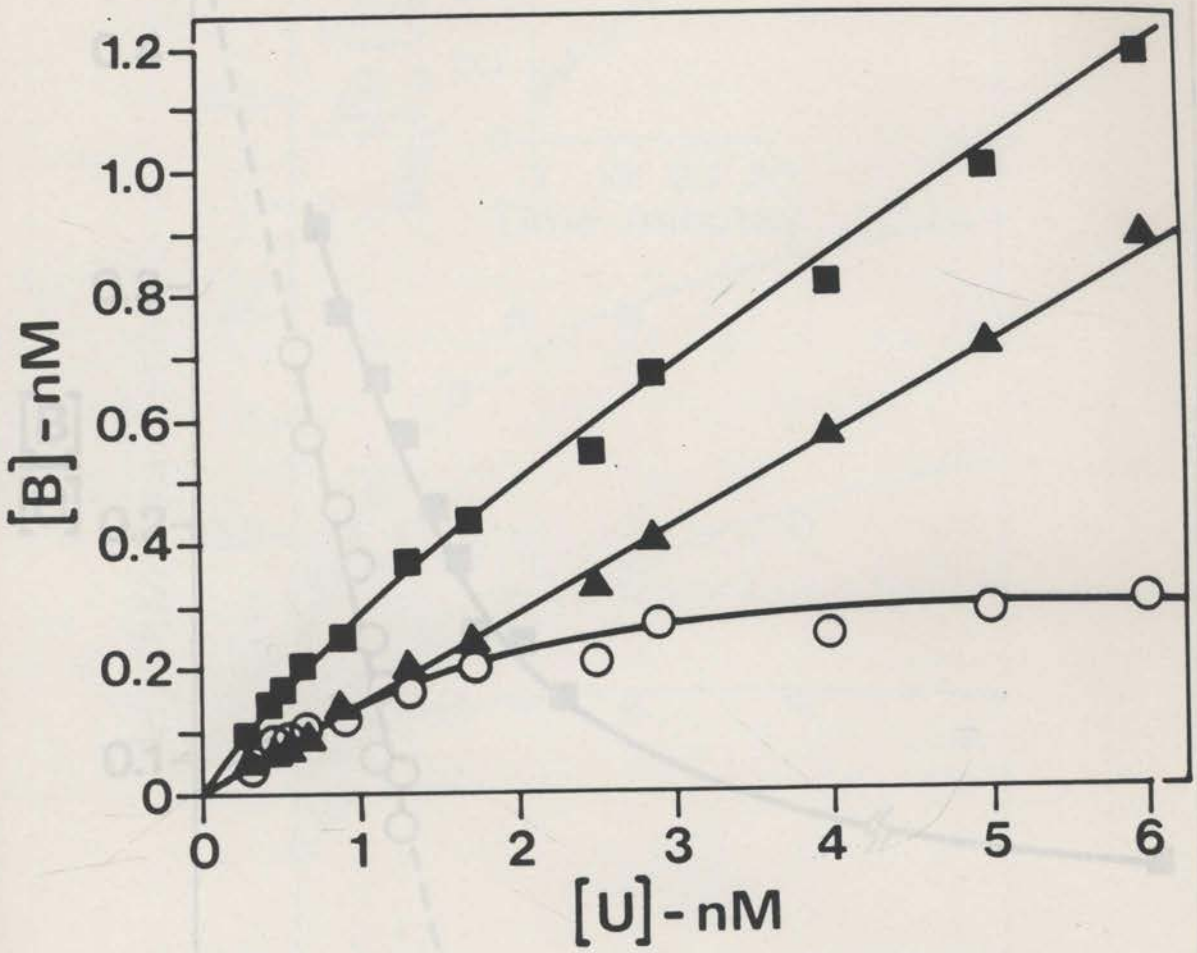

Figure 3.3: Saturation analysis of the binding of [^{3}H]TAM to AEBS in PMF from MCF 7 cells. PMF was incubated for 16 h at 4°C with increasing concentrations of [^{3}H]TAM in the absence (total binding) or presence (non-specific binding) of 1 µM unlabelled TAM. Charcoal/dextran adsorption (30 min at 4°C) was used to separate bound and unbound [^{3}H]TAM. [B] is plotted against [U]. Specific binding (○) was determined as the difference between total binding (■) and non-specific binding (▲).

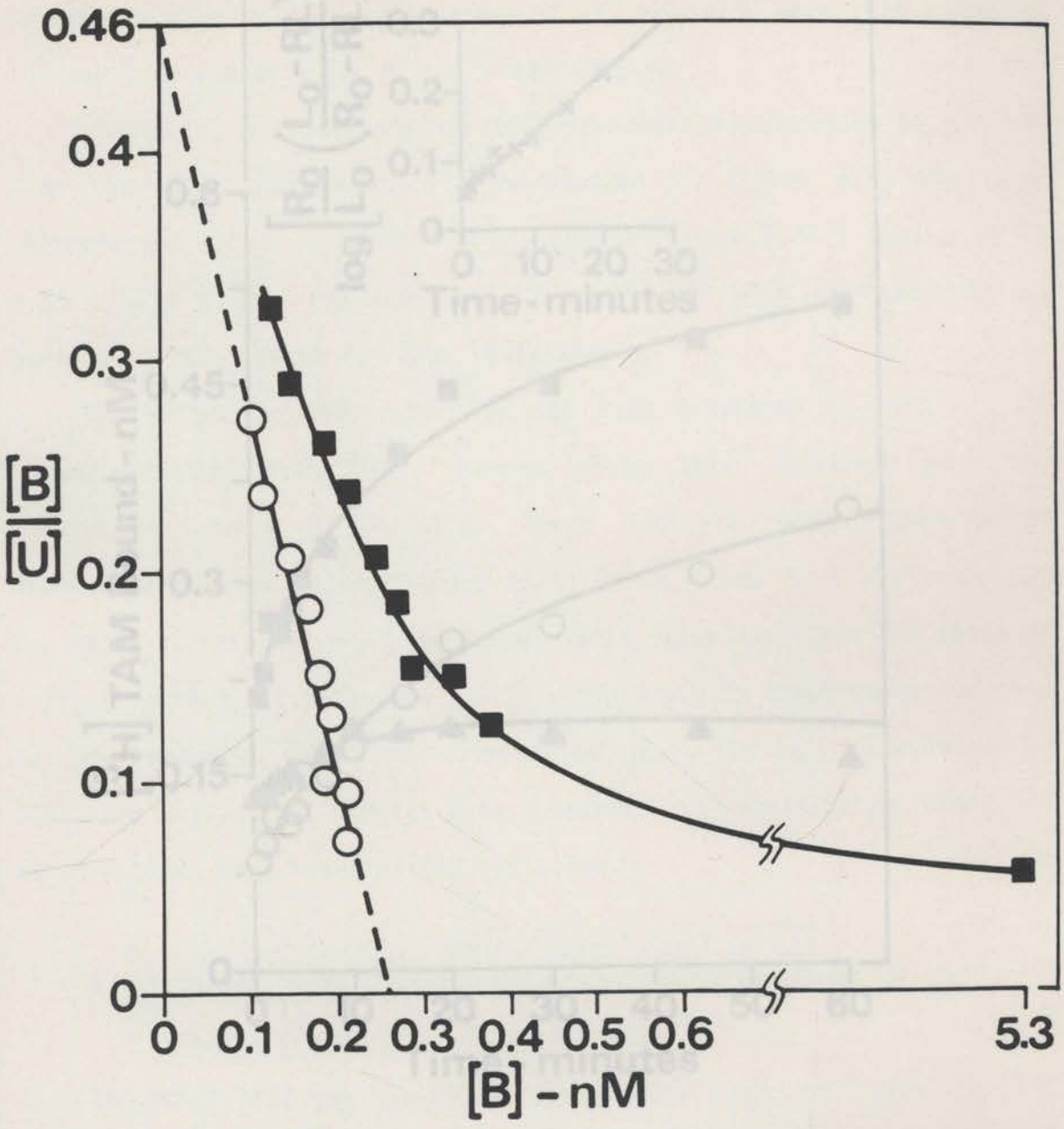

Figure 3.4: Scatchard plot of the interaction between [^{3}H]TAM and the AEBS in PMF from MCF 7 cells. [^{3}H]TAM was added in increasing concentrations to PMF from MCF 7 cells, in the presence of 1 μM E_2 to eliminate binding to ER. Non-specific binding was measured in the presence of 1 μM unlabelled TAM. Charcoal/dextran was used to separate bound and unbound ligand as described in Section 2.4.1, and the binding data were analysed as in Section 2.4.2. Data are plotted before (■) and after (○) correction for non-specific binding.

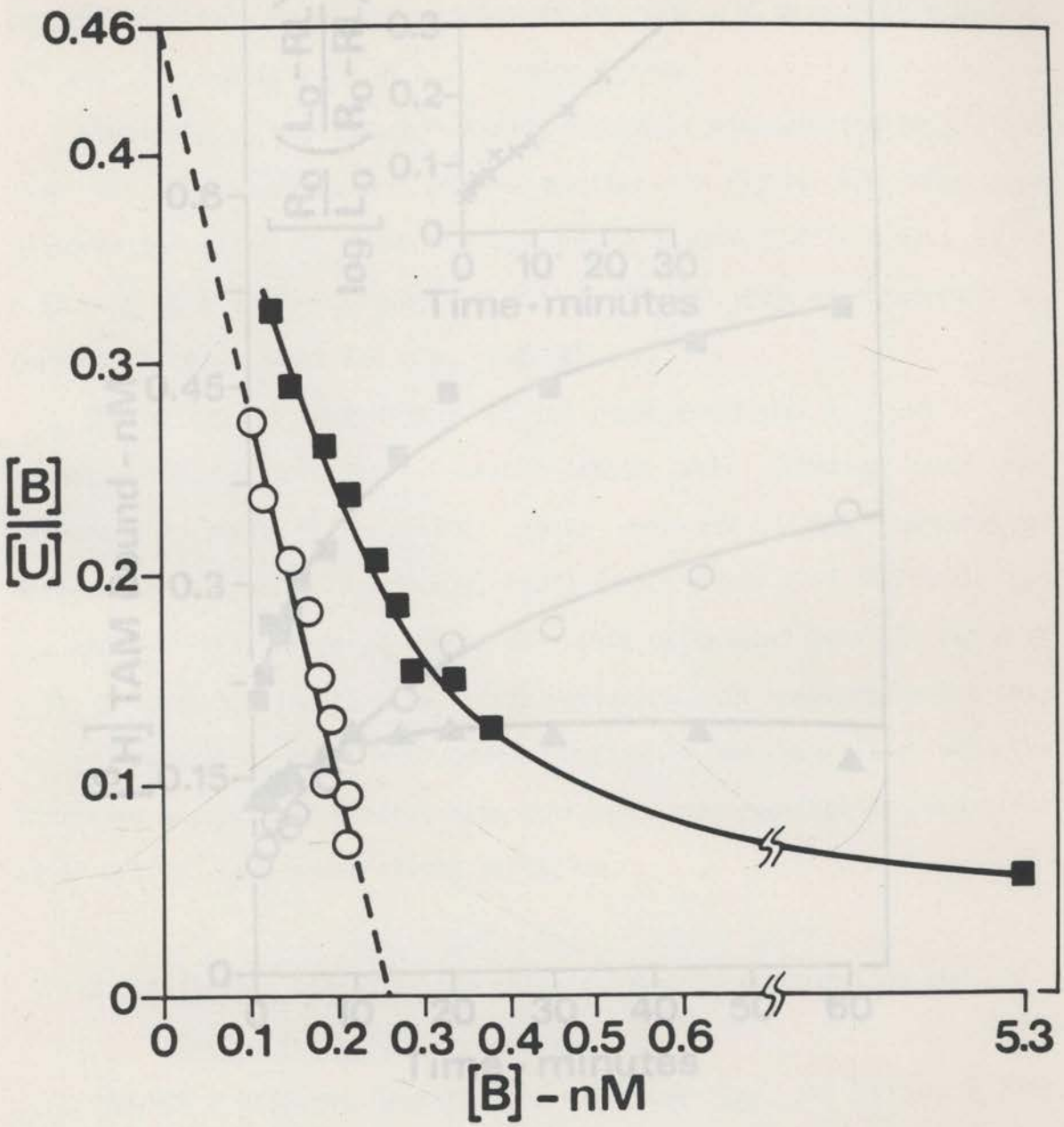

Figure 3.4: Scatchard plot of the interaction between [^{3}H]TAM and the AEBS in PMF from MCF 7 cells. [^{3}H]TAM *was added in increasing concentrations to PMF from MCF 7 cells, in the presence of 1 μM* E_2 *to eliminate binding to ER. Non-specific binding was measured in the presence of 1 μM unlabelled TAM. Charcoal/dextran was used to separate bound and unbound ligand as described in Section 2.4.1, and the binding data were analysed as in Section 2.4.2. Data are plotted before (■) and after (○) correction for non-specific binding.*

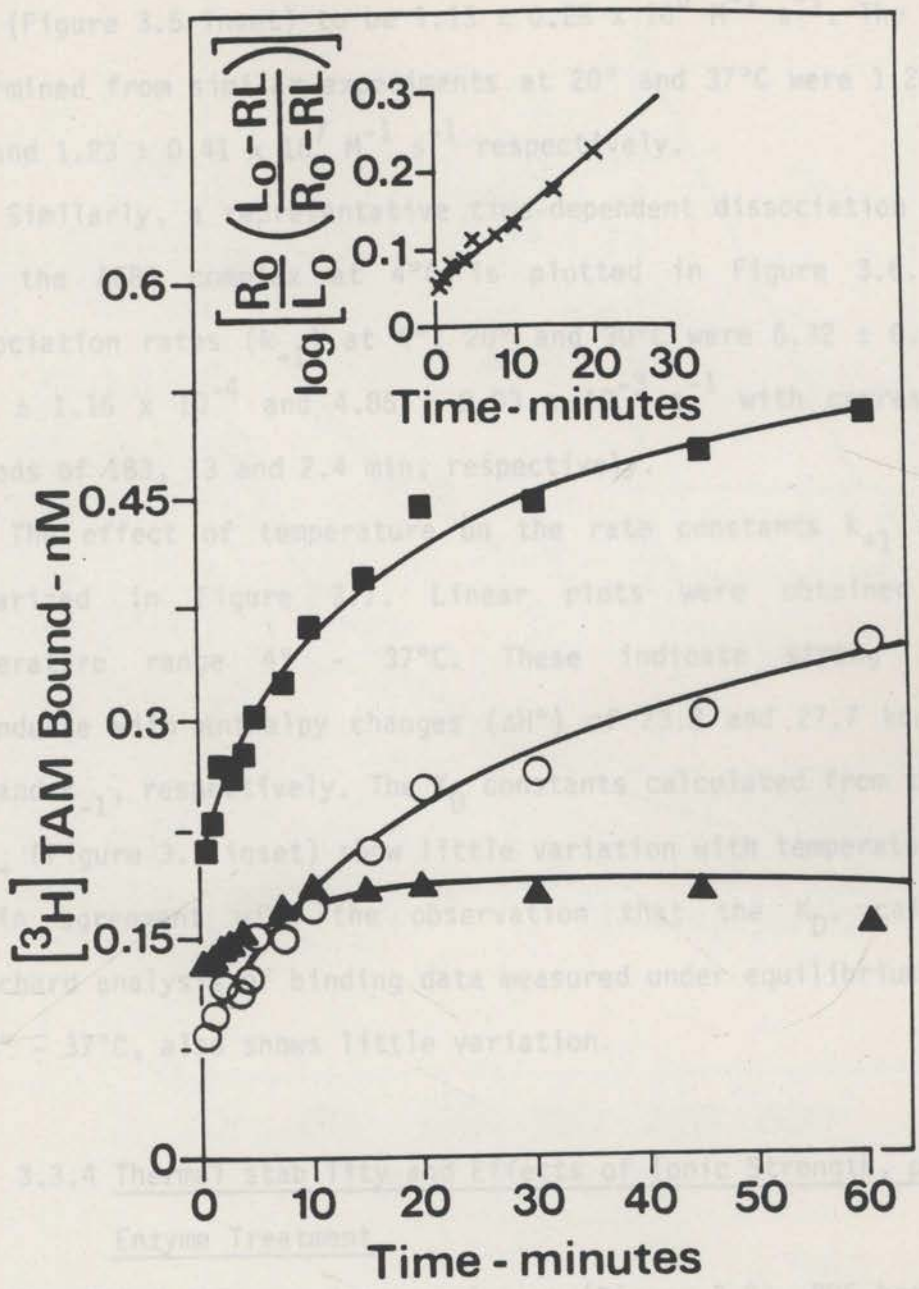

Figure 3.5: Association at 4°C of [^{3}H]TAM with AEBS in PMF from MCF 7 cells. PMF was incubated at 4°C with 5 nM [^{3}H]TAM in the absence or presence of 1 µM unlabelled TAM. At the indicated times aliquots of the reaction mixture were removed and the reaction stopped by charcoal/ dextran adsorption for 30 min at 4°C. Total (■), non-specific (○), and specific (▲) binding are presented. The inset represents the association rate of specific [^{3}H]TAM binding in a linear format, calculated assuming second order kinetics for the formation of the [^{3}H]TAM-AEBS complex.

data (Figure 3.5 inset) to be 1.13 ± 0.28 x 10^5 M^{-1} s^{-1}. The k_{+1} values determined from similar experiments at 20° and 37°C were 1.24 ± 0.07 x 10^6 and 1.23 ± 0.41 x 10^7 M^{-1} s^{-1} respectively.

Similarly, a representative time-dependent dissociation of [^{3}H]TAM from the AEBS complex at 4°C is plotted in Figure 3.6. The mean dissociation rates (k_{-1}) at 4°, 20° and 30°C were 6.32 ± 0.57 x 10^{-5}, 8.62 ± 1.15 x 10^{-4} and 4.85 ± 0.93 x 10^{-3} s^{-1} with corresponding $t_{\frac{1}{2}}$ periods of 183, 13 and 2.4 min, respectively.

The effect of temperature on the rate constants k_{+1} and k_{-1} is summarized in Figure 3.7. Linear plots were obtained over the temperature range 4° - 37°C. These indicate strong temperature dependence with enthalpy changes ($\Delta H°$) of 23.8 and 27.7 kcal/mole for k_{+1} and k_{-1}, respectively. The K_D constants calculated from the ratio of k_-/k_+ (Figure 3.7 inset) show little variation with temperature and this is in agreement with the observation that the K_D, calculated by Scatchard analysis of binding data measured under equilibrium conditions at 4° - 37°C, also shows little variation.

3.3.4 Thermal stability and Effects of Ionic Strength, pH and Enzyme Treatment

The MCF 7 AEBS was heat-resistant (Figure 3.8). PMF heated to 37°C for 24 h showed no loss of specific binding, while heating at 60°C for 24 h decreased specific binding by only 35% when compared with the 4°C control. When the PMF was stored at either -20° or -70°C for up to 4 weeks there was no significant loss of specific binding.

The effects of changes in ionic strength on the binding parameters, C and K_D are shown in Figure 3.9. Affinity for the AEBS was highest at low ionic strength and fell as ionic strength increased. The binding

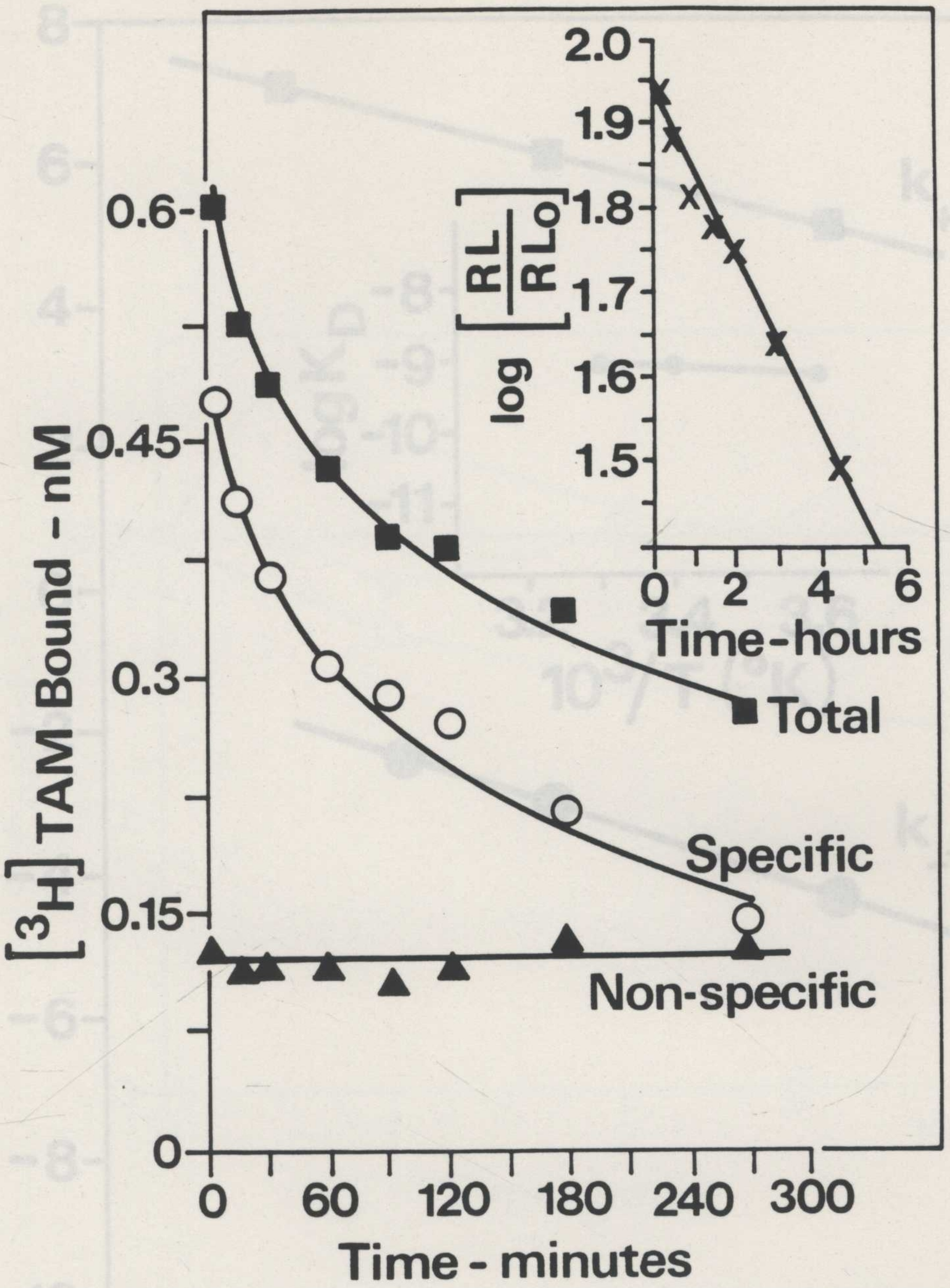

Figure 3.6: Dissociation at 4°C of [^{3}H]TAM from the AEBS in PMF of MCF 7 cells. Dissociation rates were measured by the isotopic dilution technique. PMF was incubated for 16 h at 4°C with 4 nM [^{3}H]TAM both in the absence and presence of 1 μM unlabelled TAM. Excess ligand was removed by charcoal/dextran adsorption for 30 min at 4°C. Unlabelled TAM (final concentration of 1 μM) was then added and incubation continued at 4°C. Aliquots were removed at the indicated times and bound and unbound ligand separated by charcoal/dextran adsorption for 30 min at 4°C. Total (■), non-specific (▲) and specific binding (○) are presented. The inset represents the dissociation rate of the [^{3}H]TAM-AEBS complex in a linear format, calculated assuming first order kinetics.

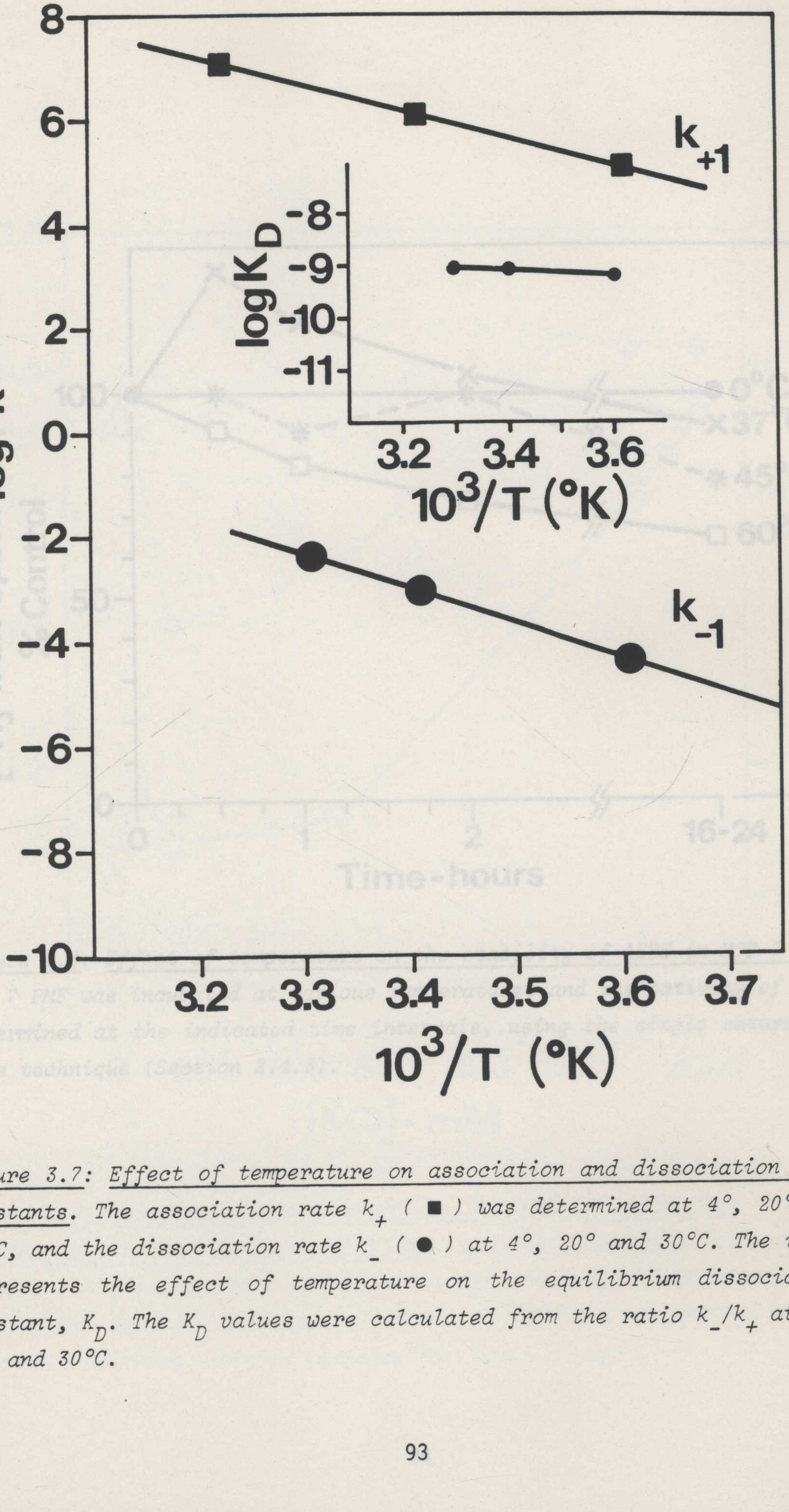

Figure 3.7: Effect of temperature on association and dissociation rate constants. The association rate k_+ (■) was determined at 4°, 20° and 37°C, and the dissociation rate k_- (●) at 4°, 20° and 30°C. The inset represents the effect of temperature on the equilibrium dissociation constant, K_D. The K_D values were calculated from the ratio k_-/k_+ at 4°, 20° and 30°C.

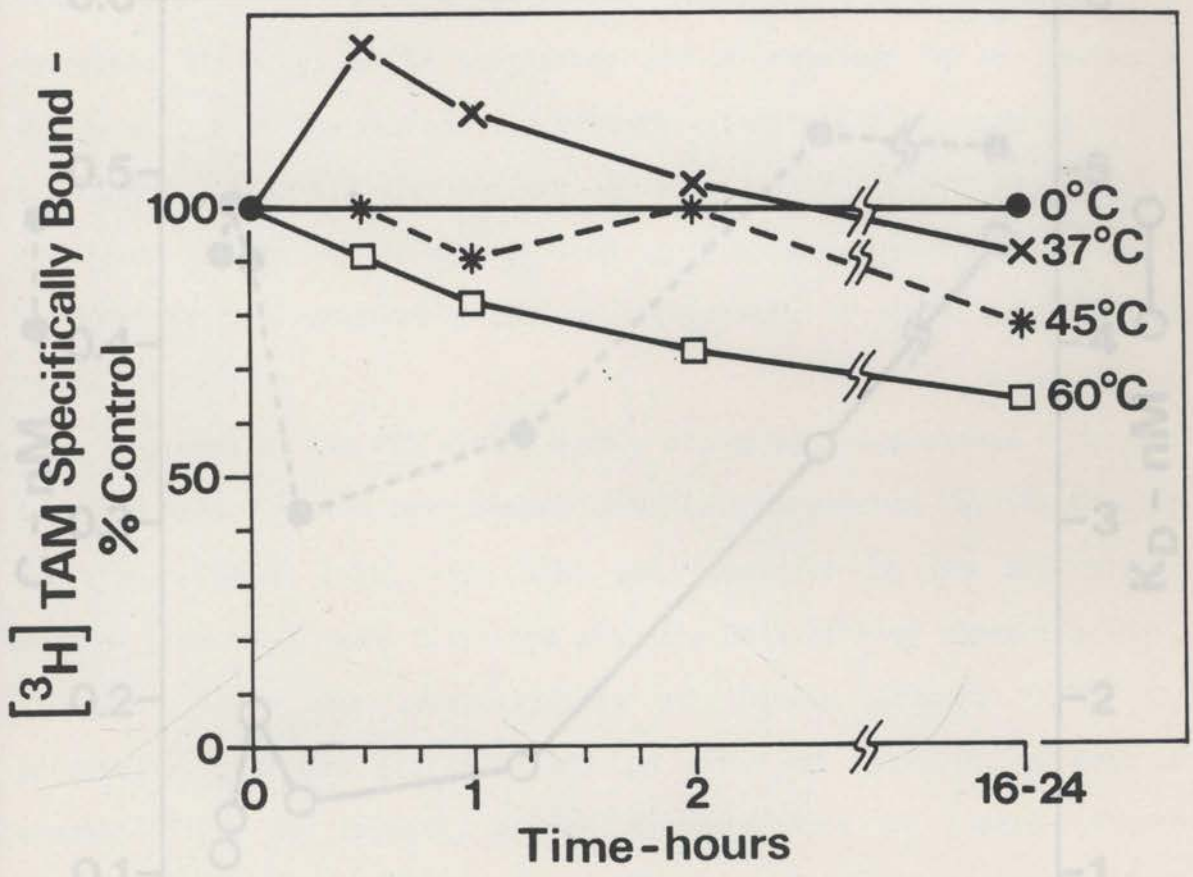

Figure 3.8: Effect of temperature on the stability of AEBS in MCF 7 PMF. MCF 7 PMF was incubated at various temperatures and the activity of AEBS determined at the indicated time intervals, using the single saturating dose technique (Section 2.4.3).

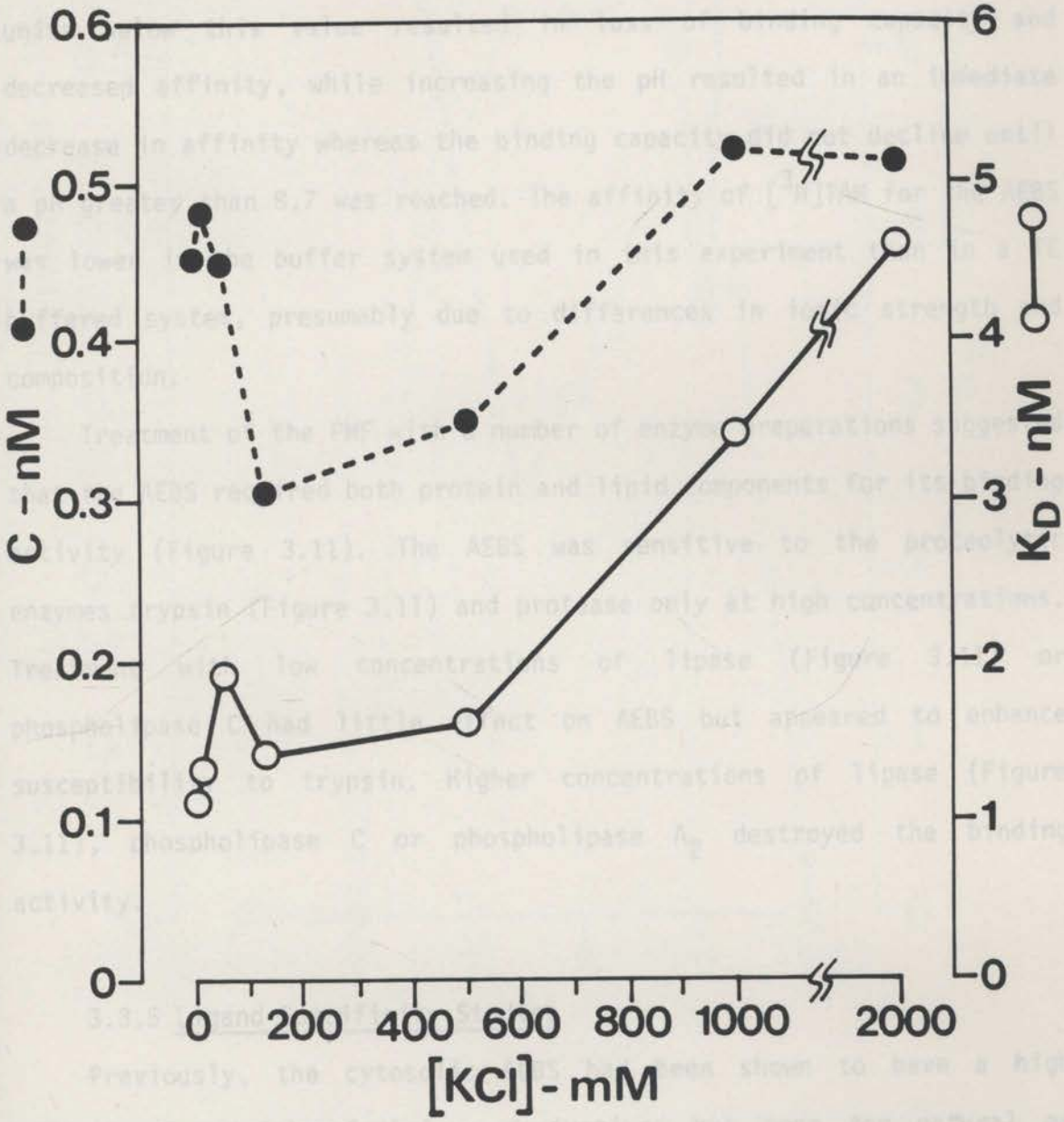

Figure 3.9: Effect of ionic strength on the binding parameters for the [^{3}H]TAM-AEBS interaction in MCF 7 PMF. Scatchard analysis was used to measure the binding capacity (C) and equilibrium dissociation constant (K_D) of the specific interaction between [^{3}H]TAM and the AEBS in PMF to which KCl had been added to increase the ionic strength.

capacity decreased as the ionic strength approached physiological levels, but at higher levels this decrease was reversed.

A narrow pH optimum around pH 7.5 was demonstrated for the binding of [^{3}H]TAM to the AEBS (Figure 3.10). Decreasing the pH more than 0.5 pH units below this value resulted in loss of binding capacity and decreased affinity, while increasing the pH resulted in an immediate decrease in affinity whereas the binding capacity did not decline until a pH greater than 8.7 was reached. The affinity of [^{3}H]TAM for the AEBS was lower in the buffer system used in this experiment than in a TE buffered system, presumably due to differences in ionic strength and composition.

Treatment of the PMF with a number of enzyme preparations suggested that the AEBS required both protein and lipid components for its binding activity (Figure 3.11). The AEBS was sensitive to the proteolytic enzymes trypsin (Figure 3.11) and protease only at high concentrations. Treatment with low concentrations of lipase (Figure 3.11) or phospholipase C had little effect on AEBS but appeared to enhance susceptibility to trypsin. Higher concentrations of lipase (Figure 3.11), phospholipase C or phospholipase A_2 destroyed the binding activity.

3.3.5 Ligand Specificity Studies

Previously, the cytosolic AEBS had been shown to have a high affinity for triphenylethylene derivatives but none for natural or synthetic steroids (Sutherland et al., 1980). To investigate the requirements for binding to the MCF 7 microsomal AEBS, structural analogues of TAM with modifications of the side chain and/or the aromatic portion of the molecule were tested for their ability to

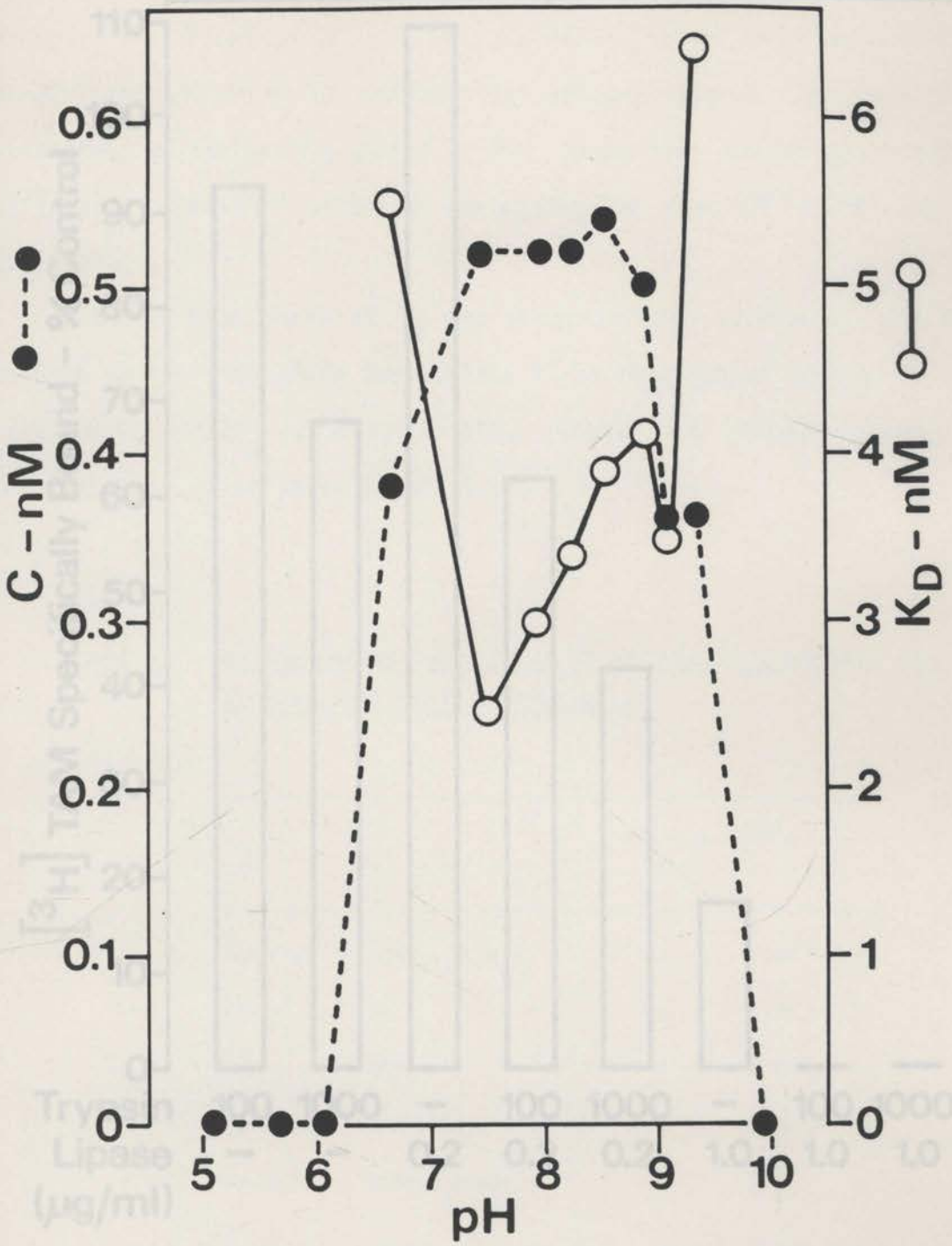

Figure 3.10: Effect of pH on the binding parameters for the [^{3}H]TAM-AEBS interaction in MCF 7 PMF. Scatchard analysis was used to measure the binding capacity (C) and equilibrium dissociation constant (K_D) of the interaction between [^{3}H]TAM and the AEBS in PMF prepared in a buffer of constant composition and ionic strength but differing pH. The pH was that measured in the final reaction mixture at 4°C.

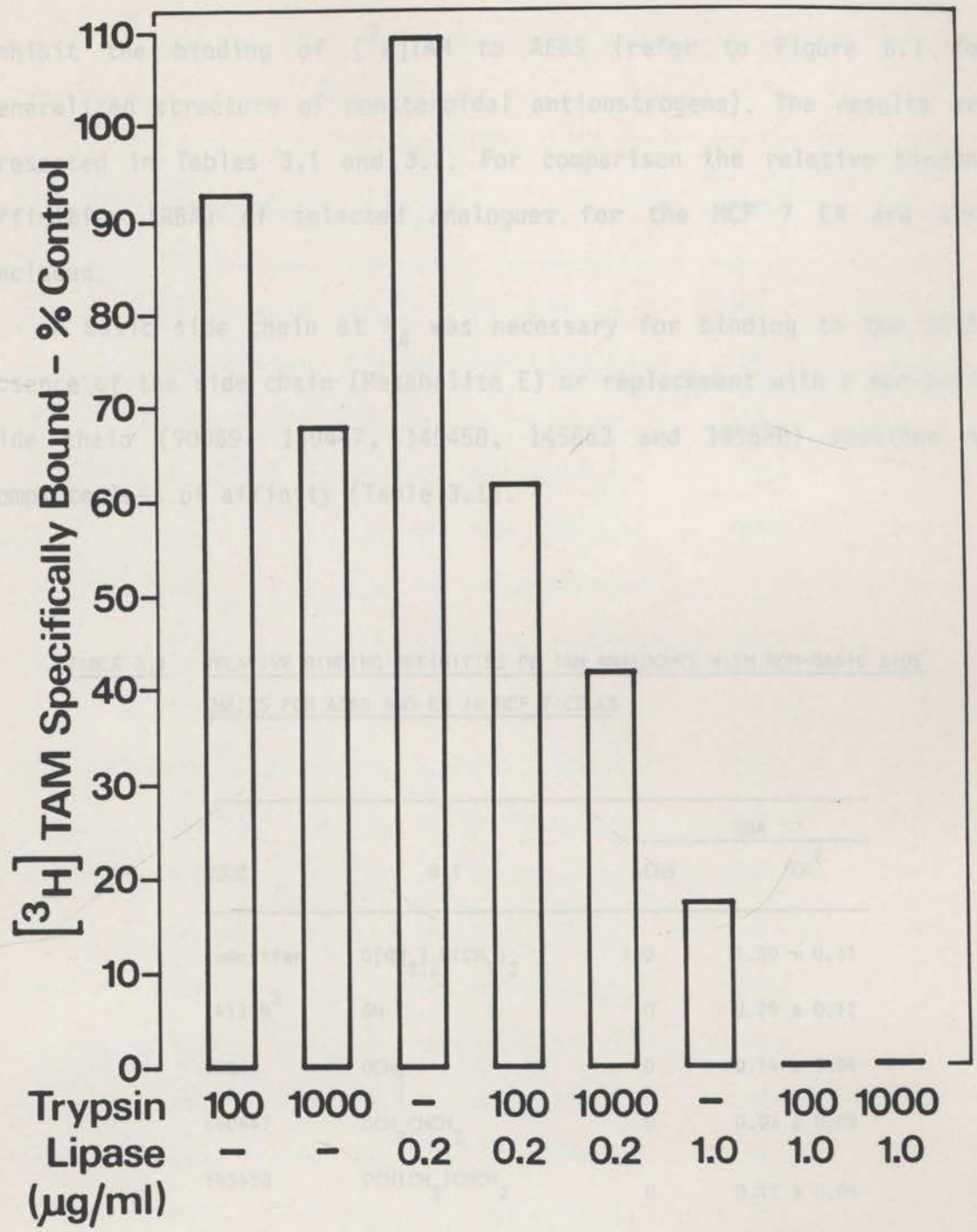

Figure 3.11: Effect of enzyme treatment on the ability of MCF 7 PMF to bind [^{3}H]TAM. Enzymes at the final concentrations shown were incubated with PMF for 1 h at 37°C, except when lipase and trypsin were both added, in which case a 30 min incubation with lipase was followed by addition of trypsin and a further 30 min incubation period. The concentration of specifically bound [^{3}H]TAM was then determined by the single saturating dose technique.

inhibit the binding of [^{3}H]TAM to AEBS (refer to Figure 6.1 for generalized structure of nonsteroidal antioestrogens). The results are presented in Tables 3.1 and 3.2. For comparison the relative binding affinities (RBA) of selected analogues for the MCF 7 ER are also included.

A basic side chain at R_4 was necessary for binding to the AEBS. Absence of the side chain (Metabolite E) or replacement with a non-basic side chain (90069, 140447, 145458, 145663 and 145680) resulted in complete loss of affinity (Table 3.1).

TABLE 3.1 RELATIVE BINDING AFFINITIES OF TAM ANALOGUES WITH NON-BASIC SIDE CHAINS FOR AEBS AND ER IN MCF 7 CELLS

CODE	R_4[1]	RBA	
		AEBS	ER[2]
Tamoxifen	$O[CH_2]_2N(CH_3)_2$	100	1.30 ± 0.31
141389[3]	OH	0	0.79 ± 0.12
90069	OCH_3	0	0.14 ± 0.04
140447	OCH_2CHCH_2	0	0.09 ± 0.03
145458	$OCH(CH_3)CHCH_2$	0	0.11 ± 0.04
145663	$OCH(CH_3)CH(OH)CH_2OH$	0	0.30 ± 0.09
145680	$OCH_2CH(OH)CH(CH_3)OH$	0	1.36 ± 0.33

[1] R_1 = H, R_2 = C_2H_5, R_3 = H in all cases. Refer to Figure 6.1; [2] E_2 has an RBA for ER of 100, from Murphy and Sutherland (1985); [3] Metabolite E.

TABLE 3.2 RELATIVE BINDING AFFINITIES OF TAM ANALOGUES FOR AEBS AND ER IN MCF 7 CELLS

ICI CODE NUMBER	R_1[1]	R_2[1]	R_3[1]	R_4[1]	RBA (%) AEBS	RBA (%) ER[2]
TAM (trans)	H	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	100	1.3 ± 0.3
47699 (cis)	H	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	98	0.05
45960	H	C_2H_5	H	$O[CH_2]_2N(C_2H_5)_2$	46 ± 4	
47108	H	C_2H_5	H	$O[CH_2]_3N(CH_3)_2$	36 ± 1	
47590	H	C_2H_5	H	$O[CH_2]_4N(C_2H_5)_2$	54 ± 7	
94230	H	C_2H_5	H	$OCH_2CH(OH)CH_2N(CH_3)_2$	2 ± 0.3	3.5 ± 0.7
47399	H	C_2H_5	H	$O[CH_2]_2$-c-$N(CH_2CH_2)_2O$	219 ± 14	
TAM-N-oxide	H	C_2H_5	H	$O[CH_2]_2\overset{O}{\uparrow}N(CH_3)_2$	5 ± 3	
55548[3]	H	C_2H_5	H	$O[CH_2]_2NCH_3$	19 ± 2	2 ± 1
79280[4]	OH	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	80 ± 7	41 ± 3
129351	OH	C_2H_5	H	$O[CH_2]_2$-c-NC_4H_8	106 ± 9	
129817	OH	C_2H_5	CH_3	$O[CH_2]_2N(CH_3)_2$	49 ± 14	
133312	H	C_2H_5	CH_3	$O[CH_2]_2N(CH_3)_2$	96 ± 6	
132802	H	C_2H_5	CH_3O	$O[CH_2]_2N(CH_3)_2$	35 ± 5	
148067	H	C_2H_5	F	$O[CH_2]_2N(CH_3)_2$	97 ± 11	
46414	H	CH_3	H	$O[CH_2]_3N(CH_3)_2$	32 ± 5	
49500	H	C_3H_7	H	$O[CH_2]_2N(CH_3)_2$	73 ± 7	

[1] R_1 - R_4 refer to substituents illustrated in Figure 6.1; [2] E_2 has an RBA for ER of 100; [3] N-desmethylTAM; [4] 4-hydroxyTAM.

The effect of increasing side chain length appeared to depend on the nature of the amino substituents. Thus, an increase in chain length

from 2 to 3 methylene units (TAM vs 47108, Table 3.2), resulted in a 64 - 70% decrease in affinity whereas an increase from 2 to 4 methylene units (45960 vs 47590, Table 3.2) had little effect on affinity. Altering the substituents on the N atom (TAM vs 45960, TAM-N-oxide, 55548 and 47399; 79280 vs 129351; Table 3.2) also resulted in changes in affinity. The terminal alkyl amino groups affected binding affinity in the order -c-$N(CH_2CH_2)_2O$ > -c-NC_4H_8 > $-N(CH_3)_2$ > $-N(C_2H_5)_2$ > $-NHCH_3$ >> -N-oxide. TAM and its geometric cis-isomer had equal affinity (TAM vs 47699, Table 3.2). Hydroxylation of a basic side chain caused a large reduction in RBA (47108 vs 94230).

Changes in substituents on the triphenylethylene portion of the molecule at positions R_1, R_2 and R_3 resulted in various effects on affinity. Hydroxylation at R_1 (79280 vs TAM, 129817 vs 133312) resulted in a 20 - 45% decrease in affinity. Replacement of the $-C_2H_5$ group by $-C_3H_7$ (49500 vs TAM) at position R_2 resulted in significantly decreased affinity whereas a $-CH_3$ group at this position (47108 vs 46414) had no effect. At position R_3 a $-CH_3$ substituent either lowered affinity (129817 vs 79280) or had no effect (133312 vs TAM). A methoxy substituent lowered affinity (132802 vs TAM) but a fluoro substituent had no effect (148067 vs TAM).

3.4 DISCUSSION

The interactions of [^{3}H]TAM with the microsomal AEBS described in this study are consistent with a simple reversible receptor - ligand system, in which association and dissociation are bi- and mono-molecular processes respectively. The values for the equilibrium dissociation constant, K_D, and the kinetic rate constants, k_{+1} and k_{-1}, are similar to those reported by other workers for the interaction of [^{3}H]TAM with

ER at 4°C (Borgna and Rochefort, 1980). However, the differences in intracellular concentration, subcellular distribution and ligand specificity demonstrate the separate nature of the AEBS and the oestrogen binding site of the ER. Dissimilarities have also been noted in thermal stability and sensitivity to proteolytic enzymes (Gulino and Pasqualini, 1982; Kon, 1983; Sudo et al., 1983). In addition, the observation that the cytosol AEBS is not depleted in the immature rat uterus following translocation of the cytosol ER to the nuclear compartment (Murphy and Sutherland, 1981a; Gulino and Pasqualini, 1982; Sudo et al., 1983) suggests that these two binding sites are situated on separate molecules.

Data from differential centrifugation studies with appropriate enzyme markers for subcellular organelles demonstrated that AEBS was predominantly microsomal. When account is taken of the trivial amount (< 4% of total) of AEBS in purified nuclei, and the likelihood that AEBS measured in the mitochondrial and crude nuclear fractions was due to contamination with microsomes and unbroken cells respectively, it appears that AEBS is confined almost exclusively to the microsomes. The almost parallel distribution of AEBS and NADPH cytochrome c reductase activity in Figures 3.1 and 3.2 suggests that within the microsomal fraction AEBS is located in the endoplasmic reticulum. The observation that within the microsomal subfractions (Figure 3.2) AEBS activity tends to parallel the distribution of RNA suggests that the AEBS is more concentrated in the rough (ribosome-bearing) endoplasmic reticulum than in the smooth endoplasmic reticulum. Further studies are required to understand the basis of this apparent association and to conclusively establish the subcellular localization of the AEBS. An alternative hypothesis that could be investigated is that the AEBS is associated

with a sub-population of microsomal vesicles, derived perhaps from the plasma membrane or some other organelle, with sedimentation properties similar to those of the (rough) endoplasmic reticulum.

Subcellular fractionation techniques used in the present study were adaptations of those which have been well characterized and validated for the various organelles in rat liver. Although these techniques were effective as judged by the separation of organelles in MCF 7 cells, it would be desirable in subsequent studies to establish the validity of making the assumption that the enzyme markers for specific rat liver organelles are also appropriate for the same organelles in MCF 7 cells. For example, 5'-nucleotidase activity is largely confined to the plasma membrane in rat liver but it is not known whether this is also the case in MCF 7 cells, or whether significant 5'-nucleotidase activity is also present in other organelles.

A lack of significant amounts of AEBS in the cytosol and the KCl wash of microsomes rules out the possibility that AEBS is a high molecular weight lipoprotein or soluble protein loosely adsorbed by the microsomes, and suggests that reports of cytoplasmic AEBS resulted from microsomal contamination of these preparations (Sutherland and Foo, 1979; Sutherland and Murphy, 1980; Sutherland et al., 1980; Murphy and Sutherland, 1981a,b; Faye et al., 1980, 1983; Gulino and Pasqualini, 1980; Kon, 1983). Prolonged high speed centrifugation, low speed centrifugation in the presence of 10 mM Ca^{2+}, and filtration through 0.22 μm filters are procedures which remove both microsomal material and AEBS from cytosol.

The data on the subcellular localization of the AEBS are substantially in agreement with those published subsequent to the completion of this study by Miller and Katzenellenbogen (1983). These

authors found that the AEBS was concentrated in the microsomal fraction (180,000 x g_{av} pellet). However, within the various subcellular fractions the AEBS did not parallel the distribution of the microsomal marker enzyme glucose-6-phosphatase, which was concentrated within the mitochondrial fraction (12,000 x g pellet). Although the authors suggest this may be due to microsomal heterogeneity, a more likely explanation is that the techniques used failed to separate substantial amounts of microsomal material from the mitochondrial fraction (as evidenced by the presence of glucose-6-phosphatase) and also that the subsequent estimate of AEBS in this fraction was artifactually low. This artifact would have arisen because the AEBS assay technique used employed a 12,800 x g_{av} centrifugation step to pellet charcoal/dextran. Such a step would also pellet a significant proportion of the mitochondrial fraction, presumably including TAM binding (AEBS) components. In comparison with the present study, Miller and Katzenellenbogen found an approximately three-fold lower concentration of AEBS in the 12,000 x g post-mitochondrial fraction (390 ± 50 fmol/mg PMF protein) and a significantly higher K_D (3.7 ± 0.9 nM).

The data from effects of various enzyme treatments on the AEBS are compatible with this binding site being an integral membrane protein of the endoplasmic reticulum. The site was resistant to the effects of trypsin except after prior treatment with low concentrations of lipase or phospholipase C, which presumably removed protecting membrane lipids from the vicinity of the binding sites. The apparent loss of binding capacity at higher concentrations of lipase or phospholipase C may imply a requirement for lipid for the proper binding function of the AEBS, either as a binding co-factor or in the maintenance of structural integrity. The apparent loss of binding affinity only at high ionic

strengths also suggests that the AEBS is in a protected environment, resistant to salting-out effects. The thermal stability of the AEBS supports this conclusion. Unlike the microsomal AEBS, the cytosolic form has been reported not to be susceptible to lipase or phospholipase action (Sutherland et al., 1980; Kon, 1983). Otherwise, the properties of AEBS from these two subcellular fractions appear to be very similar.

The present study has allowed definition of some of the structural determinants of binding to the AEBS which reveal major differences in specificity between this site and the oestrogen binding site of ER. It is clear that the basic aminoether side chain of TAM analogues is a major determinant of binding to the AEBS, but is not an essential requirement for binding to the ER. Compounds with non-basic side chains (e.g. 145680) bind to ER with the same affinity as TAM, but have no detectable affinity for the AEBS. Binding to AEBS thus seems to require the presence of an ionizable group. Binding to ER can take place even when no side chain is present (Section 1.6.2) although it is apparent from this study that structural changes in the side chain may influence affinity for both binding sites.

Changes in binding capacity and affinity with pH are unlikely to be due to effects on the ionizable groups in the side chain. The N-atom of the aminoether side chain of TAM (pk_a = 10.8, Robertson et al., 1982a) would not be significantly deprotonated over the pH range studied. Thus changes in affinity with pH may be due to the protonation/deprotonation of ionic groups within the binding site or to indirect effects on binding site conformation.

The triphenylethylene moiety is also necessary for optimal binding. Near maximal binding has also been observed with diphenyldihydronaphthalene derivatives bearing aminoether side chains (e.g. nafoxidine,

Sutherland et al., 1980) but binding was generally markedly reduced when stilbene, bibenzyl, triphenylethanol (MER-25) or benzothiophene (LY 117018) derivatives and analogues of the cytochrome P-450 inhibition SKF 525A were tested (Chapter 6). E_2 and other natural and synthetic steroids have no affinity for the AEBS and hydroxylation of triphenylethylenes at position R_1 (Figure 6.1), which corresponds to the C3 phenolic group of E_2, results in a decreased affinity for the AEBS but increased affinity for ER (Borgna and Rochefort, 1980; Jordan et al., 1983). The effect of cis-trans isomerism on binding affinity (TAM vs 47699) further emphasises the very different ligand specificities of the two sites.

With regard to TAM analogues, optimal affinity for the AEBS is shown by those analogues bearing basic ether side chains of 2 methylene units terminating in dimethylamino or pyrrolidino-groups. Of all the substituents tested at positions R_1, R_2 and R_3, those of TAM itself produced the optimal binding affinity.

The ligand specificity data of this study on TAM analogues are in agreement with those published by other authors for the cytosolic and microsomal MCF 7 AEBS (Sutherland et al., 1980; Faye et al., 1983; Miller and Katzenellenbogen, 1983; Wakeling et al., 1984). All these studies showed that natural and synthetic steroids had no affinity for AEBS and that 4-hydroxyTAM (79280) had similar affinity to that of TAM. In contrast to the present study, however, Miller and Katzenellenbogen found that cis-TAM (47699) had significantly higher affinity than TAM (RBA = 267 ± 20). Other data from this laboratory (Sutherland et al., 1982; Murphy et al., 1983) show that the RBA of TAM analogues and other compounds for MCF 7 cytosol AEBS are not significantly different to those for the MCF 7 microsomal AEBS.

These studies do not indicate what the functional significance of the AEBS could be. Certainly the high intracellular concentration of AEBS in combination with its high affinity indicate that the AEBS has the potential to set up a concentration gradient into the cell. This is supported by studies *in vivo* (Major *et al.*, 1976) in which [^{3}H]TAM was administered to rats and shown to be preferentially concentrated in the microsomal fraction of the uterus, a tissue known to contain relatively high concentrations of AEBS (see Section 4.3.1).

There appears to be a divergence between ability to bind to the AEBS and antioestrogenic activity, at least as assessed by antagonism of oestrogen-induced uterotrophic activity in the immature rat. It is known that the presence of a basic aminoether side chain results in anti-oestrogenic activity among triphenylethylene derivatives and removal of the side chain produces compounds with full oestrogen agonist properties (Jordan and Gosden, 1982). Although this change from antagonist to agonist activity correlates with a loss of affinity for the AEBS, other triphenylethylene derivatives with non-basic side chains (e.g. U 23469) are also antioestrogenic (Hayes *et al.*, 1981) but do not bind strongly to the AEBS (Sudo *et al.*, 1983). In addition the *cis*-isomer of TAM has high affinity for AEBS, but displays predominantly oestrogenic rather than antioestrogen activity (Jordan *et al.*, 1981b). Such findings argue against a functional role for AEBS in mediating antioestrogenic effects. This point is discussed further in Chapter 8.

Unfortunately, the usefulness of MCF 7 cells as a source of AEBS for further studies on ligand specificity, subcellular distribution and purification is severely limited by the inconvenience and costliness of culturing sufficient cells to provide the large amounts of material required. However, the AEBS was also found to be present in high

concentration in rat liver (Section 4.3.1) and subsequent studies focus upon the properties of AEBS in this tissue.

CHAPTER FOUR

RAT LIVER MICROSOMAL ANTIOESTROGEN BINDING SITES

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4.1 INTRODUCTION

Several studies have reported the presence of high affinity binding sites, apparently specific for triphenylethylene antioestrogens, in the cytosol fractions of a variety of tissues. Such sites have been detected in chick oviduct (Sutherland and Foo, 1979), guinea pig uterus (Gulino and Pasqualini, 1980), foetal guinea pig uterus, heart and lung (Gulino and Pasqualini, 1982), rat uterus (Faye et al., 1980; Sutherland et al., 1980; Murphy and Sutherland, 1981a) and in rat liver and kidney (Sutherland et al., 1980). These studies primarily investigated the parameters of [^{3}H]TAM and [^{3}H]CI 628 binding and, to a limited extent, ligand specificity. Although these properties appeared similar to those of the AEBS in the MCF 7 human breast cancer cell line (Chapter 3), there was insufficient information to determine whether or not there were distinct AEBS in different tissues, or to what extent species/tissue differences occurred.

Subsequent to the realization that the AEBS was located in the microsomal fraction of MCF 7 cells (Section 3.3.1, Murphy et al., 1983, Watts et al., 1984), investigations in this laboratory showed that this was also true of all other tissues examined. In every case the cytosolic AEBS represented but a small fraction of the cellular total. The properties of AEBS had not been investigated in microsomal fractions from tissues of any of the above species and the present study was therefore undertaken to establish the tissue distribution and detailed biochemical properties of the microsomal AEBS in the rat.

Because rat liver was found to contain a very high AEBS concentration relative to other rat tissues and MCF 7 cells, this tissue promised to be a convenient source of material for purification and other studies. A further advantage of rat liver was that the subcellular

distribution of AEBS could be investigated in detail, given that methods for the subcellular fractionation of liver are better established than for any other tissue. The present Chapter focuses on the properties of the rat liver microsomal AEBS and compares them to those of the MCF 7 microsomal AEBS.

4.2 METHODS

4.2.1 Buffers

Unless otherwise stated the buffer used in all experiments was TSK. [^{3}H]TAM and unlabelled ligands were in TSKB (Section 2.1.3).

4.2.2 Subcellular Fractionation of Rat Tissues

Various rat tissues were removed from 18 h starved male or female Sprague-Dawley or Fischer rats (200 - 250 g), homogenized in TSK (10 or 20% w/v) and post-mitochondrial fractions (PMF) prepared as described for rat liver (Section 2.2.3.1).

KCl-washed rat liver microsomal suspensions in TSK were prepared as described in Section 2.2.3.1, and were routinely diluted 1:50 (v/v) prior to measurement of [^{3}H]TAM binding activity (i.e. to 0.02 - 0.08 mg microsomal protein/ml).

Subcellular fractionation of rat liver using differential centrifugation was carried out as described in Section 2.2.3.

Self-generating Percoll gradients were used to fractionate rat liver PMF prepared from a 1:10 (w/v) homogenate in 0.25 M sucrose. 0.5 ml of PMF was mixed with 11 ml of 30% Percoll (Pharmacia) in 0.25 M sucrose, pH 7.4, in 16 x 76.2 mm tubes. Parallel tubes containing Density Marker Beads (Pharmacia) were used to measure gradient density. Centrifugation was at 60,000 x g_{av} in a 50 Ti rotor for 45 min.

Fractions were collected from the bottom of the tube and assayed for protein, AEBS (single saturating dose technique), 5'-nucleotidase and NADPH cytochrome c reductase activity as described in Sections 2.3, 2.4.3 and 2.2.4.

Separation of rough (RM) and smooth (SM) microsomes was carried out as described in Section 2.2.3.2. Rough microsomes, stored at -20°C for < 24 h were used to prepare free ribosomes and stripped membrane fractions by treatment with puromycin/KCl based on the method of Adelman et al. (1974). Rough microsomes were suspended in buffer (0.25 M sucrose, 500 mM KCl, 5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5) to a concentration of 1 - 2 mg microsomal protein/ml. Puromycin was then added to a concentration of 1 mM and the suspension incubated at 22°C for 1.5 h. 5 ml of this preparation were then underlayed with 2.5 ml 2 M sucrose in 10 mM Tris-HCl, 25 mM KCl and 5 mM $MgCl_2$, pH 7.4, and centrifuged at 180,000 x g_{av} for 16 h in a 50 Ti rotor at 4°C. Membranes stripped of ribosomes were recovered as a band at the 0.25 - 2.0 M sucrose interface, and ribosomes as the pelleted material.

4.2.3 Binding of TAM to AEBS

All binding assays were performed in the presence of 1 µM E_2, to eliminate interaction of TAM with ER. Saturation analysis, single saturating dose and competitive binding assays were performed as described in Section 2.4. Incubation was either for 16 - 20 h at 0 - 4°C or for 1 - 2 h at 22°C. Results obtained at these two temperatures were not significantly different.

Previously published data have shown that rat liver microsomes do not metabolise tamoxifen during 24 h at 0°C (Sudo et al., 1983). However, concentrated microsomal preparations (2 - 88 mg microsomal

protein/ml) at 37°C in the presence of NADPH show significant metabolism (Robertson et al., 1982b; Ruenitz et al., 1984; Lyman and Jordan, 1985b) but this is abolished in the absence of NADPH (Dr Peter Ruenitz, personal communication). These findings indicate that the metabolism of tamoxifen is unlikely to be a complicating factor in interpretation of the present experimental results at 0 - 22°C, obtained using very low concentrations of washed microsomes (0.01 - 0.04 mg microsomal protein/ml) and in the absence of NADPH.

The association and dissociation rates of [^{3}H]TAM binding to the microsomal AEBS were measured at 0° and 22°C, as described in Section 2.4.4. The association rate constant (k_+) was calculated for the first 60 min and 14 min at 0°C and 22°C respectively, and the dissociation rate constant (k_-) was calculated for the first 60 min and 30 min at 0° and 22°C respectively. The AEBS lost no activity under these conditions.

4.2.4 Thermal Stability and Effects of pH, Ionic Strength and Enzyme Treatment

These techniques are described in Section 2.4.6.

4.3 RESULTS

4.3.1 Tissue Distribution

In order to identify tissues containing high levels of AEBS suitable for further characterization and purification, saturable [^{3}H]TAM binding was measured in the post-mitochondrial fractions of a range of rat tissues (Table 4.1). High affinity (K_D = 1 - 2 nM), saturable binding sites for tamoxifen were present in a number of tissues e.g. liver, stomach, ovary and brain (Figure 4.1), with very high concentrations being found in liver. Little or no detectable

binding was found in lung, heart, skeletal muscle or small intestine. Since liver contained almost 30-fold higher concentrations of AEBS than any other tissue, this organ was selected for further study.

TABLE 4.1 TISSUE DISTRIBUTION OF AEBS IN POST-MITOCHONDRIAL FRACTIONS OF RAT TISSUES

TISSUE	CONCENTRATION (fmol/mg microsomal protein)	pmol/g tissue	K_D (nM)
Liver	30,500 ± 4900[a]	780 ± 100	0.9 ± 0.1
Colon	1,060 ± 546	38 ± 16	2.9 ± 1.1
Stomach	870 ± 183	19 ± 4	1.3 ± 0.2
Ovary	820 ± 357	19 ± 9	2.3 ± 0.5
Uterus	730 ± 226	11 ± 4	1.6 ± 0.4
Kidney	650 ± 145	21 ± 8	1.6 ± 0.4
Brain	610 ± 115	15 ± 6	1.2 ± 0.4
Oesophagus	530 ± 110	9 ± 1	2.1 ± 1.5
Spleen	390	20	1.2
Lung	< 250	3	4.9
Heart	0	0	-
Skeletal Muscle	0	0	-
Small Intestine	0	0	-

[a] Value for liver is for KCl-washed microsomes.

4.3.2 Subcellular Distribution

Figure 4.2 shows the distribution of AEBS and the subcellular organelle enzyme markers between the cytosol, mitochondrial, microsomal and nuclear fractions of rat liver. The AEBS was concentrated in the microsomal fraction and, throughout the fractions, paralleled closely the distribution of the endoplasmic reticulum marker enzyme, NADPH cytochrome c reductase. The microsomal fraction contained 78.4 ± 2.1% of the total recovered AEBS. The cytosol had < 1% of the detectable AEBS, but appreciable quantities were detected in crude nuclear and mitochondrial fractions. This was probably due to contamination by microsomal components, as evidence by similar proportions of NADPH

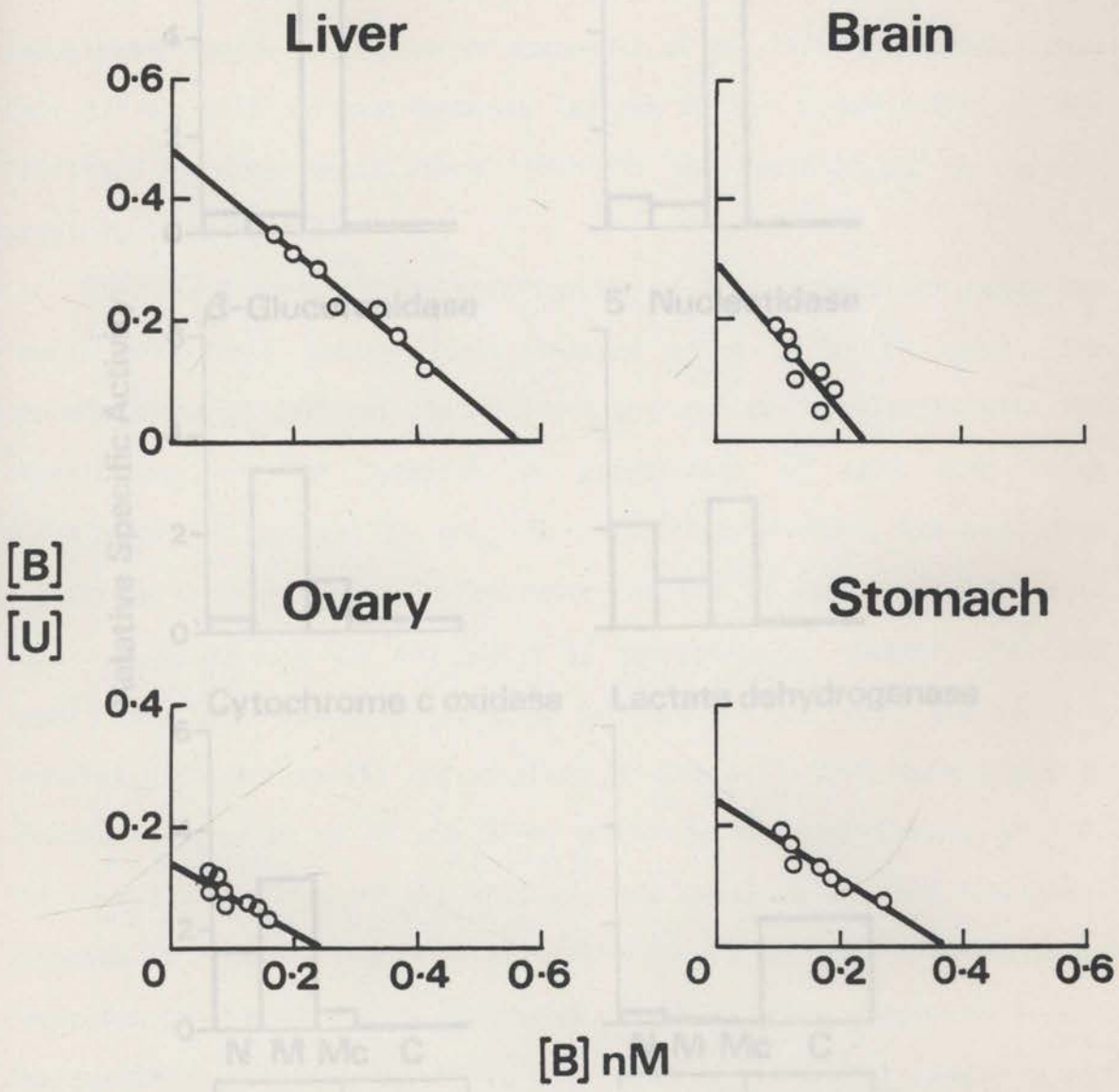

Figure 4.1: Scatchard plots of binding of [^{3}H]TAM to selected rat tissues. Rat tissues were homogenized in TSK buffer (1:10 w/v), and post-mitochondrial supernatants (PMF) were prepared by centrifugation of the homogenates at 10,000 x g for 20 min. Brain, stomach and ovary PMF were diluted 2-fold and liver PMF 50-fold with TSK prior to assay. [^{3}H]TAM was added in increasing concentrations, in the presence of 1 µM E_2 to eliminate binding to ER. Non-specific binding was measured in the presence of 100 nM unlabelled TAM. Charcoal/dextran was used to separate bound and unbound ligand as described in Section 2.4.1. and the binding data were analysed as in Section 2.4.2.

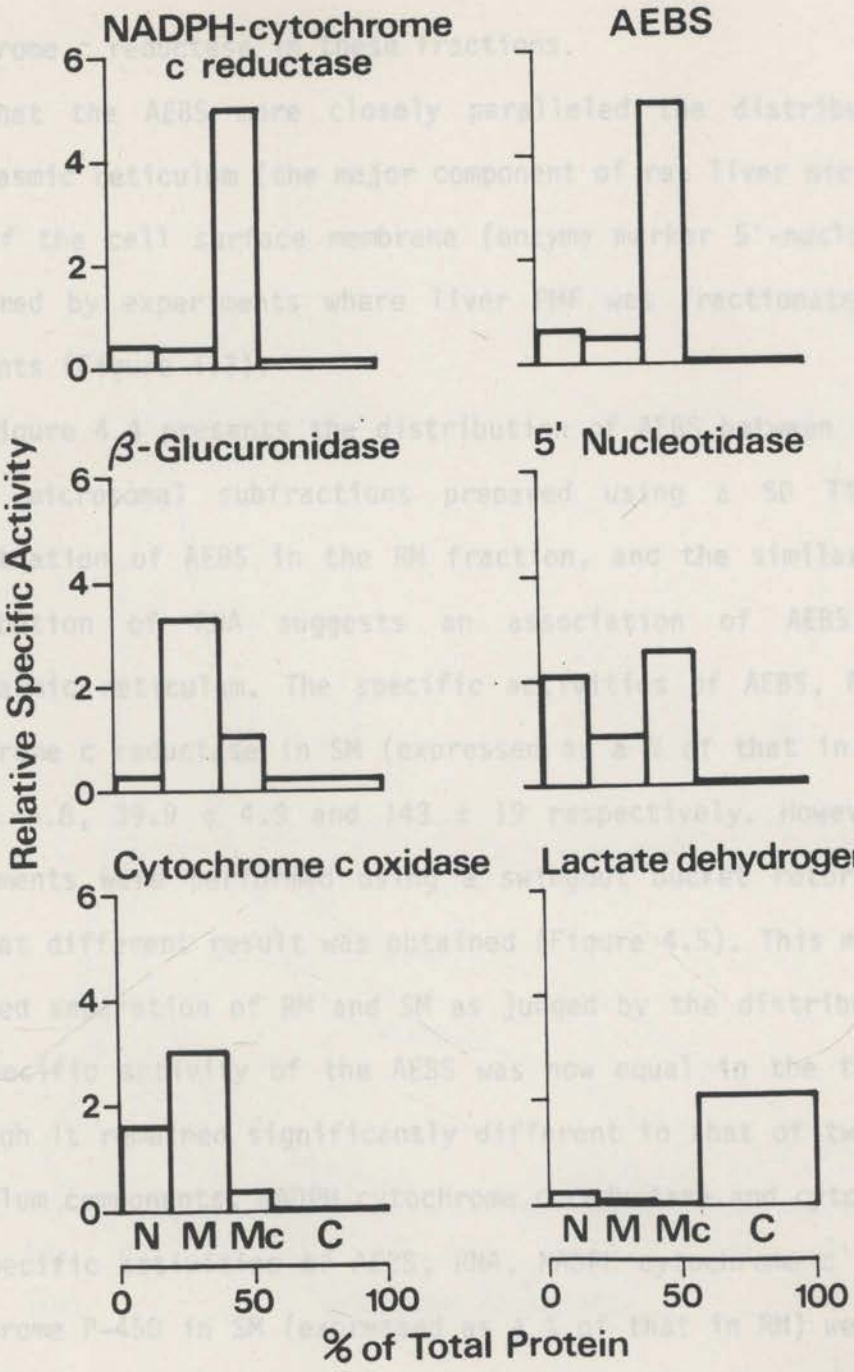

Figure 4.2: Subcellular localization of AEBS in rat liver. Rat liver homogenates were separated by differential centrifugation into nuclear (N), mitochondrial (M), microsomal (Mc) and cytosol (C) fractions as described in Section 2.2.3.2. The distribution of marker enzymes for plasma membranes (5'-nucleotidase), endoplasmic reticulum (NADPH cytochrome c reductase), lysosomes (β-glucuronidase), mitochondria (cytochrome c oxidase) and cytosol (lactate dehydrogenase) and the distribution of AEBS in each fraction is shown.

cytochrome c reductase in these fractions.

That the AEBS more closely paralleled the distribution of the endoplasmic reticulum (the major component of rat liver microsomes) than that of the cell surface membrane (enzyme marker 5'-nucleotidase) was confirmed by experiments where liver PMF was fractionated on Percoll gradients (Figure 4.3).

Figure 4.4 presents the distribution of AEBS between the rough and smooth microsomal subfractions prepared using a 50 Ti rotor. The concentration of AEBS in the RM fraction, and the similarity with the distribution of RNA suggests an association of AEBS with rough endoplasmic reticulum. The specific activities of AEBS, RNA and NADPH cytochrome c reductase in SM (expressed as a % of that in the RM) were 62.1 ± 4.8, 39.9 ± 4.9 and 143 ± 19 respectively. However, when the experiments were performed using a swingout bucket rotor (SW 50.1) a somewhat different result was obtained (Figure 4.5). This method gave an improved separation of RM and SM as judged by the distribution of RNA. The specific activity of the AEBS was now equal in the two fractions, although it remained significantly different to that of two endoplasmic reticulum components, NADPH cytochrome c reductase and cytochrome P-450. The specific activities of AEBS, RNA, NADPH cytochrome c reductase and cytochrome P-450 in SM (expressed as a % of that in RM) were 92 ± 6, 22 ± 2, 189 ± 8 and 252 ± 10 respectively. The RM and SM fractions contained 66 ± 4% and 36 ± 4% of the total AEBS activity.

When RM were stripped of bound ribosomes, and separated into stripped membrane and ribosomal fractions, AEBS was found only in the membrane fraction but with a 47 ± 8% reduction in specific activity compared with the starting material.

Microsomes extracted with 1 M KCl, 0.5 M acetic acid, or low

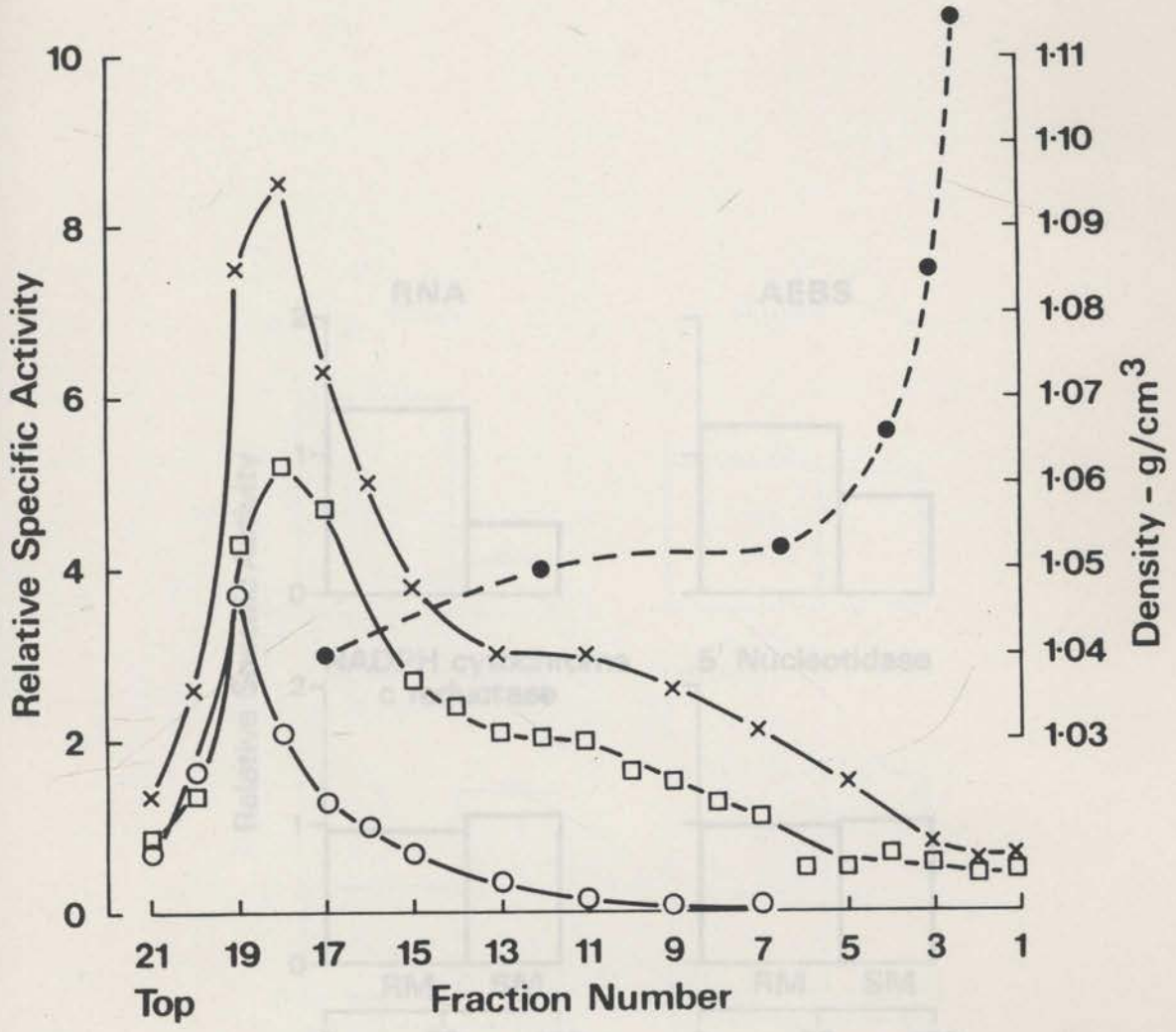

Figure 4.3: Subcellular fractionation of rat liver post-mitochondrial fraction on a Percoll density gradient. Rat liver PMF was mixed with 30% Percoll in 0.25 M sucrose and centrifuged at 60,000 x g_{av} for 45 min in a 50 Ti rotor. Fractions, collected from the bottom of the tubes, were assayed for AEBS (□) (single saturating dose technique), NADPH cytochrome c reductase (X) (marker enzyme for endoplasmic reticulum), and 5'-nucleotidase (O) (marker enzyme for plasma membrane) activities as described in Section 2.2.4. Gradient density (●) was determined by Density Marker Beads.

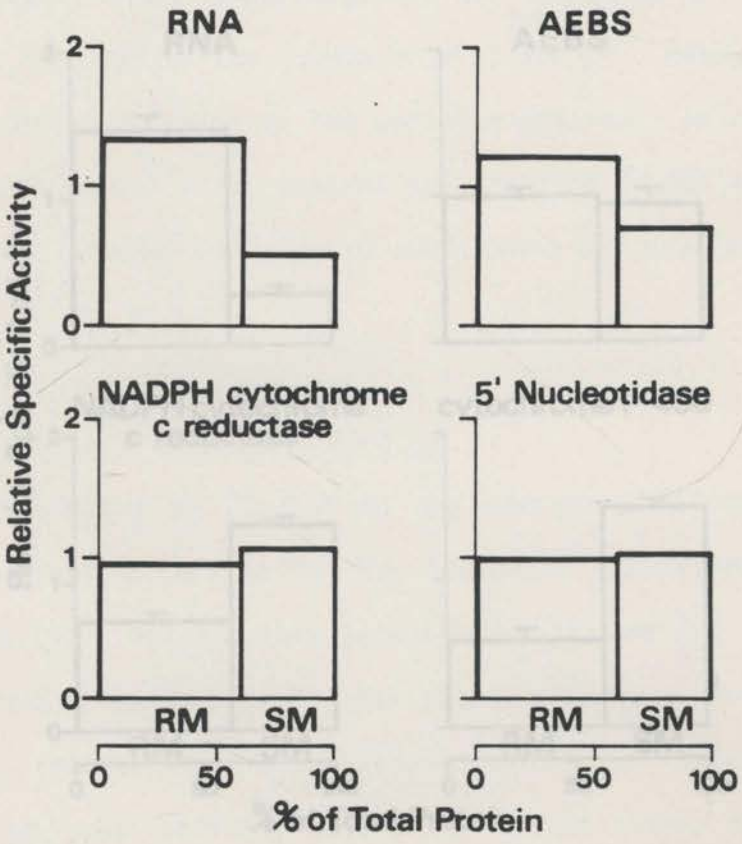

Figure 4.4: Distribution of AEBS between subfractions of rat liver microsomes (I). Microsomes were separated on a discontinuous sucrose gradient containing CsCl, using a 50 Ti rotor into rough (RM) and smooth (SM) membrane fractions as described in Section 2.2.3.2. The distribution of marker enzymes for plasma membranes (5'-nucleotidase), endoplasmic reticulum (NADPH cytochrome c reductase), and the distribution of AEBS and RNA is shown.

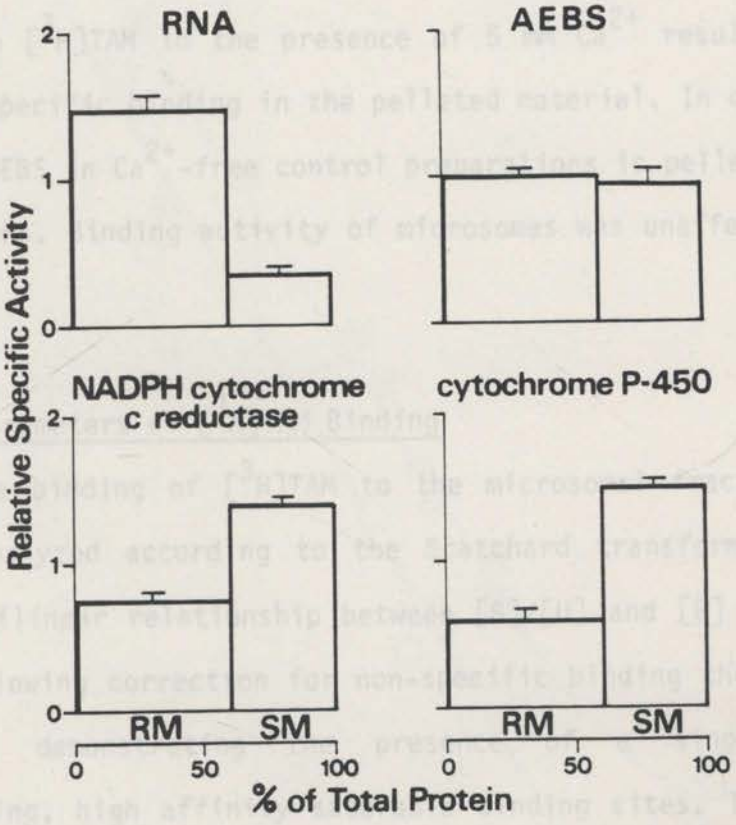

Figure 4.5: Distribution of AEBS between subfractions of rat liver microsomes (II). Microsomes were separated on a discontinuous sucrose gradient containing CsCl, using a SW 50.1 Ti rotor into rough (RM) and smooth (SM) membrane fractions as described in Section 2.2.3.2. The distribution of marker enzymes for plasma membranes (5'-nucleotidase), endoplasmic reticulum (NADPH cytochrome c reductase), and the distribution of AEBS, RNA and cytochrome P-450 is shown.

concentrations of the detergent sodium deoxycholate (< 0.05% w/v) retained AEBS. The AEBS was, however, solubilized by high concentrations of sodium cholate (see Chapter 5).

Low concentrations of Ca^{2+} or Mg^{2+} (< 10 mM) appeared to inhibit binding of [^{3}H]TAM to AEBS. However, this effect resulted from divalent cation-induced aggregation of microsomal vesicles (Schenkman and Cinti, 1978) which are subsequently pelleted when dextran-charcoal suspensions are centrifuged during the AEBS assay. AEBS activity was retained by these aggregates. Thus, centrifugation at 10,000 x g of microsomes labelled with [^{3}H]TAM in the presence of 5 mM Ca^{2+} resulted in > 90% recovery of specific binding in the pelleted material. In contrast, less than 10% of AEBS in Ca^{2+}-free control preparations is pelleted under the same conditions. Binding activity of microsomes was unaffected by 10 mM EDTA or EGTA.

4.3.3 Parameters of [^{3}H]TAM Binding

When the binding of [^{3}H]TAM to the microsomal fraction from rat liver was analyzed according to the Scatchard transformation (Figure 4.6), a curvilinear relationship between [B]/[U] and [B] was apparent. However, following correction for non-specific binding the relationship was linear, demonstrating the presence of a single class of non-interacting, high affinity saturable binding sites. The K_D of this interaction, which was not significantly different in the RM, SM or stripped-membrane fractions, was 0.9 ± 0.1 nM (range 0.6 - 1.6 nM) when measured at 0° - 22°C. The AEBS concentration in the microsomal fraction was 30.5 ± 4.9 pmol/mg protein (0.78 ± 0.10 nmol/g liver).

Measurement of the kinetic rate constants of the reaction showed that the association (k_+) and dissociation (k_-) rates of the binding of

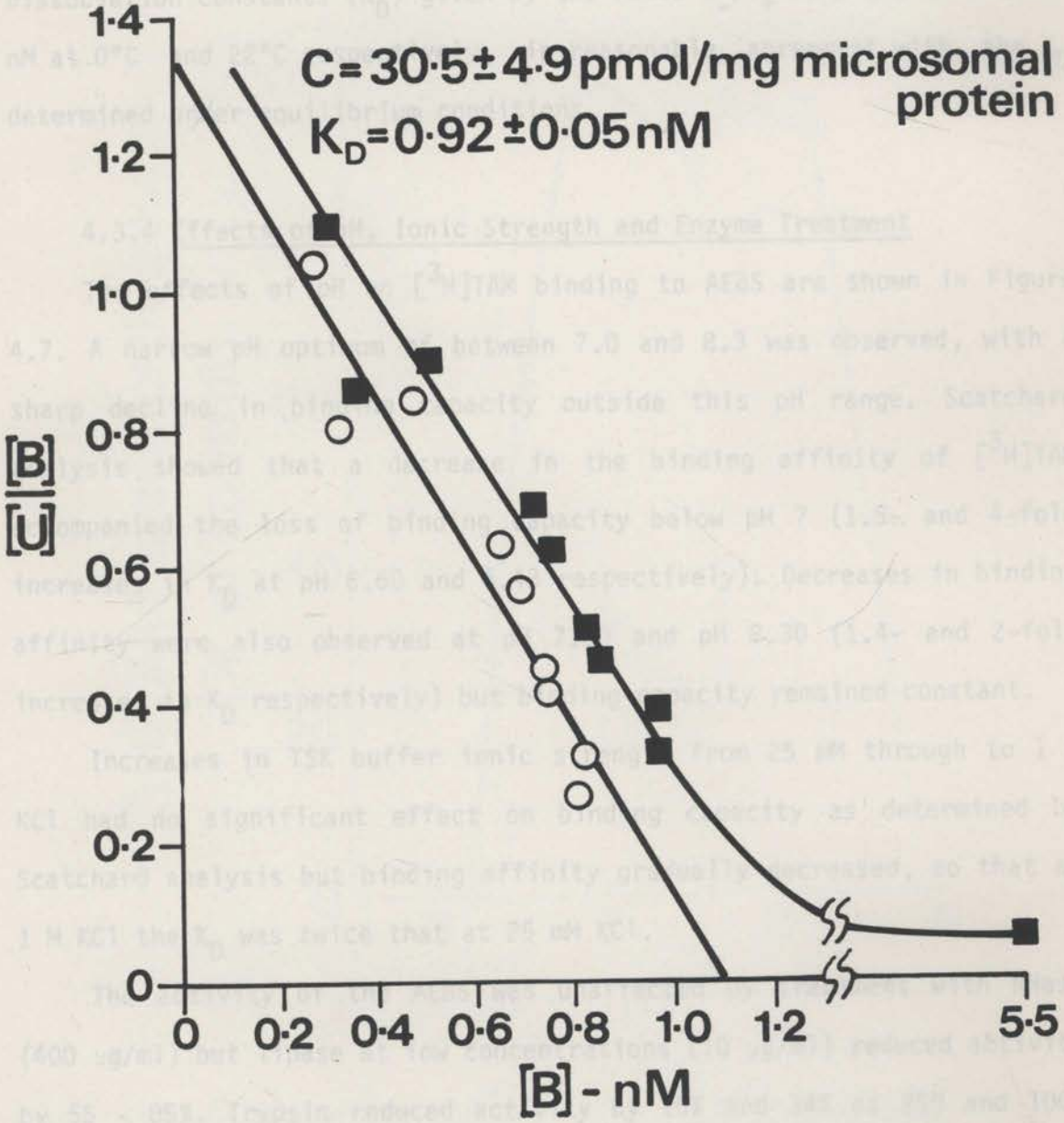

Figure 4.6: Binding of [^{3}H]TAM to rat liver microsomal AEBS. Scatchard plot of the interaction between [^{3}H]TAM and the AEBS in rat liver microsomes. Data are plotted before (■) and after (○) correction for non-specific binding.

[^{3}H]TAM to rat liver microsomal AEBS were compatible with second- and first-order reactions, respectively. The rate constants measured at 0°C and 22°C were k_+: 5.43 ± 1.08 x 10^4 M^{-1} s^{-1} and 3.35 ± 0.95 x 10^5 M^{-1} s^{-1}; and k_-: 2.65 ± 0.67 x 10^{-5} s^{-1} ($t_{\frac{1}{2}}$ = 435 min at 0°) and 2.39 ± 0.08 x 10^{-4} s^{-1} ($t_{\frac{1}{2}}$ = 48 min at 22°C). The calculated equilibrium dissociation constants (K_D) given by the ratio k_-/k_+ were 0.5 nM and 0.7 nM at 0°C and 22°C respectively, in reasonable agreement with the K_D determined under equilibrium conditions.

4.3.4 Effects of pH, Ionic Strength and Enzyme Treatment

The effects of pH on [^{3}H]TAM binding to AEBS are shown in Figure 4.7. A narrow pH optimum of between 7.0 and 8.3 was observed, with a sharp decline in binding capacity outside this pH range. Scatchard analysis showed that a decrease in the binding affinity of [^{3}H]TAM accompanied the loss of binding capacity below pH 7 (1.5- and 4-fold increases in K_D at pH 6.60 and 6.43 respectively). Decreases in binding affinity were also observed at pH 7.80 and pH 8.30 (1.4- and 2-fold increases in K_D respectively) but binding capacity remained constant.

Increases in TSK buffer ionic strength from 25 mM through to 1 M KCl had no significant effect on binding capacity as determined by Scatchard analysis but binding affinity gradually decreased, so that at 1 M KCl the K_D was twice that at 25 mM KCl.

The activity of the AEBS was unaffected by treatment with RNase (400 µg/ml) but lipase at low concentrations (10 µg/ml) reduced activity by 55 - 85%. Trypsin reduced activity by 15% and 34% at 250 and 1000 µg/ml respectively, but in the presence of detergent (1% w/v sodium cholate) trypsin had a greater effect and all binding activity was lost at a concentration of 50 µg/ml.

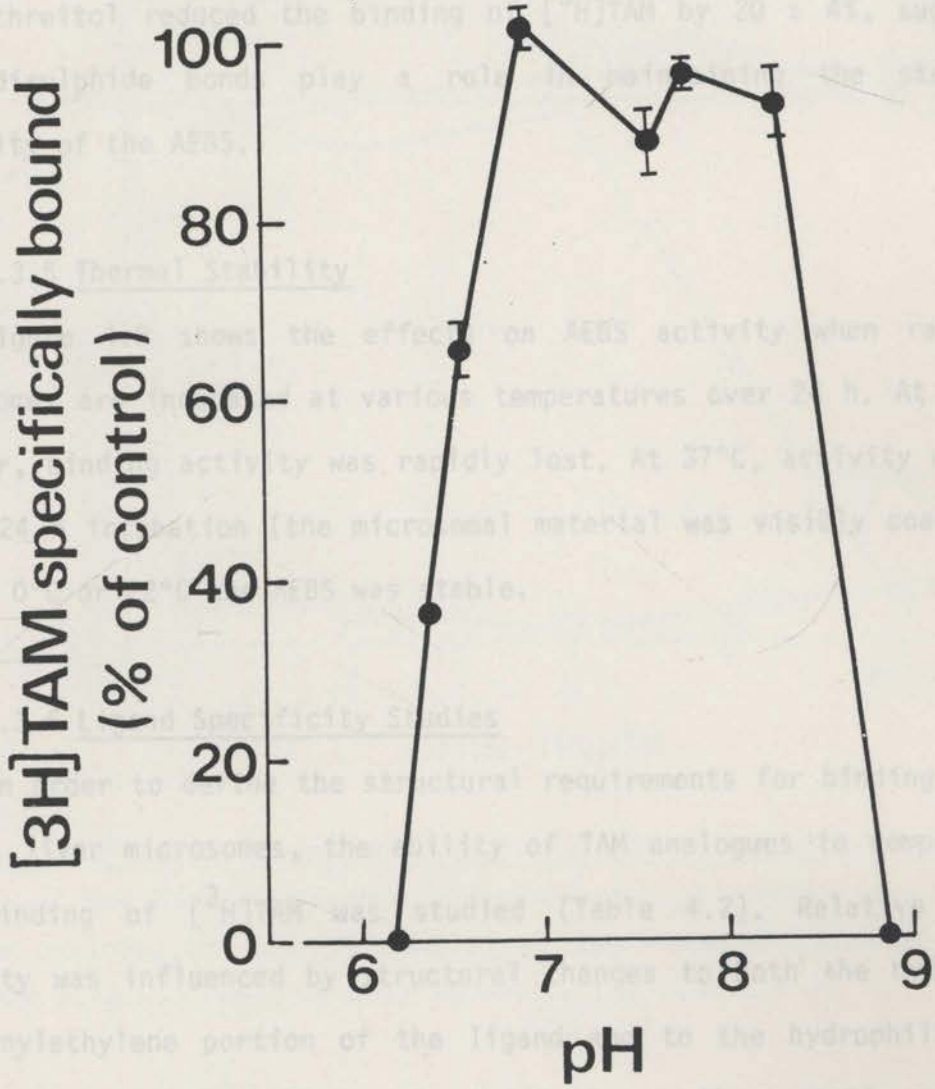

Figure 4.7: Effect of pH on [^{3}H]TAM binding to rat liver microsomal AEBS. The specific [^{3}H]TAM binding capacity of rat liver microsomes at various pH was measured by Scatchard analysis. The pH indicated is that in the final assay incubation mixture, measured at 22°C, and results are expressed as a percentage of control binding at pH 7.28.

Investigation of other factors that might influence AEBS activity showed that molybdate, which protects against steroid receptor inactivation possibly because of its action as a phosphatase inhibitor, had no effect on AEBS binding activity or stability at concentrations up to 10 mM. Phenylmethylsulfonylfluoride, a protease inhibitor, also had no effect. However, treatment of microsomal suspensions with 1 mM dithiothreitol reduced the binding of [^{3}H]TAM by 20 ± 4%, suggesting that disulphide bonds play a role in maintaining the structural integrity of the AEBS.

4.3.5 Thermal Stability

Figure 4.8 shows the effects on AEBS activity when rat liver microsomes are incubated at various temperatures over 24 h. At 60°C or greater, binding activity was rapidly lost. At 37°C, activity was lost after 24 h incubation (the microsomal material was visibly coagulated) but at 0°C or 22°C the AEBS was stable.

4.3.6 Ligand Specificity Studies

In order to define the structural requirements for binding to AEBS in rat liver microsomes, the ability of TAM analogues to compete with the binding of [^{3}H]TAM was studied (Table 4.2). Relative binding affinity was influenced by structural changes to both the hydrophobic triphenylethylene portion of the ligand and to the hydrophilic basic amino side chain. The effects of substitution on the aromatic portion of the ligand were evident when TAM, 79280, 129817, 132802, 148067 and 133312 were compared. The introduction of the F, OH, OCH_3 or CH_3 substituents, which are electron-donating because of resonance effects, led to a 70 - 80% reduction in affinity.

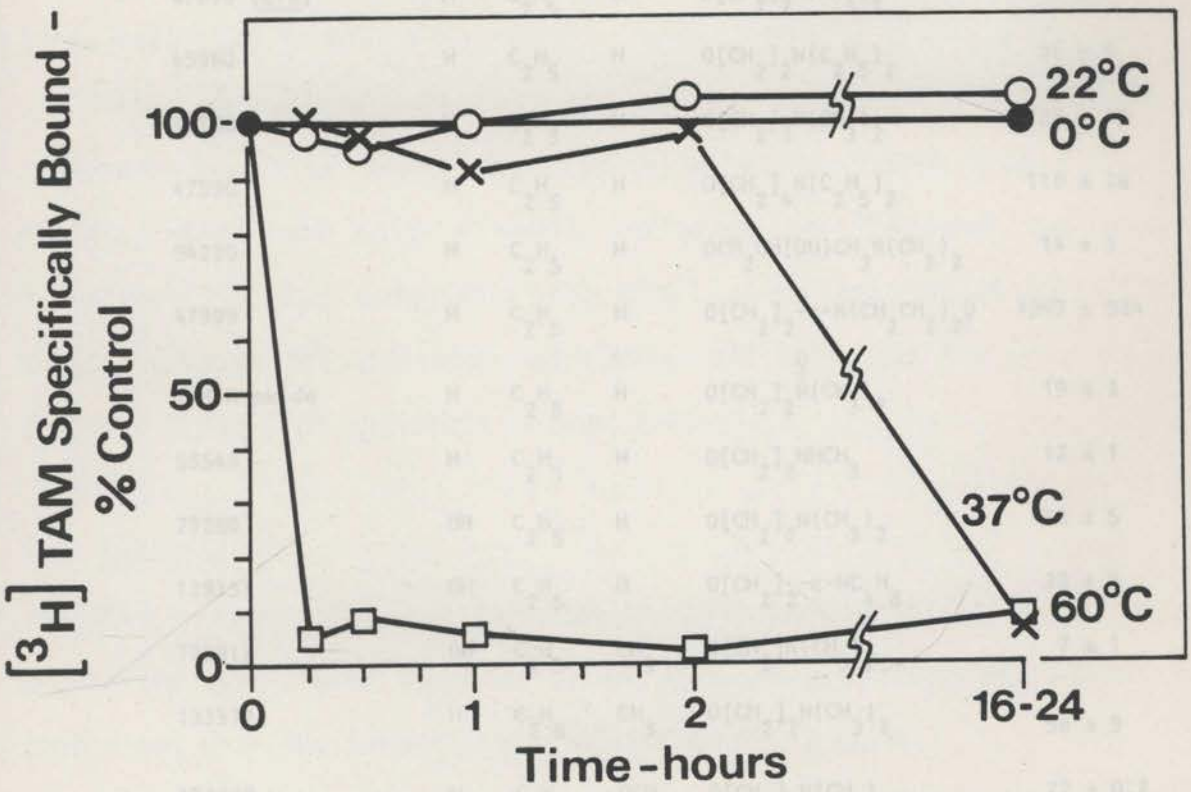

Figure 4.8: Effect of temperature on the stability of AEBS in rat liver microsomes. Rat liver microsomes were incubated at various temperatures, and the activity of AEBS determined at the indicated time intervals, using the single saturating dose technique (Section 2.4.3).

TABLE 4.2 RELATIVE BINDING AFFINITIES (RBA) OF TAM ANALOGUES FOR RAT LIVER MICROSOMAL AEBS

ICI CODE NUMBER	R_1[1]	R_2[1]	R_3[1]	R_4[1]	RBA(%)
TAM (trans)	H	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	100
47699 (cis)	H	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	73 ± 7
45960	H	C_2H_5	H	$O[CH_2]_2N(C_2H_5)_2$	21 ± 5
47108	H	C_2H_5	H	$O[CH_2]_3N(CH_3)_2$	127 ± 15
47590	H	C_2H_5	H	$O[CH_2]_4N(C_2H_5)_2$	119 ± 26
94230	H	C_2H_5	H	$OCH_2CH(OH)CH_2N(CH_3)_2$	14 ± 3
47399	H	C_2H_5	H	$O[CH_2]_2$-c-$N(CH_2CH_2)_2O$	1993 ± 924
TAM-N-oxide	H	C_2H_5	H	$O[CH_2]_2\overset{O}{\uparrow}N(CH_3)_2$	19 ± 3
55548[2]	H	C_2H_5	H	$O[CH_2]_2NHCH_3$	12 ± 1
79280[3]	OH	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	30 ± 5
129351	OH	C_2H_5	H	$O[CH_2]_2$-c-NC_4H_8	28 ± 5
129817	OH	C_2H_5	CH_3	$O[CH_2]N(CH_3)_2$	7 ± 1
133312	H	C_2H_5	CH_3	$O[CH_2]_2N(CH_3)_2$	58 ± 9
132802	H	C_2H_5	OCH_3	$O[CH_2]_2N(CH_3)_2$	22 ± 0.2
148067	H	C_2H_5	F	$O[CH_2]_2N(CH_3)_2$	31 ± 3
46414	H	CH_3	H	$O[CH_2]_3N(CH_3)_2$	163 ± 29
49500	H	C_3H_7	H	$O[CH_2]_2N(CH_3)_2$	42 ± 10

[1] R_1 - R_4 refer to substituents illustrated in Figure 6.1; [2] N-desmethylTAM; [3] 4-hydroxyTAM.

The side chain terminal amino group played a major role in determining binding affinity. The TAM analogue carrying the terminal cyclic amino group -$N(CH_2CH_2)_2O$ (47399) had increased affinity, whereas

analogues with $-N(C_2H_5)_2$ (45960), $-NHCH_3$ (55548), or N-oxide terminal groups had lower affinities than TAM ($-N(CH_3)_2$).

Changes in the length of the hydrocarbon portion of the side chain also produced changes in RBA, the magnitude of which were apparently dependent upon the terminal amino group (TAM vs 47108; 45960 vs 47590). The presence of a side chain hydroxyl group (47108 vs 94230) caused a large reduction in RBA.

Other structural factors influencing binding affinity were: cis/trans isomerism about the triphenylethylene double bond (TAM vs 47699), which had only a small influence on RBA; and the vinyl substituent at R_2. Compounds with ethyl or methyl (46414 vs 47108) substituents at this position had similar affinity, but the presence of a propyl group (TAM vs 49500) reduced RBA.

4.4 DISCUSSION

The present study defines the subcellular localization, binding properties and ligand specificity of AEBS in rat liver and allows a comparison with the properties of AEBS from MCF 7 cells.

Distribution studies of AEBS in rat tissues showed that the liver had the highest concentration. The site was not restricted to classical oestrogen target tissues, even though other studies have shown that, in some tissues, levels of AEBS are under oestrogenic control (rat liver: Winneker and Clark, 1983; rat and guinea pig uterus: Faye et al., 1980; Gulino and Pasqualini, 1983). Qualitatively similar data on the tissue distribution of microsomal AEBS in the rat have subsequently been presented by Sudo et al. 1983. However, the present study found significantly higher AEBS concentrations in liver perhaps, because of variations in assay conditions as discussed in Section 3.4, and also

because of the use of lower liver microsomal protein concentrations than those used by Sudo et al., which reduces interference from low affinity or non-specific binding components.

Rat liver AEBS was shown to be located almost exclusively in the microsomal fraction, and not the cytosol as previously reported for this and other rat tissues (Faye et al., 1980; Sutherland et al., 1980; Murphy and Sutherland, 1981a; Winneker and Clark, 1983). Less than 1% of total liver AEBS was present in the cytosol, while > 60% of the activity associated with crude nuclei was removed following purification through 2.2 M sucrose. It is still unclear whether the remainder represents microsomal contamination, or a distinct nuclear binding site for TAM. Such results, however, bring into question the validity of hormonal regulation studies on AEBS, where inadequate subcellular separation techniques were employed or only a portion of the total cellular AEBS was measured. The nuclear AEBS identified by Murphy et al. (1984b) in cockerel liver is almost certainly microsomal in origin. Kon (1985a) has also described the presence of small amounts of AEBS in purified rat liver nuclei.

The subcellular localization of the rat liver AEBS is in agreement with observations that it is also predominantly microsomal in rat uterus (Sudo et al., 1983) and MCF 7 cells (Section 3.3.1; Miller and Katzenellenbogen, 1983; Watts et al., 1984).

Within the nuclear, mitochondrial, microsomal and cytosol fractions the AEBS most closely paralleled the distribution of NADPH cytochrome c reductase, suggesting a possible location of AEBS in the endoplasmic reticulum. Within the microsomal fraction, which in the liver consists mainly of endoplasmic reticulum, discontinuous gradient techniques using a fixed angle rotor (Figure 4.4) showed that AEBS was concentrated in

the RM fraction, which is comprised largely of rough (ribosome-bearing) endoplasmic reticulum. Although this suggested the possibility that AEBS might be involved in biochemical features unique to this organelle e.g. protein-synthetic or -transport mechanisms, subsequent experiments using a modified technique (Figure 4.5) showed the AEBS to be evenly distributed between RM and SM. Thus, a specific association of AEBS with rough endoplasmic reticulum seems unlikely. The sedimentation properties of AEBS, which differed from the endoplasmic reticulum components NADPH cytochrome c reductase and cytochrome P-450, do not, however, exclude a location on endoplasmic reticulum. Microsomal fractionations on linear density gradients show heterogeneous density distributions and sedimentation profiles of endoplasmic reticulum constituents (Beaufay et al., 1974).

Katzenellenbogen et al. (1985a) also found that the majority of AEBS was associated with the rough microsomal fraction, when prepared by the method of Adelman et al. (1974). The proportions of AEBS in the rough, smooth and ribosome fractions were 55 ± 4, 32 ± 2, and 13 ± 1% respectively. However, the specific activity of AEBS in these fractions was not indicated.

The endoplasmic reticulum is the site of cytochrome P-450-mediated metabolism of TAM and other triphenylethylenes. TAM binds directly to cytochrome P-450 (Ruenitz et al., 1984) and it has been suggested that the AEBS may be associated with this protein (Ruenitz and Bagley, 1985b). This possibility is discussed in more detail in Chapter 6, but from the present study it is clear that the AEBS and cytochrome P-450 are quite differently distributed within the microsomal fraction.

Apart from endoplasmic reticulum, the microsomal fraction contains small amounts of elements derived from the plasma membrane, the Golgi

complex, fragments of lysosomes, peroxisomes and the outer mitochrondrial membrane, and free ribosomes. The AEBS is unlikely to be located on any of these components. AEBS did not parallel the enzyme markers for plasma membrane, lysosomes or mitochondria between the nuclear, mitochondrial, microsomal or cytosol fractions or within the microsomal fraction. Catalase, a marker enzyme for peroxisomes, sediments predominantly in the mitochondrial and cytosol fractions whereas the Golgi complex (marker enzyme galactosyltransferase) sediments mainly in the microsomal fraction (Amar-Costesec et al., 1974). However, separation of microsomes into RM and SM fractions using similar techniques to the present study caused the Golgi complex to be concentrated in the latter fraction, as shown by 17-fold difference in the specific activity of galactosyl transferase (Fleischer and Kervina, 1974). Free ribosomes are also unlikely to contain AEBS. Although dense, ribosomes sediment very slowly and thus the majority remain in the cytosol fraction. Any remaining free ribosomes contaminating the microsomal or RM and SM fractions are transferred to the high-speed supernatant fractions during washing procedures. These supernatants and the cytosol have minimal AEBS content.

Many studies have noted the presence of low levels of ER in the plasma membrane and microsomal fractions of oestrogen target tissues (as reviewed by Szego and Pietras, 1984). In rat uterine microsomes, one study has shown ER is present at relatively high concentrations (Watson and Muldoon, 1985), representing about 20% of the total rat uterine extranuclear E_2 binding capacity (90 fmol/mg microsomal protein, approximately 12% of the level of rat uterine AEBS, Table 4.1). What relationship (if any) such sites have to the microsomal AEBS is unknown. Furthermore, the validity of these observations has been called into

question by immunocytochemical techniques using monoclonal antibodies to ER. These studies have shown that, in all cases, specific staining for ER is confined to the nucleus. None has been observed in the cell membranes (Section 1.5).

The reduction in specific activity of AEBS following removal of ribosomes from rough membranes may indicate that their removal renders AEBS more susceptible to degradation, or that the presence of ribosomes is essential for binding site integrity. The failure of 1 M KCl or 0.5 M acetic acid to solubilize AEBS suggests that it is not a loosely adsorbed or extrinsic (peripheral) membrane protein. Furthermore, the effective solubilization of AEBS only at relatively high concentration of detergent suggest that AEBS is not a luminal protein of the microsomal vesicles. Lower concentrations of detergent cause the vesicles to become permeable which allows luminal proteins to escape. The sensitivity of the AEBS to enzyme treatment is consistent with it being a membrane-bound protein, resistant to proteolytic attack because of the presence of protecting lipids which can be removed by detergent. The sensitivity to lipase suggests that lipids are also required for binding activity, either as co-factors or in the maintenance of appropriate binding site structure.

At 0°C, rat liver AEBS association and dissociation rate constants for [^{3}H]TAM binding have similar values to those of the MCF 7 AEBS. However, at 22°C these rate constants are two- to five-fold lower in rat liver. The values for MCF 7 AEBS are k_+: 5.62 x 10^4 M^{-1} s^{-1} and 1.59 x 10^6 M^{-1} s^{-1} at 0°C and 22°C respectively and k_-: 2.95 x 10^{-5} s^{-1} ($t_{\frac{1}{2}}$ = 392 min) and 1.32 x 10^{-3} s^{-1} ($t_{\frac{1}{2}}$ = 8.75 min) at 0°C and 22°C respectively (calculated from data presented in Section 3.3.3). The effects of pH on the binding of [^{3}H]TAM to rat liver were similar to

those previously observed for MCF 7 AEBS. However, the binding affinity of rat liver AEBS was somewhat less sensitive to increasing ionic strength. The lower thermal stability of the rat liver AEBS may indicate differences either in AEBS protein structure, or in the stability of other elements of the rat liver microsomes.

When ligand specificity was compared between rat liver and MCF 7 AEBS, both quantitative and, in some cases qualitative differences were apparent (Tables 3.2 and 4.2). In general, the rat liver AEBS has more rigorous requirements with regard to ligand structure. Alterations to the ligand, especially to the terminal amino group and to the hydrophobic triphenylethylene portion of the molecule, cause larger changes in binding affinity for liver AEBS than for MCF 7 AEBS (e.g. TAM vs 79280, 47399 and 148067). In contrast, binding affinity for the MCF 7 AEBS is more sensitive to changes involving the alkyl portion of the alkyl amino ether side chain (TAM vs 47108 and 94230), but not when the terminal group is $-N(C_2H_5)_2$ (45960 vs 47590). TAM analogues with non-basic side chains had no affinity for either rat or MCF 7 AEBS (Table 6.2). The structural specificity of the AEBS is investigated and discussed in more detail in Chapter 6.

The similarity of the rat liver and MCF 7 AEBS with regard to ligand binding, subcellular distribution and other properties suggests that these two binding sites are essentially equivalent. The rat liver is a convenient source of AEBS for further investigation of its properties and purification.

CHAPTER FIVE

DETERGENT SOLUBILIZATION OF THE ANTIOESTROGEN BINDING SITE

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5.1 INTRODUCTION

Solubilization of membrane bound proteins in general, and membrane-bound receptors in particular, is a pre-requisite for a full understanding of their biochemistry. In the solubilized state such proteins can in principle be purified and their properties studied in isolation from the influence of other membrane lipid and protein components. Such studies often demonstrate the requirement for protein-lipid or protein-protein interactions within the membrane for the full expression of biological activity. In the case of the AEBS solubilization studies were considered desirable for several reasons. Apart from information that could be obtained regarding the physical and biochemical properties of the AEBS protein, which might aid in establishing function or identity with other known endoplasmic reticulum proteins, solubilization with subsequent purification would allow unambiguous binding specificity studies to be performed in the absence of other potentially interfering membrane receptors or binding proteins, perhaps resolving inconsistencies in species/tissue binding specificity data (Chapter 6). Solubilization studies were also initiated with a view to the ultimate production of purified material which would enable production of antibodies to the AEBS, providing the basis for a more generally useful assay than the radio-ligand techniques used at present. Hopefully, such a technique would lead to improved understanding of the tissue and subcellular distribution and physiological or pharmacological function of the AEBS.

Previous observations (Section 4.3.2) had established that the AEBS was probably an integral (intrinsic) membrane protein rather than a loosely bound peripheral (extrinsic) or luminal protein of the endoplasmic reticulum. Integral membrane proteins are characterizsed by

their tight association with the membrane, and penetrate into the phospholipid bilayer making contact with hydrophobic regions. Because of the presence of highly hydrophobic domains on these proteins they are generally insoluble, aggregating in aqueous solution in the absence of lipid, and can only be solubilized under conditions that disturb hydrophobic interactions with consequent disruption of the membrane. Organic solvents, detergents or, more rarely, chaotropic agents have been used for this purpose. Although organic solvents such as 2-chloroethanol, n-butanol and chloroform-methanol mixtures have proved to be useful in the solubilization of a small proportion of membrane proteins especially those tightly associated with lipid (Boyan and Clement-Cormier, 1984), the method suffers from the frequent loss of functional activity that occurs when proteins are re-introduced into aqueous solution. Thus the present study concentrated upon detergent solubilization of the AEBS.

The properties of detergents and their use in membrane solubilization have been extensively reviewed (Helenius and Simons, 1975; Helenius et al. 1979; Hjelmeland and Chrambach, 1984). In outline, the process of membrane solubilization occurs in stages as the detergent to membrane ratio is increased. As sites of decreasing affinity become bound by detergent monomers, membrane permeability increases, followed by lysis and then membrane solubilization with the formation of protein-detergent, lipid-protein-detergent and lipid-detergent micelles. Further increases in detergent ideally result in maximal exchange of lipid from around proteins (delipidation), with individual protein molecules contained within detergent micelles. In this state the proteins are potentially separable by procedures such as ion-exchange, gel filtration and hydrophobic interaction chromatography. In practise, individual

detergents may fail to solubilize particular proteins from the membrane, or fail to disrupt protein-lipid or protein-protein interactions in solution. A further common problem is irreversible loss of biological activity or the need for detergent removal and reconstitution with other membrane components, phospholipids in particular, to restore activity. Optimal conditions for solubilization with retention of biological activity have to be determined empirically. Factors to be considered include the type of detergent and concentrations used, membrane concentration and conditions of ionic strength, pH and temperature.

Such an approach was adopted for the AEBS, resulting in successful solubilization with sodium cholate. In addition, the properties of the solubilized binding site were investigated, and techniques were investigated for photo-affinity covalent labelling of the AEBS to enable monitoring of the protein during detergent solubilization and subsequent purification procedures.

5.2 METHODS

Preparation of rat liver microsomes and the determination of [^{3}H]TAM binding are as described in Sections 2.2.3.1 and 2.4 respectively.

5.2.1 Detergent Solubilization of Microsomes

Microsomes were prepared as described above and resuspended in TSK buffer. Detergents were then added as concentrated solutions to the desired final concentration. After 30 min at 0°C with occasional gentle stirring, the detergent treated material was centrifuged at 130,000 x g_{av} for 1 h in a 50 Ti rotor. Solubilized material was considered to be that remaining in the supernatant. When required, the pellet was

resuspended in TSK buffer. Prior to the [^{3}H]TAM binding assay the supernatant and pellet suspensions were diluted with TSK, typically between 10- and 50-fold.

5.2.2 Gel Filtration Chromatography

Microsomes were solubilized in TSK containing 1% (w/v) sodium cholate and 1 M NaCl. 5 ml of the 130,000 x g_{av} supernatant was chromatographed in the presence of the same buffer on a Fractogel HW-65F column (85 x 1.5 cm) at 4°C. A high flow rate (60 ml/h) was used to minimize AEBS degradation. The absorbance of the fractions (2 ml) was determined at 280 nm. After dilution, 5 nM [^{3}H]TAM was added and specific [^{3}H]TAM binding was measured as the difference between [^{3}H]TAM bound in the absence and presence of 1 μM unlabelled TAM.

Calibration proteins, fractionated separately under the same conditions, were thyroglobulin, ferritin, BSA and ovalbumin (M_r 669,000, 440,000, 67,000 and 43,000 respectively). The elution volumes of these proteins were not significantly altered by the presence of detergent.

5.2.3 Hydrophobic Interaction Chromatography

Rat liver microsomes were solubilized in TSK containing 1% (w/v) cholate and 1 M NaCl, and centrifuged at 130,000 x g_{av} for 1 h. Aliquots (1 ml) of this preparation were applied to columns, each containing 1 ml of hydrophobic agaroses of increasing hydrocarbon chain length (Sigma Chemical Co.): agarose - $NH(CH_2)_nCH_3$, phenyl-agarose, and agarose - $NH(CH_2)_nNH_2$ (n = 0 - 12). For the latter alkyl-amino series of agaroses, the applied sample was adjusted to 4 M NaCl to increase the strength of the hydrophobic interaction. The columns were washed with 1 ml of buffer and the combined eluates were assayed for AEBS (single saturating dose

technique, Section 2.4.3) and protein recovery.

5.2.4 Polyethylene Glycol Precipitation

PEG 6000 as a 40% (w/v) solution in TSK containing 1% sodium cholate/1 M NaCl was added to the 130,000 x g_{av} supernatant of microsomes solubilized in the same buffer as described above. After 15 min at 0°C protein was pelleted in an Eppendorf Microfuge (5 min). The supernatant was retained for protein and AEBS assays and the pellet washed with TSK, resuspended in the appropriate buffer and assayed for protein and AEBS using the single saturating dose technique with both charcoal and pelleting procedures (Section 2.4.3) for separation of bound and unbound ligand.

5.2.5. Photoaffinity Labelling

Rat liver microsomal suspensions diluted to a protein concentration of 0.1 - 0.2 mg/ml were labelled with 2 nM [^{3}H]fluphenazine or [^{3}H]TAM in the presence of either BSA (1 mg/ml) or DMF (5% v/v). After removal of unbound ligand with dextran/charcoal, 2 ml aliquots of the labelled suspension were placed in 1 cm quartz cuvettes, bubbled with N_2 gas when DMF was present (to remove O_2 and hence minimize photo-oxidation of ligand and protein) and irradiated at 254 nm (Model UV G-54 Mineral Light Lamp) at a distance of 5 cm, either at 0° or 22°C. The contents of the cuvette were agitated at 1 min intervals. At various time points aliquots were removed and ethanol (100%) was added to precipitate protein and remove reversibly bound ligand. After three ethanol washes with centrifugation the pellets were solubilized in 0.5 M NaOH and aliquots neutralized with HCl before liquid scintillation counting.

5.3 RESULTS

5.3.1 Detergent Solubilization of AEBS

As the first step towards purification of AEBS, conditions were defined for its detergent solubilization. Because membrane proteins may lose biological activity in the presence of detergents, a range of detergents was evaluated for their ability to solubilize AEBS while retaining [^{3}H]TAM binding activity (Table 5.1). An initial solubilizing detergent concentration of 1% (w/v) was chosen, corresponding to a detergent:membrane protein ratio of 5:1 (w/w). It was found that detergents at concentrations above their critical micellar concentrations interfered with the assay of bound [^{3}H]TAM by preventing the charcoal adsorption of free [^{3}H]TAM, presumably because the unbound ligand was associated with the detergent micelles. Therefore, the solubilization of AEBS using detergents with low critical micellar concentrations (such as Triton-X 100, Brij 35 and 58, and the Calbiochem zwitterionic detergents 3 - 12, 3 - 14 and 3 - 16) was impractical because of the need for excessive sample dilution or detergent removal prior to the assay of AEBS. Such detergents were not tested for their ability to solubilize AEBS at lower detergent concentrations.

Table 5.1 shows that of the 9 detergents tested only the bile salts sodium cholate and sodium deoxycholate were able to solubilize AEBS in an active form. All other detergents either failed to solubilize AEBS and/or caused an apparent destruction of binding activity. [^{3}H]TAM binding to AEBS in cholate or deoxycholate solubilized supernatants could only be detected following partial detergent removal by dilution, that is, in the solubilized state there was an apparent (but reversible) loss of AEBS binding activity. At low cholate concentrations ($\leq$ 0.5% w/v), binding was not significantly affected but at higher

TABLE 5.1 SOLUBILIZATION OF AEBS BY VARIOUS DETERGENTS

Rat liver microsomes were treated at 0 - 4°C for 30 min with 1% (w/v) detergent (unless otherwise stated). After centrifugation at 130,000 x g_{av} for 1 h, the supernatants and resuspended pellets were diluted and assayed for specific [^{3}H]TAM binding by the single saturating dose assay (Section 2.4.3). Results are expressed as % of specific [^{3}H]TAM binding in the original microsomes.

DETERGENT	SPECIFIC BINDING IN SUPERNATANT (%)	SPECIFIC BINDING IN PELLET (%)
Zwittergent 3 - 08	8	38
Zwittergent 3 - 10	0	21
β-D-octyl glucoside	5	0
CHAPS	0	12
CHAPSO	0	N.D.
Digotonin	0	N.D.
Taurodeoxycholic acid	0	17
Sodium deoxycholate (1.5%)	33	2
Sodium cholate (0.6%)	8	N.D.
Sodium cholate (1%)	29	46
Sodium cholate (2%)	61	N.D.

N.D. = not determined.

concentrations affinity for [^{3}H]TAM was rapidly lost, and the apparent binding capacity fell to zero at detergent concentrations > 1% (Figure 5.1). However, binding activity could subsequently be fully restored by reducing cholate concentrations by dilution to < 0.5% (w/v). The dissociation constant for [^{3}H]TAM binding to AEBS was unaffected following this reconstitution of activity.

Although deoxycholate was partially effective at solubilizing AEBS, this detergent was of limited usefulness as, unlike cholate, substantial binding activity was lost during solubilization and the solubilized activity was unstable (75% loss of activity after 2 days at -20°C in the

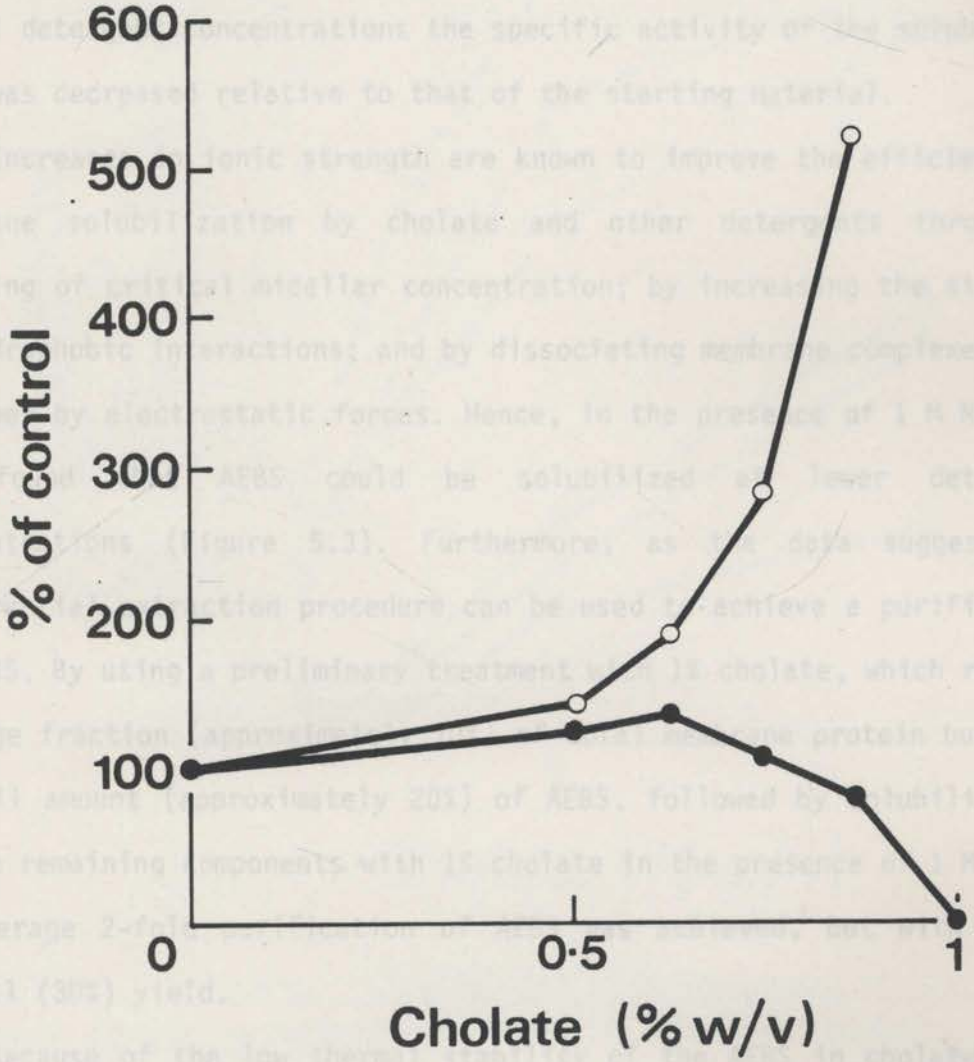

Figure 5.1: Effect of cholate concentration on binding of [^{3}H]TAM to AEBS. Sodium cholate was added in increasing concentrations to rat liver microsomes. The final detergent concentration in the assay system is shown. The K_D (○) and binding capacity (●) were calculated by Scatchard analysis of the binding data for [^{3}H]TAM.

presence of deoxycholate cf. 0 - 20% loss in the presence of cholate). Furthermore, binding activity appeared to be dependent upon the presence of deoxycholate and no activity was apparent when solubilized microsomes were diluted to deoxycholate concentration below 0.08% (w/v).

Figure 5.2 shows the ability of cholate to solubilize AEBS and protein at different concentrations of microsomes. Efficiency of extraction for both decreases with increasing microsomal concentration. At all detergent concentrations the specific activity of the solubilized AEBS was decreased relative to that of the starting material.

Increases in ionic strength are known to improve the efficiency of membrane solubilization by cholate and other detergents through a lowering of critical micellar concentration; by increasing the strength of hydrophobic interactions; and by dissociating membrane complexes held together by electrostatic forces. Hence, in the presence of 1 M NaCl it was found that AEBS could be solubilized at lower detergent concentrations (Figure 5.3). Furthermore, as the data suggests, a differential extraction procedure can be used to achieve a purification of AEBS. By using a preliminary treatment with 1% cholate, which removes a large fraction (approximately 70%) of total membrane protein but only a small amount (approximately 20%) of AEBS, followed by solubilization of the remaining components with 1% cholate in the presence of 1 M NaCl, an average 2-fold purification of AEBS was achieved, but with a low overall (30%) yield.

Because of the low thermal stability of the AEBS in cholate/NaCl-solubilized microsomes (approximately 20% and > 80% loss of activity after 30 min at 22° and 37°C respectively), all solubilization and subsequent procedures were performed at 0 - 4°C (< 20% loss of activity after 24 h at 4°C). Glycerol, useful as a protective agent in the

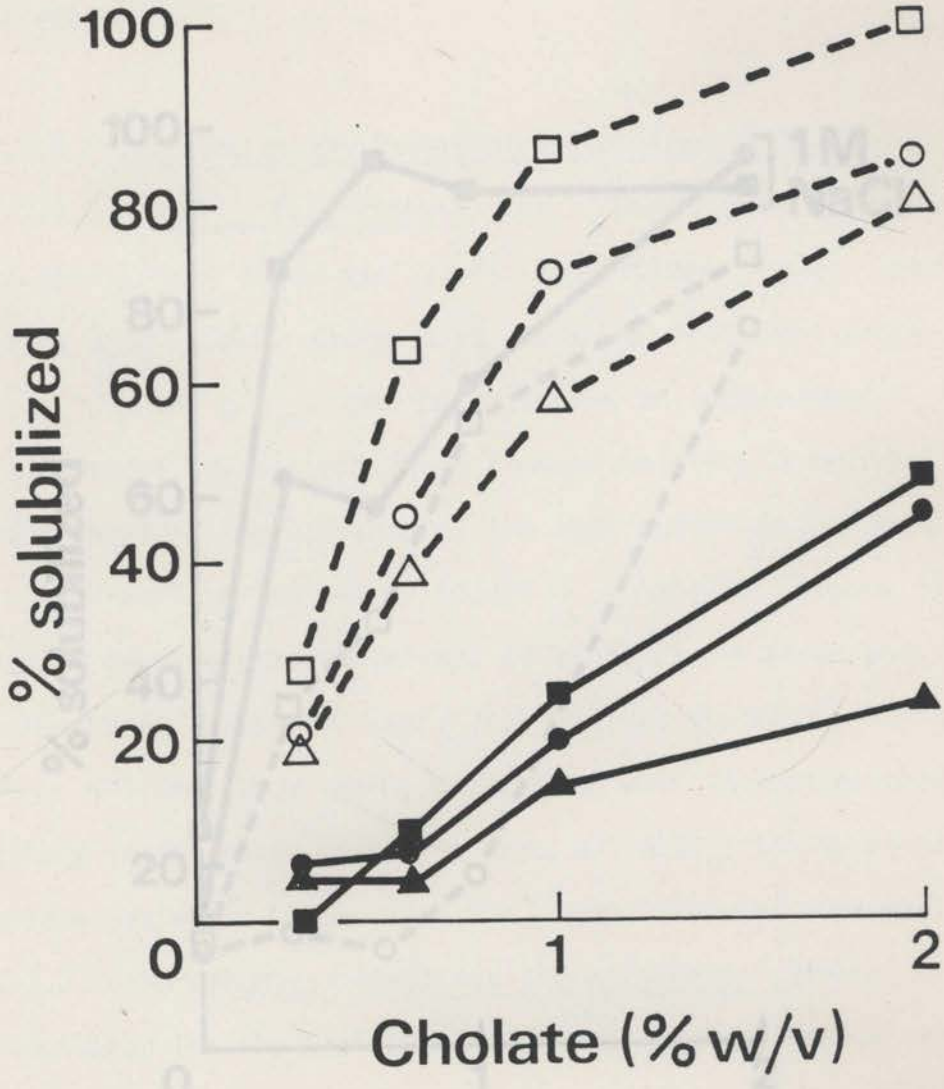

Figure 5.2: Efficiency of cholate solubilization at different microsomal protein concentrations. Total protein (open symbols, dashed lines) and AEBS (closed symbols, solid lines) were measured in 130,000 x g_{av} supernatants after solubilization of microsomes with increasing concentrations of cholate. In this experiment the microsomal protein concentrations were 6.1 mg/ml (△ , ▲) 3.0 mg/ml (○ , ●) and 0.6 mg/ml (□ , ■). Recoveries in the supernatant are expressed as a percentage of the initial quantities of microsomal protein or AEBS.

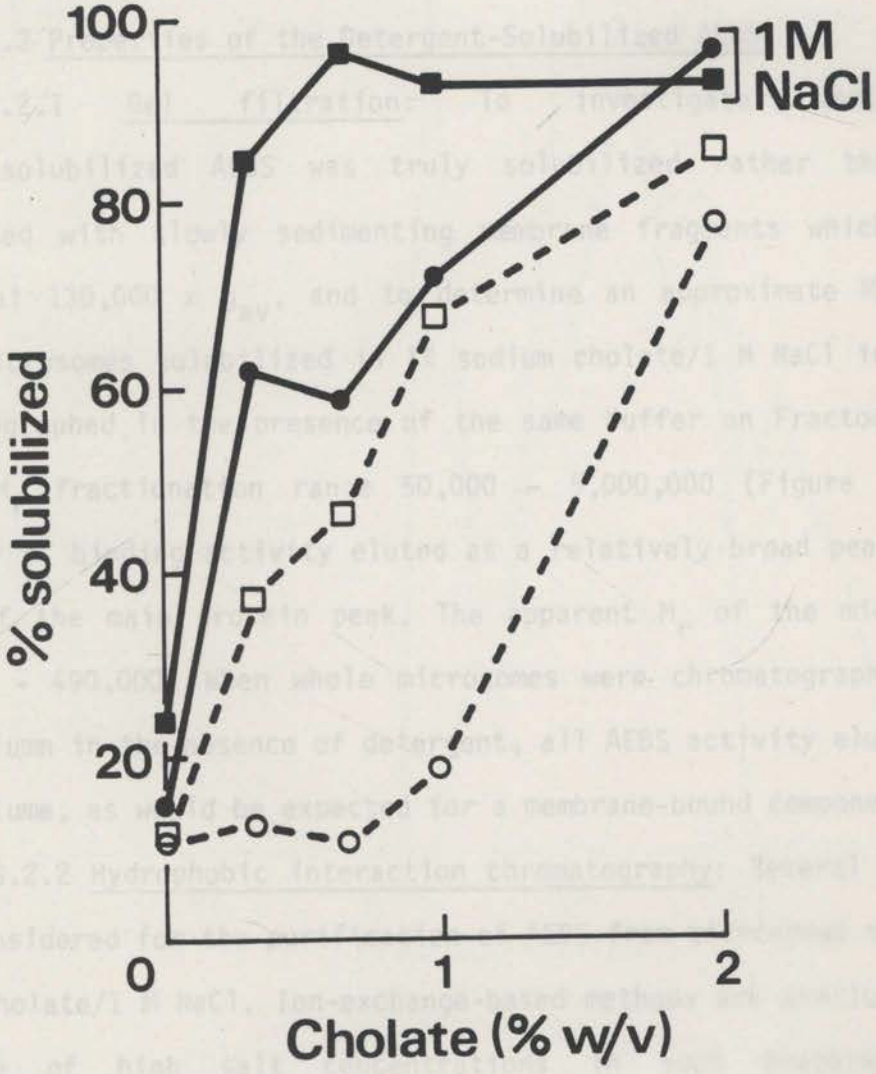

Figure 5.3: Effect of NaCl on cholate solubilization of microsomes. Increasing concentrations of sodium cholate were added to rat liver microsomes (1 mg/ml protein) in the presence (closed symbols) or absence (open symbols) of 1 M NaCl. Solubilized AEBS (○ , ●) and protein (□ , ■) were assayed (as in Sections 2.4.3 and 2.3) in the supernatants after centrifugation at 130,000 x g_{av} for 1 h.

solubilization of several other membrane proteins, had no beneficial effects on the stability of the AEBS. The influence of pH on solubilization could not readily be investigated because of practical limitations imposed by the narrow pH range for [^{3}H]TAM binding to the AEBS (Figure 4.7).

5.3.2 Properties of the Detergent-Solubilized AEBS

5.3.2.1 Gel filtration: To investigate whether the cholate-solubilized AEBS was truly solubilized rather than merely associated with slowly sedimenting membrane fragments which did not pellet at 130,000 x g_{av}, and to determine an approximate M_r for the AEBS, microsomes solubilized in 1% sodium cholate/1 M NaCl in TSK were chromatographed in the presence of the same buffer on Fractogel HW TSK 65 F, M_r fractionation range 50,000 - 5,000,000 (Figure 5.4). The majority of binding activity eluted as a relatively broad peak slightly ahead of the main protein peak. The apparent M_r of the mid-peak was 440,000 - 490,000. When whole microsomes were chromatographed on the same column in the absence of detergent, all AEBS activity eluted at the void volume, as would be expected for a membrane-bound component.

5.3.2.2 Hydrophobic interaction chromatography: Several procedures were considered for the purification of AEBS from microsomes solubilized in 1% cholate/1 M NaCl. Ion-exchange-based methods are precluded by the presence of high salt concentrations in such preparations. An unfortunate consequence of the loss of AEBS binding activity in the presence of detergent (Section 5.3.1) is that binding-site directed affinity chromatography techniques are also not feasible as a means of AEBS purification. Furthermore, although lectin-sugar interactions can take place in the presence of detergents, a variety of lectin-agaroses

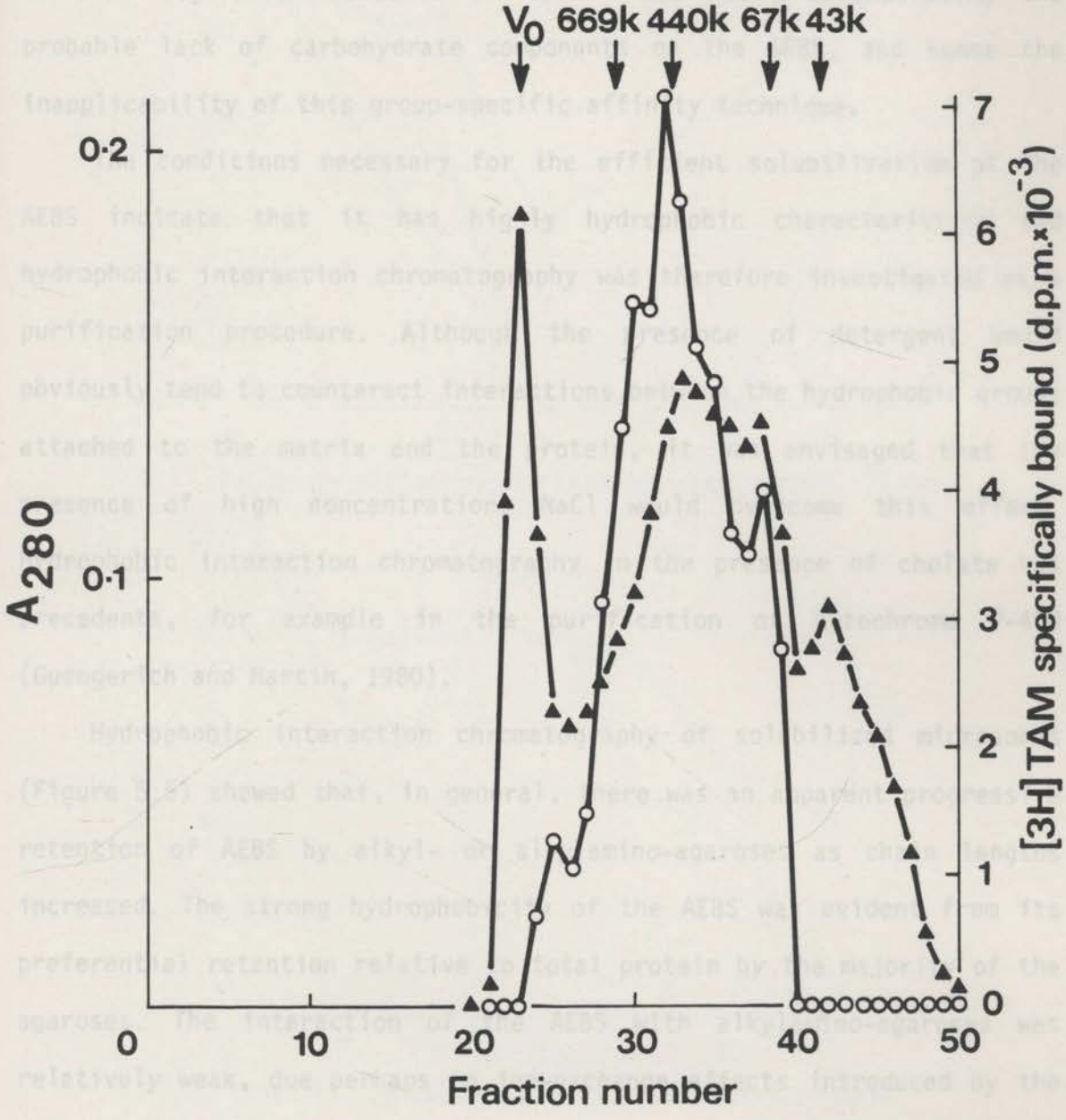

Figure 5.4: Gel filtration of cholate-solubilized AEBS. Rat liver microsomes, solubilized in TSK buffer containing 1% cholate and 1 M NaCl, were chromatographed in the same buffer on a Fractogel HW TSK 65 F column. Fractions were assayed for absorbance at 280 nm (▲) and, after dilution, for specific [^{3}H]TAM binding (O) as described in Section 2.4.3. V_o represents the column void volume (approximate M_r 5 x 10^6). The calibration proteins were thyroglobulin, ferritin, BSA and ovalbumin (M_r 669,000, 440,000, 67,000 and 43,000 respectively).

(concanavalin-A-, castor bean-, lentil-, and wheatgerm-agaroses) failed to bind significant amounts of solubilized AEBS, demonstrating the probable lack of carbohydrate components on the AEBS, and hence the inapplicability of this group-specific affinity technique.

The conditions necessary for the efficient solubilization of the AEBS indicate that it has highly hydrophobic characteristics and hydrophobic interaction chromatography was therefore investigated as a purification procedure. Although the presence of detergent would obviously tend to counteract interactions between the hydrophobic groups attached to the matrix and the protein, it was envisaged that the presence of high concentrations NaCl would overcome this effect. Hydrophobic interaction chromatography in the presence of cholate has precedents, for example in the purification of cytochrome P-450 (Guengerich and Martin, 1980).

Hydrophobic interaction chromatography of solubilized microsomes (Figure 5.5) showed that, in general, there was an apparent progressive retention of AEBS by alkyl- or alkylamino-agaroses as chain lengths increased. The strong hydrophobicity of the AEBS was evident from its preferential retention relative to total protein by the majority of the agaroses. The interaction of the AEBS with alkylamino-agaroses was relatively weak, due perhaps to ion-exchange effects introduced by the terminal amino residue, with consequent weakening of hydrophobic interactions. Chromatography in the presence of 4 M NaCl was necessary to reverse these effects. Little or no activity remained in the eluates from hexyl-, octyl- decyl-, phenyl- or dodecylamino-agarose columns. However, subsequent experiments failed to show that AEBS could be eluted from these five agaroses in an active form. Treatment with buffers containing 5 - 10% cholate with or without 1 M NaCl, or 50% ethylene

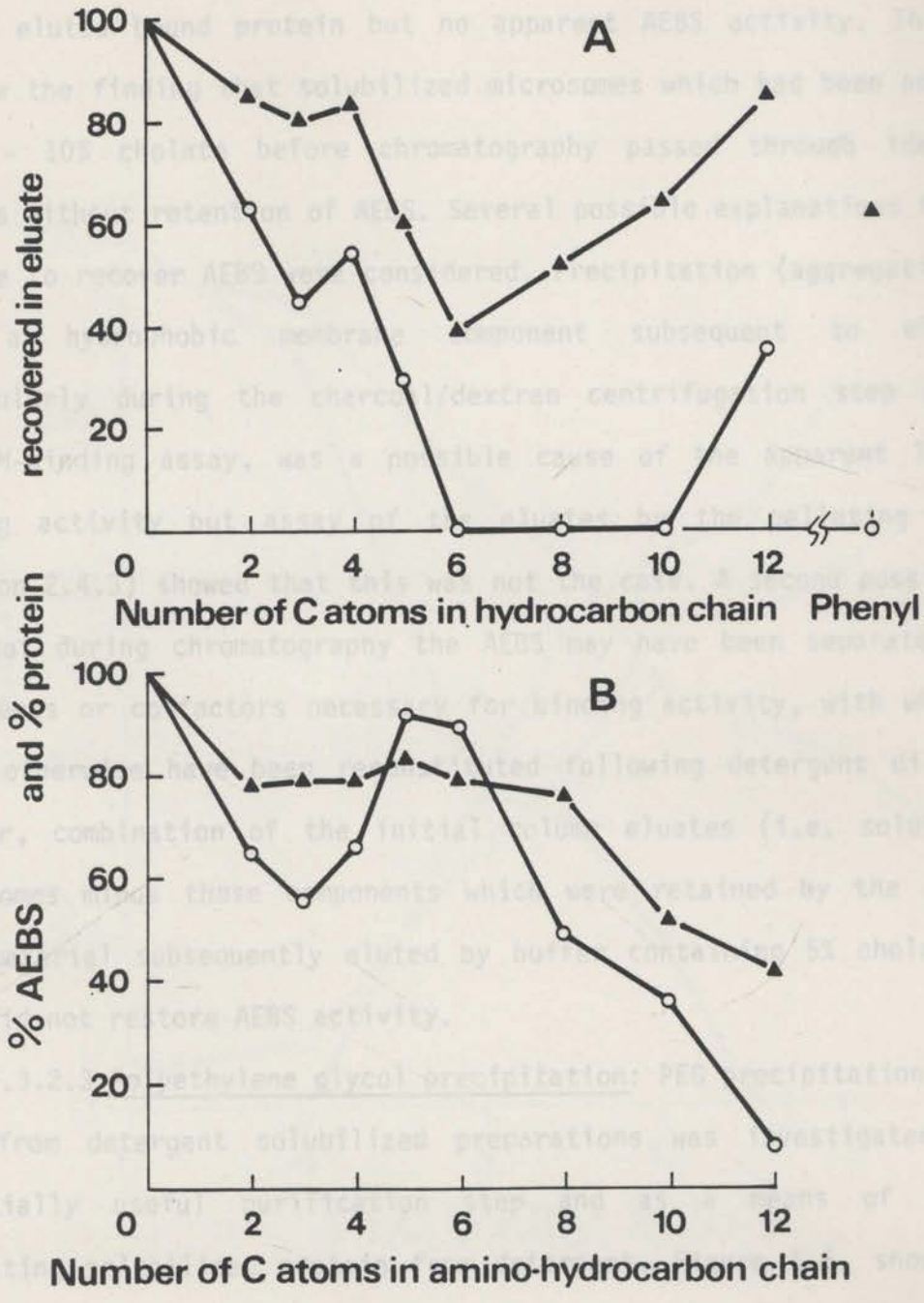

Figure 5.5: Hydrophobic chromatography of detergent solubilized AEBS. Aliquots of rat liver microsomes solubilized with 1% cholate in the presence of 1 M NaCl were passed through columns containing hydrophobic agaroses of increasing hydrocarbon chain length: agarose-NH[CH_2]$_n$$CH_3$ and phenyl-agarose (Figure 5.5A); or agarose -NH[CH_2]$_n$$NH_2$ (Figure 5.5B). For the latter series of agaroses the applied sample was adjusted to 4 M NaCl to increase the strength of the hydrophobic interaction. The eluates were assayed for AEBS (○) and protein (▲) recovery.

glycol eluted bound protein but no apparent AEBS activity. This was despite the finding that solubilized microsomes which had been adjusted to 5 - 10% cholate before chromatography passed through identical columns without retention of AEBS. Several possible explanations for the failure to recover AEBS were considered. Precipitation (aggregation) of such a hydrophobic membrane component subsequent to elution, particularly during the charcoal/dextran centrifugation step of the [^{3}H]TAM-binding assay, was a possible cause of the apparent loss of binding activity but assay of the eluates by the pelleting method (Section 2.4.3) showed that this was not the case. A second possibility was that during chromatography the AEBS may have been separated from components or co-factors necessary for binding activity, with which it would otherwise have been reconstituted following detergent dilution. However, combination of the initial column eluates (i.e. solubilized microsomes minus those components which were retained by the column) with material subsequently eluted by buffer containing 5% cholate/1 M NaCl did not restore AEBS activity.

5.3.2.3 Polyethylene glycol precipitation: PEG precipitation of the AEBS from detergent solubilized preparations was investigated as a potentially useful purification step and as a means of rapidly separating solubilized protein from detergent. Figure 5.6 shows that PEG 6000 completely precipitates the AEBS between 4 - 14% (w/v); that precipitation is selective (approximately 40% of protein remains in solution after complete removal of the AEBS); and that the majority of binding activity is able to be recovered from the pellet (maximum recovery from the 18% pellet), but with only a marginal increase in specific activity (a 4 - 18% cut resulted in an approximate 1.4-fold purification). The pellets were insoluble in TSK buffer but were fully

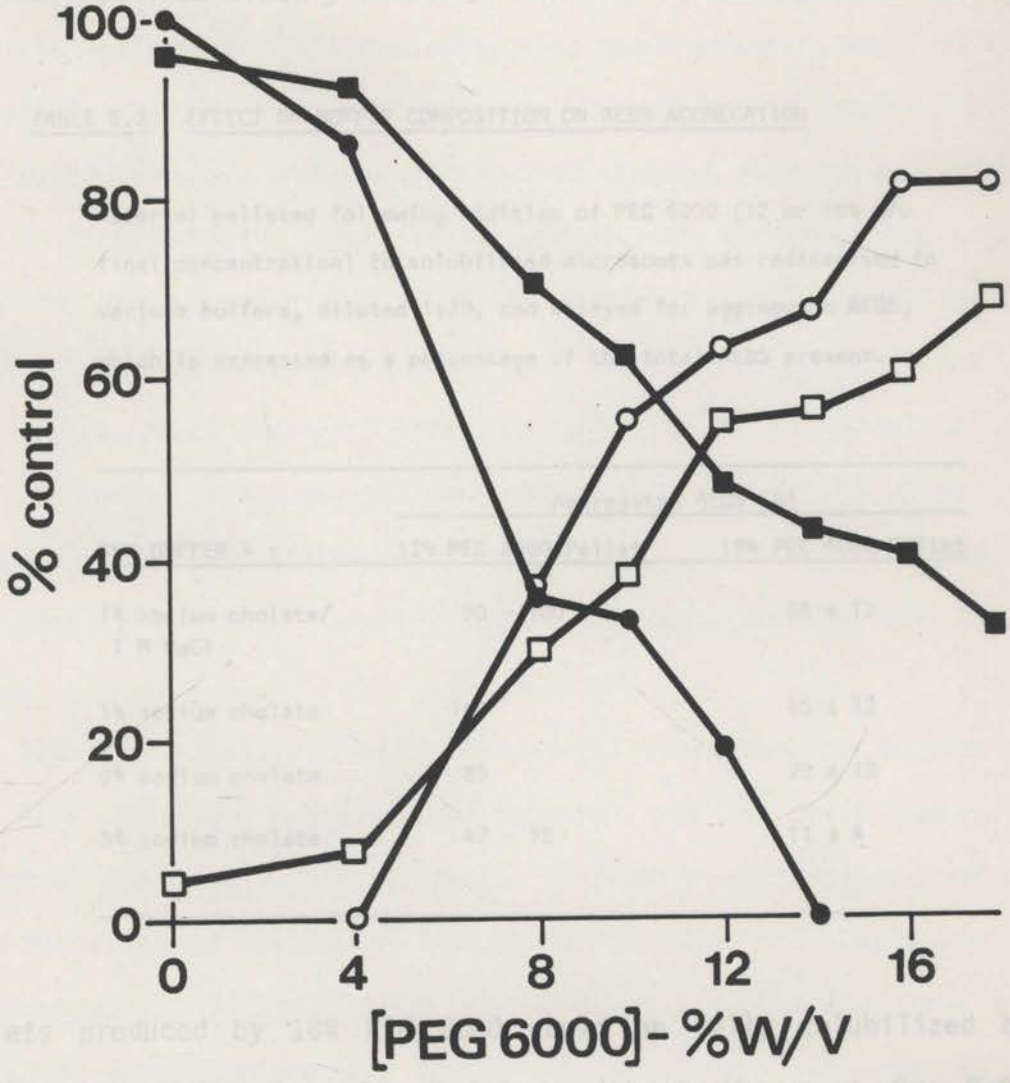

Figure 5.6: Polyethylene glycol precipitation of the AEBS. Microsomes solubilized in TSK containing 1% (w/v) sodium cholate and 1 M NaCl (2.4 mg protein/ml) were treated with increasing concentrations of PEG 6000. After centrifugation the pellets were resuspended in TSK and assayed for protein (□) and AEBS (○ , single saturating dose, pelleting technique, Section 2.4.3). The supernatant was also assayed for AEBS (● , single saturating dose, charcoal technique, Section 2.4.3) and protein (■). Results are expressed as a percentage of the values for the solubilized starting material.

soluble in TSK containing 1% sodium cholate/1 M NaCl, although subsequent dilution to reduce detergent concentration resulted in the majority of AEBS sedimenting during the charcoal assay procedure. This material retained binding activity. Further investigations showed that

TABLE 5.2 EFFECT OF BUFFER COMPOSITION ON AEBS AGGREGATION

Material pelleted following addition of PEG 6000 (12 or 18% w/v final concentration) to solubilized microsomes was redissolved in various buffers, diluted 1:20, and assayed for aggregated AEBS, which is expressed as a percentage of the total AEBS present.

	Aggregated AEBS (%)	
TSK BUFFER + :	12% PEG 6000 Pellet	18% PEG 6000 Pellet
1% sodium cholate/ 1 M NaCl	90 - 100	66 ± 12
1% sodium cholate	100	45 ± 12
2% sodium cholate	85	29 ± 15
5% sodium cholate	47 - 78	11 ± 4

pellets produced by 18% PEG 6000 could be fully solubilized by TSK buffers containing 1 - 5% cholate, without the need for NaCl (in contrast to intact microsomes; Section 5.3.1), and with the added advantage that the AEBS did not aggregate to such a great extent during subsequent dilution and assay (Table 5.2). However, the AEBS in solubilized pellets of material precipitated by 12% PEG 6000 remained prone to aggregation. This suggests that at low concentrations of PEG 6000 the AEBS is separated from components (phospholipids?) necessary to prevent aggregation after detergent removal.

5.3.3. Photoaffinity Labelling

Because of the loss of [^{3}H]TAM binding affinity in the solubilized state and the consequent difficulty in following AEBS through subsequent purification procedures, covalent techniques were investigated as a possible means of providing a label that would identify the site even in procedures causing loss of binding activity. Such a label would be of particular use, for example, in sodium dodecyl sulfate polyacrylamide gel electrophoresis. Tamoxifen aziridine, an electrophilic analogue, which has been successfully used to covalently label the ER (Robertson et al., 1981; Katzenellenbogen et al., 1983b), was initially considered for this purpose. However, covalent labelling of rat liver microsomal AEBS using the tritium labelled compound could not be demonstrated. Monoma et al. (1984) have also reported the same result using MCF 7 microsomal AEBS which, however, could reversibly bind TAM aziridine (RBA = 8%).

The high affinity binding of several phenothiazine derivatives to the AEBS (Section 6.3.8) suggested the use of these compounds as potential photoaffinity labels. Under activating conditions phenothiazines at micromolar concentrations have previously been shown to covalently label calmodulin (Weiss et al., 1980; Prozialeck et al., 1981) and various microsomal membrane-bound enzymes and proteins (Akera and Brody, 1972; Testylier et al., 1984).

Selective labelling of AEBS might be achieved if lower concentrations of ligand were used, and initial experiments gave promising results. Irradiation of rat liver microsomes labelled with 2 nM [^{3}H]fluphenazine for 30 min at 0°C, in the presence of BSA, resulted in up to 40% of bound label being converted to a non-ethanol extractable form, which was presumed to be covalently bound. Time-course studies of

[^{3}H]fluphenazine covalent labelling, in the absence of O_2 in DMF-containing buffer (Figure 5.7A), showed that labelling occurred rapidly in the first five minutes, followed by a more gradual increase. A lower level of covalent binding was found in the absence of BSA.

To determine stability of the AEBS-ligand complex, microsomes prelabelled with [^{3}H]TAM were U.V.-irradiated at 22°C in the presence of DMF. In retrospect, results from this experiment would have been difficult to interpret given the known ability of TAM to undergo U.V.-induced cyclisation to a phenanthrene structure (Adam, 1981), thereby resulting in possible loss of binding affinity for AEBS. However, the experiment led to the completely unexpected finding that a significant fraction of reversibly bound [^{3}H]TAM apparently became covalently bound (Figure 5.7A). A similar degree of covalent labelling was observed in the presence of BSA at 0°C (Figure 5.7B), although the rate of formation of the covalent complex was decreased. At 22°C (Figure 5.7B) this rate was increased, although the AEBS in the absence of ligand was correspondingly less stable to U.V.-irradiation at this temperature (Figure 5.7B).

In these experiments [^{3}H]TAM specifically bound to AEBS represented 72 - 74% of total bound ligand. Parallel experiments in which 100 nM unlabelled TAM was present showed that non-specifically bound [^{3}H]TAM was also able to form an irreversible label and at a similar rate to total bound [^{3}H]TAM.

Figure 5.7 shows that the degree of covalent labelling is somewhat greater in the presence of BSA compared to DMF. BSA is a useful scavenger molecule during photoaffinity labelling procedures, reacting at multiple sites with dissociated or excess photolabel and hence preventing non-specific reactions on membrane sites (Ruoho et al.,

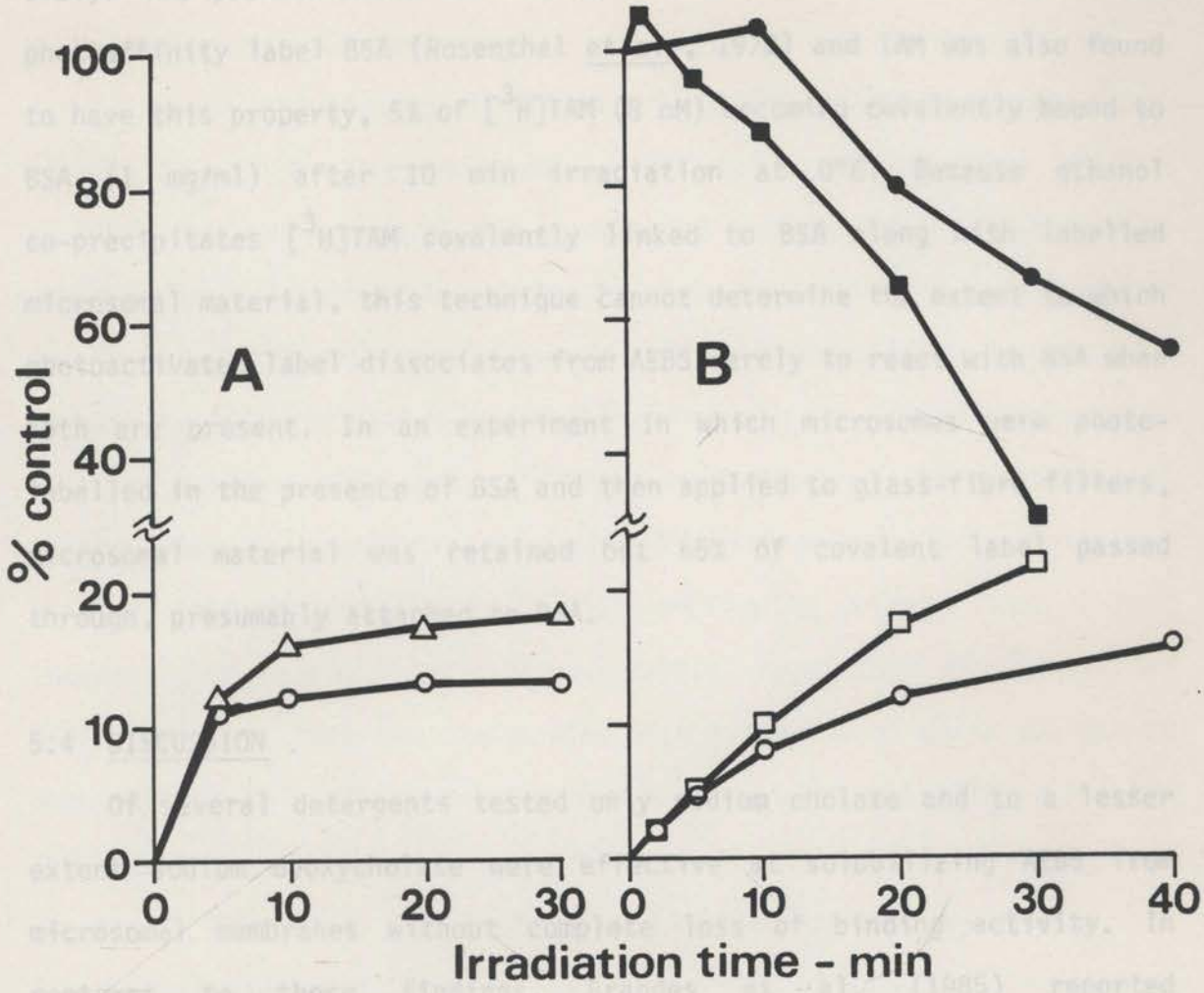

Figure 5.7: Photo-labelling of rat liver microsomes with [^{3}H]fluphenazine and [^{3}H]TAM. (A) Rat liver microsomes in TSK buffer containing 5% DMF were labelled with 2 nM [^{3}H]fluphenazine (△) or [^{3}H]TAM (○). After charcoal/dextran removal of unbound ligand, N_2 was bubbled through the labelled microsomal suspensions which were then irradiated at 0°C as described in Section 5.2.5. Covalent binding determined after ethanol extraction is expressed as a percentage of the total binding in the prelabelled non-irradiated microsomes. (B) Microsomes were labelled with [^{3}H]TAM and irradiated as above, but in the presence of BSA instead of DMF and without N_2 gassing, at 0°C (○) or 22°C (□). AEBS stability at 0°C (●) or 22°C (■) was determined by irradiation of unlabelled microsomal suspensions followed by assay for AEBS (single saturating dose technique). AEBS content is expressed as a percentage of that in non-irradiated microsomes.

1984). The phenothiazine derivative chlorpromazine has been shown to photoaffinity label BSA (Rosenthal et al., 1978) and TAM was also found to have this property, 5% of [^{3}H]TAM (8 nM) becoming covalently bound to BSA (1 mg/ml) after 10 min irradiation at 0°C. Because ethanol co-precipitates [^{3}H]TAM covalently linked to BSA along with labelled microsomal material, this technique cannot determine the extent to which photoactivated label dissociates from AEBS merely to react with BSA when both are present. In an experiment in which microsomes were photo-labelled in the presence of BSA and then applied to glass-fibre filters, microsomal material was retained but 65% of covalent label passed through, presumably attached to BSA.

5.4 DISCUSSION

Of several detergents tested only sodium cholate and to a lesser extent sodium deoxycholate were effective at solubilizing AEBS from microsomal membranes without complete loss of binding activity. In contrast to these findings, Brandes et al. (1985) reported solubilization of the AEBS using 1% digitonin but no characterization of the solubilized material was reported. Solubilization of AEBS was efficient only at high cholate and salt concentrations, demonstrating that the AEBS is probably a strongly bound and hydrophobic membrane component. Gel filtration of cholate-solubilized microsomes showed that the AEBS is in a truly soluble form and not associated with membrane fragments, which would cause binding activity to appear at the column void volume. Whether the peak of activity at M_r 440,000 - 490,000 represents the native AEBS-detergent complex, or a complex with undissociated membrane lipid or protein components requires further investigation. The broad appearance of the peak of activity is quite

likely a consequence of both the high flow rate used and of detergent-protein interactions.

Although solubilized AEBS lost [^{3}H]TAM binding activity, this activity could be reconstituted by detergent removal. This finding suggests that binding is dependent upon the association of the AEBS with other membrane components, which is disrupted in the presence of detergent. Detergent removal would allow reassociation with these components. Alternatively, reversible binding site disaggregation into inactive subunits might occur. Inhibition of binding sites by detergent through a direct competitive mechanism does not appear likely given that binding activity is completely lost following a less than 2-fold increase in cholate concentration. Partial separation of solubilized AEBS from other membrane components, with retention of activity, can be demonstrated by differential detergent extraction, gel filtration, and PEG 6000 precipitation, although following the latter procedure the AEBS is in an insoluble and presumably aggregated form, except in the presence of detergent. However, the requirement of detergent for binding activity following deoxycholate solubilization suggests that this detergent has replaced membrane components that may normally be associated with the functional AEBS and which cholate, by implication, either fails to remove or else allows to reassociate.

The observation that [^{3}H]TAM binding activity was not recovered from hydrophobic agarose columns under conditions where it would be expected to elute could also be explained if the AEBS and other components necessary for binding activity or stability had been separated during chromatography. However, in this case attempts to reconstitute activity failed under conditions where separated membrane components were recombined. Although restoration of activity by

reconstitution into artificial membranes might be a useful approach, it seems more likely that binding of the AEBS to the hydrophobic matrix either results in denaturation of the protein or else produces an interaction so strong as to be essentially irreversible under standard elution conditions.

These studies provide only limited information as to the identity of membrane components necessary for AEBS activity/stability. It is likely that lipids are important given that the actions of lipase and phospholipase C abolish binding activity (Sections 3.3.4, 4.3.4), although this could alternatively be due to an indirect inhibitory effect of free fatty acids (produced in the case of phospholipase C by the subsequent action of endogenous endoplasmic reticulum lipases). Membrane proteins may depend upon the presence of lipids for biological activity by a number of mechanisms. Specific lipids may be required as co-factors or, by forming a single bi-layer shell around the hydrophobic region of the protein, act in the maintenance of protein conformation. More general effects of lipid composition on membrane fluidity have also been observed with consequent influence on: protein mobility, necessary for the formation of the transition state; membrane permeability and hence the access of substrates and ligands; and protein conformation which in turn influences specificity and affinity. The relevance of these effects to the AEBS is unknown, although it should be noted that the linearity of the relationship between kinetic rate constants and K_D and temperature between 4° - 37°C (Figure 3.7) shows that the AEBS is not responsive to the changes in membrane fluidity that occur upon lipid liquid-crystalline phase transitions within this temperature range.

The preliminary results obtained suggest that true photoaffinity labelling of microsomal AEBS by TAM and fluphenazine is unlikely to be

occurring to any great extent. The high levels of covalent label that become attached to BSA when labelled microsomes and BSA are irradiated together shows that the bulk of photo-labelled dissociates from the AEBS. Labelling of AEBS might still occur through a pseudophotoaffinity mechanism by which dissociated label reassociates with AEBS and then reacts covalently. Further investigation is required to directly demonstrate that the AEBS can be irreversibly labelled.

The mechanisms by which U.V.-irradiated TAM (or indeed fluphenazine) covalently label microsomal material or BSA are unclear. Presumably, in the case of TAM it could involve some reactive intermediate which is formed during rearrangement to the phenanthrene structure. Alternatively, it could involve the U.V.-induced formation of reactive side chain cleavage products containing the tritium label. Such products would rapidly dissociate from the AEBS.

These studies have demonstrated the successful detergent solubilization of the rat liver microsomal AEBS as a first step towards its purification. Properties of the solubilized protein have been defined and several potentially useful purification procedures investigated. Further purification strategies are considered in Chapter 8.

CHAPTER SIX

BINDING OF TRIPHENYLETHYLENES AND STRUCTURALLY RELATED COMPOUNDS TO THE ANTIOESTROGEN BINDING SITE

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6.1 INTRODUCTION

The structure-affinity studies reported in this Chapter were undertaken to develop an understanding of the structural requirements for binding to AEBS. Previously, such studies were limited to the triphenylethylene-related antioestrogens. TAM, 4-hydroxyTAM, cis-TAM (ICI 47699), nafoxidine (U 11,100A) and CI 628 were shown by Sutherland et al. (1980) to bind with high affinity to AEBS in MCF 7 cytosol whereas N-desmethylTAM had significantly lower affinity. Androgens (testosterone, 5α-dihydrotestosterone, R 1881); oestrogens (E_1, E_2, E_3); and progestins (progesterone, R 5020) had no affinity. Gulino and Pasqualini (1980) and Faye et al. (1980) confirmed these results for TAM, CI 628, nafoxidine and a variety of steroids in guinea pig and rat uterine cytosol. The cis-isomers of enclomiphene (zuclomiphene) and TAM were found to bind with lower affinity than the corresponding trans-isomers in MCF 7 cytosol (Murphy et al., 1981). The importance of side chain structure as a determinant of binding affinity was established by Murphy and Sutherland (1981a) using a small series of clomiphene derivatives. The presence of an alkylamino side chain was shown to be an absolute requirement for binding to AEBS. Changes to the amino substituents, alkyl chain length or linkage to the aromatic portion of the molecule all resulted in altered affinity. Gulino and Pasqualini (1982) reported the binding affinities of several TAM metabolites for AEBS in guinea pig uterine cytosol, and also showed that DES, cortisol and RU 16117 (a synthetic oestrogen with antioestrogenic properties) had no affinity.

These early observations that the AEBS could interact with triphenylethylene antioestrogens but not natural or synthetic oestrogens suggested the possibility that the AEBS might play a role in mediating

antioestrogenic activity. Although it is generally accepted that antioestrogens exert their effects through interaction with the ER, several features of their action remain unexplained i.e. the wide species and tissue variation in the properties of these drugs; the lack of correlation between activity (i.e. expression of agonist/antagonist properties) and binding affinity for ER; and oestrogen-irreversible (non-ER mediated?) activities *in vivo* (Martin, 1980, 1981), and *in vitro* (Murphy and Sutherland, 1983a; Sutherland *et al.*, 1983b). Further information was therefore required to clarify the significance of the AEBS in the mechanism of antioestrogen action, and as a prelude more detailed information on structure-affinity relationships was essential to a deeper understanding of the ligand specificity, and role of AEBS (See Section 8.2).

In this study the binding properties of a wide range of synthetic nonsteroidal antioestrogens (including analogues of TAM, clomiphene, CI 628, cyclofenil, and bibenzyl and stilbene derivatives) and structurally related compounds (including analogues of the cytochrome P-450 inhibitor SKF-525A and phenothiazine derivatives) were tested. In contrast to previously published studies, the microsomal fraction was used as a source of AEBS rather than cytosol. Furthermore, the properties of rat liver and MCF 7 AEBS were compared to assess any possible species/tissue differences. It was also intended that information obtained with respect to MCF 7 AEBS would aid in the selection of compounds useful for *in vitro* studies of the functional significance of AEBS in the control of breast cancer cell growth (Chapter 7). For selected compounds binding affinity for ER was determined using receptor extracted from MCF 7 cell nuclei.

A further aspect of this study was to determine whether binding to

the AEBS by compounds with a variety of pharmacological activities, e.g. inhibitors of calmodulin and cytochrome P-450, and dopamine-, serotonin-, histamine-, adrenergic- and cholinergic-receptor antagonists, might indicate that the AEBS had identity with known receptors or binding sites for these compounds.

6.2 METHODS

6.2.1 Materials

Analogues of TAM, clomiphene, hydroxyclomiphene (PR series), CI 628, SKF-525A, cyclofenil, and bibenzyl and stilbene derivatives (H compounds) and other compounds were obtained as described in Section 2.1.3. Ethanol and aqueous stock solutions were prepared as described in the same section. The structures of these compounds are given in Figure 6.1 and Tables 6.1 - 6.14.

6.2.2 Determination of Relative Binding Affinities

Relative binding affinities (RBA) for AEBS were determined in the presence of E_2 as described in Section 2.4.5 using either KCl-washed rat liver microsomes (Section 2.2.3.1) or MCF 7 PMF (Section 2.2.2.2). RBA for ER was determined using a nuclear extract from MCF 7 cells (Section 2.2.2.4).

6.3 RESULTS AND DISCUSSION

Figures 6.2 and 6.3 show representative competitive binding curves for a series of compounds binding to AEBS in rat liver microsomes and MCF 7 PMF respectively. All compounds which competed for specifically bound [^{3}H]TAM did so to the same extent as unlabelled TAM, with the exception of compounds of very low affinity which did not fully displace

[^{3}H]TAM at concentrations of 10 μM. Higher concentrations were not able to be tested because this was at the limit of solubility of these compounds.

6.3.1 TAM Analogues

RBA of a series of TAM analogues for MCF 7 and rat liver AEBS have been presented and discussed in previous chapters (Sections 3.3.5 and 4.5.6, respectively). These results have been brought together in Table 6.1. The side chain structures of several TAM analogues with no affinity for AEBS are shown in Table 6.2.

6.3.2 Clomiphene Analogues

RBA of a series of clomiphene analogues for rat liver and MCF 7 AEBS are shown in Table 6.3. The data for the MCF 7 microsomal AEBS are very similar to those of Murphy and Sutherland (1981a) for the AEBS present in the cytosol fraction of MCF 7 cells, supporting the conclusion that the microsomal and cytosolic sites are, in fact, the same entity.

For clomiphene analogues an alkyl amino side chain is necessary for binding to AEBS. An increase in alkyl chain length (19 vs 22) resulted in an approximate 70% decrease in affinity. Enclomiphene (19) with ethyl amino substituents had higher affinity than compound 21 with propyl amino substituents. Compound 24 with a secondary amino terminal group also had reduced affinity. H or OCH_3 substituents at position R_4 (Figure 6.1) resulted in loss of detectable affinity for AEBS (TACE, 2014, 8753, Table 6.2). An amine linkage between the side chain and the triphenylethylene portion of the molecule caused a large reduction in affinity for MCF 7 AEBS but only a minor reduction for rat liver AEBS (19 vs 23).

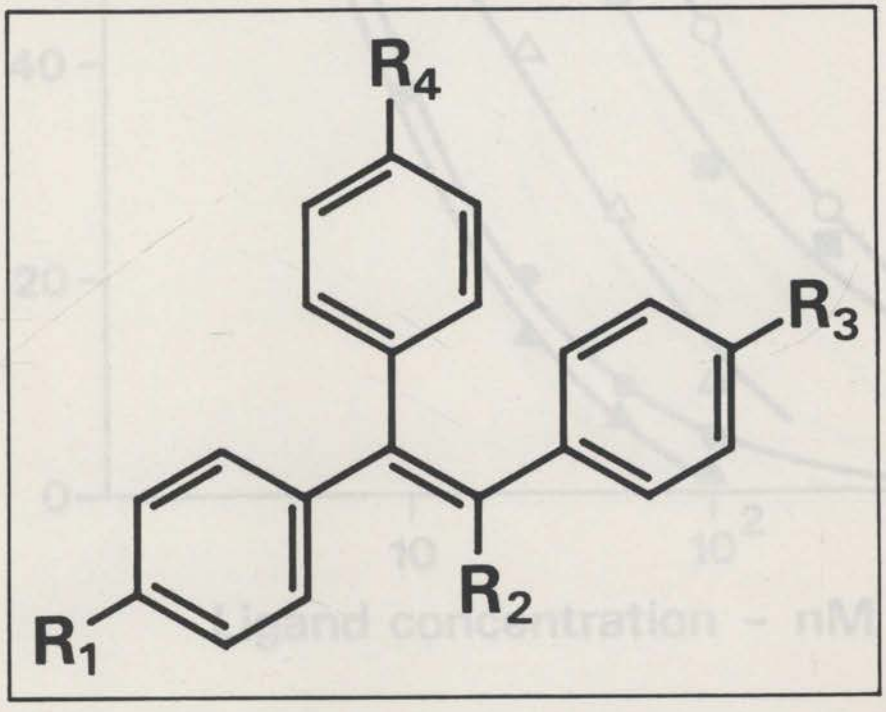


Figure 6.1: Generalized structure of substituted triphenylethylenes.

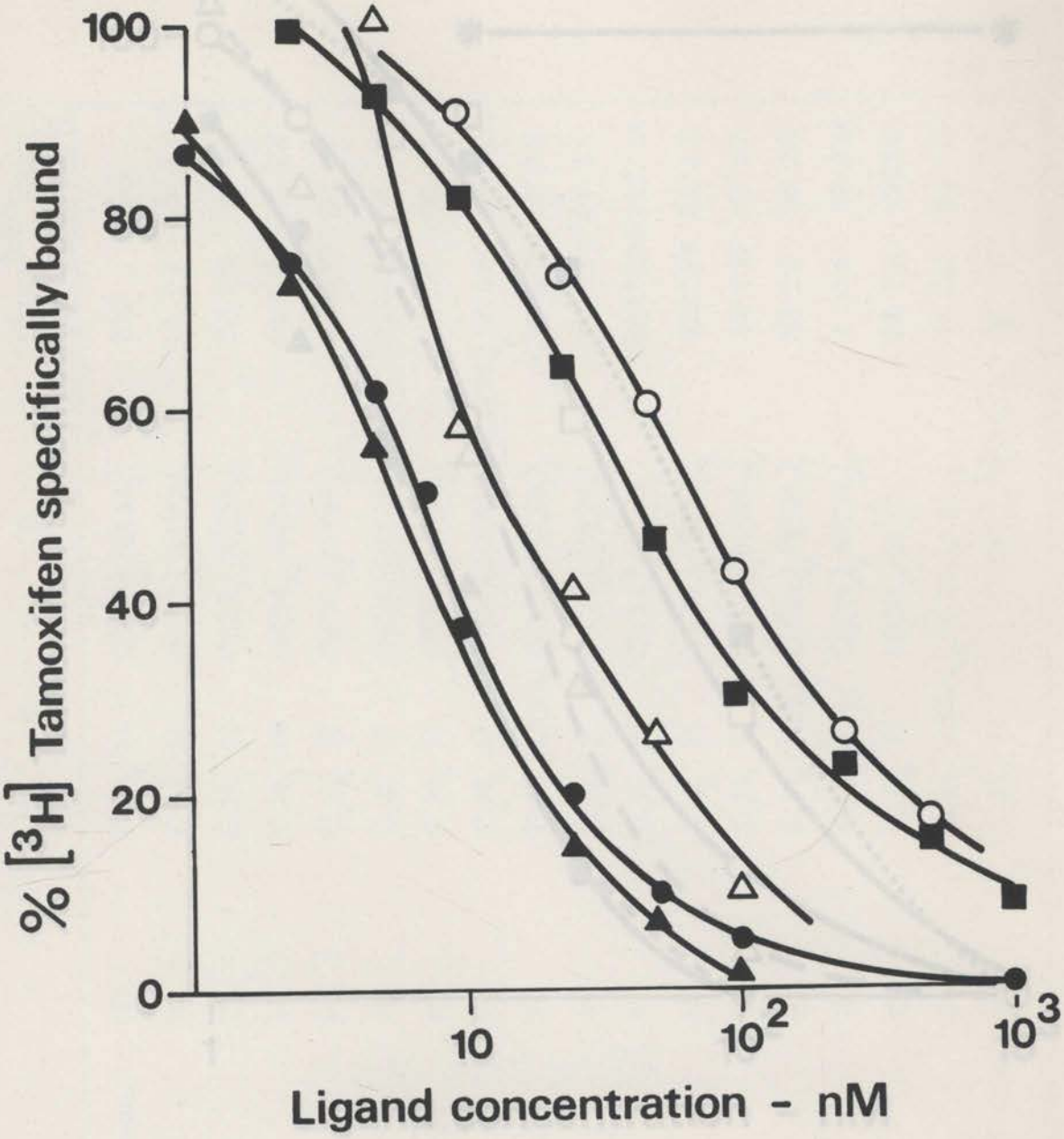


Figure 6.2: Competitive binding assay for the rat liver microsomal AEBS. Rat liver microsomes were labelled with 5 nM [^{3}H]TAM together with increasing concentrations of unlabelled ligands as described in Section 2.4.5. Data are the amount of [^{3}H]TAM specifically bound (expressed as a percentage of the total specifically bound [^{3}H]TAM in the absence of added ligand) vs the concentration of added ligand. TAM (●), N-desmethylTAM (○), 4-hydroxyTAM (■), enclomiphene (▲), 9599 (△).

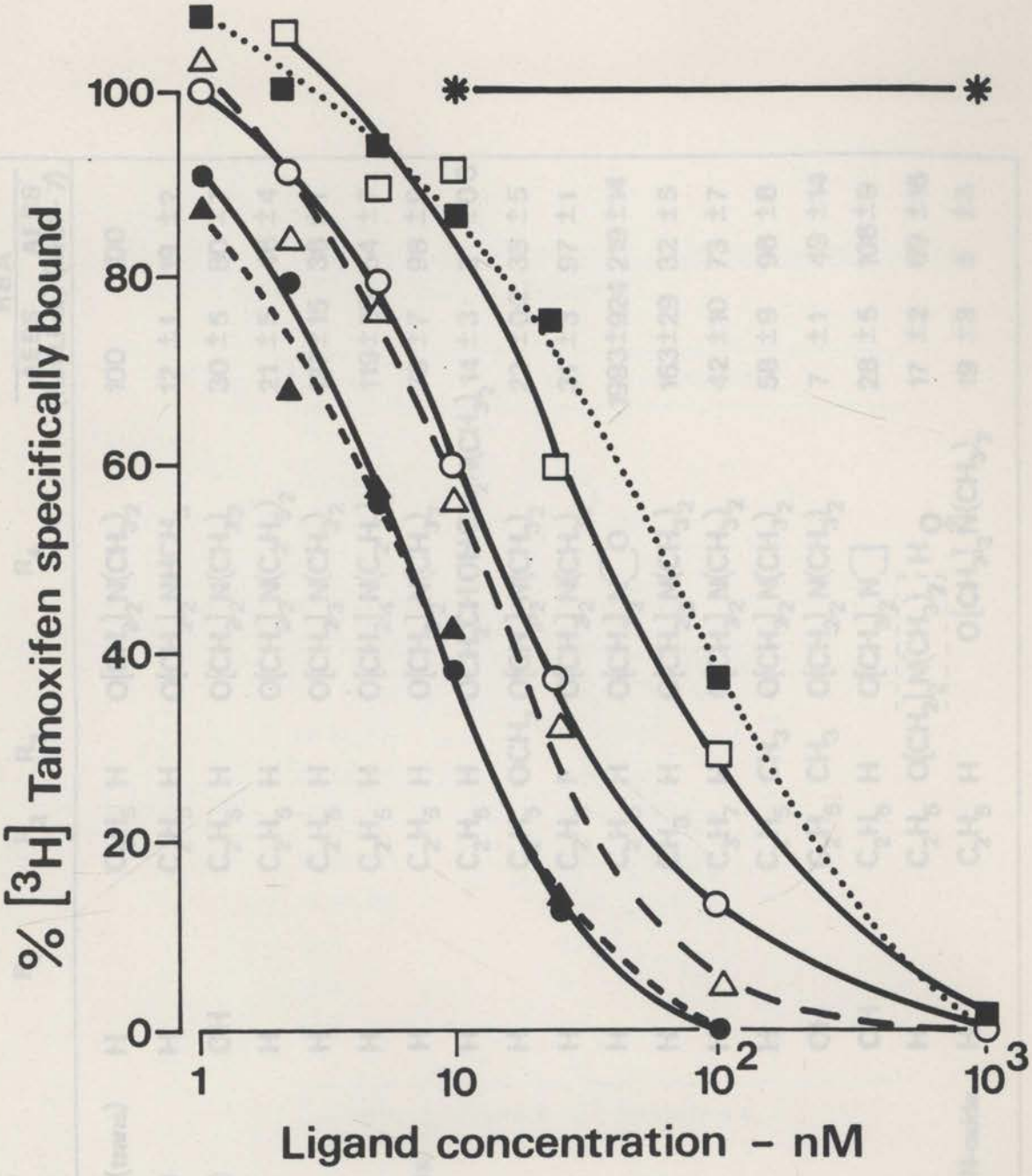


Figure 6.3: Competitive binding assay for the MCF 7 microsomal AEBS. MCF 7 PMF was labelled with 5 nM [^{3}H]TAM together with increasing concentrations of unlabelled ligands as described in Section 2.4.5. Data are the amount of [^{3}H]TAM specifically bound (expressed as a percentage of the total specifically bound [^{3}H]TAM in the absence of added ligand) vs the concentration of added ligand. TAM (●), enclomiphene (▲), zuclomiphene (○), 6866 (△), 10222 (□), desmethylTAM (■) TACE, and steroidal progestins, oestrogens, androgens (✱).

TABLE 6.1 RELATIVE BINDING AFFINITIES OF TAM ANALOGUES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS[1]

Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
1	Tamoxifen (trans)	H	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	100	100
2	55,548(a)	H	C_2H_5	H	$O[CH_2]_2NHCH_3$	12 ±1	19 ±2
3	79,280(b)	OH	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	30 ±5	80 ±7
4	45,960	H	C_2H_5	H	$O[CH_2]_2N(C_2H_5)_2$	21 ±5	46 ±4
5	47,108	H	C_2H_5	H	$O[CH_2]_3N(CH_3)_2$	127±15	36 ±1
6	47,590	H	C_2H_5	H	$O[CH_2]_4N(C_2H_5)_2$	119±26	54 ±7
7	47,699 (cis)	H	C_2H_5	H	$O[CH_2]_2N(CH_3)_2$	73 ±7	98 ±9
8	94,230	H	C_2H_5	H	$OCH_2CHOHCH_2N(CH_3)_2$	14 ±3	2 ±0·3
9	132,802	H	C_2H_5	OCH_3	$O[CH_2]_2N(CH_3)_2$	22 ±0·2	35 ±5
10	148,067	H	C_2H_5	F	$O[CH_2]_2N(CH_3)_2$	31 ±3	97 ±1
11	47,399	H	C_2H_5	H	$O[CH_2]_2N$ (morpholino)	1993±924	219±14
12	46,414	H	CH_3	H	$O[CH_2]_3N(CH_3)_2$	163±29	32 ±5
13	49,500	H	C_3H_7	H	$O[CH_2]_2N(CH_3)_2$	42 ±10	73 ±7
14	133,312	H	C_2H_5	CH_3	$O[CH_2]_2N(CH_3)_2$	58 ±9	96 ±6
15	129,817	OH	C_2H_5	CH_3	$O[CH_2]_2N(CH_3)_2$	7 ±1	49 ±14
16	129,351	OH	C_2H_5	H	$O[CH_2]_2N$ (pyrrolidino)	28 ±5	106±9
17	133,055	H	C_2H_5	$O[CH_2]_2N(CH_3)_2$	H O	17 ±2	69 ±16
18	Tamoxifen N-oxide	H	C_2H_5	H	$O[CH_2]_2\overset{\uparrow}{N}(CH_3)_2$	19 ±3	5 ±3

[1] R_{1-4} refer to Figure 6.1; [a] N-desmethylTAM; [b] 4-hydroxyTAM.

TABLE 6.2 SIDE CHAIN STRUCTURES OF TAM (ICI), CI 628 (CN) AND ENCLOMIPHENE ANALOGUES WITH NO AFFINITY FOR AEBS

COMPOUND	SIDE CHAIN STRUCTURE[a]
ICI 90069	OCH_3
ICI 140447	OCH_2CHCH_2
ICI 141389[b]	OH
ICI 145458	$OCH(CH_3)CHCH_2$
ICI 145663	$OCH(CH_3)CH(OH)CH_2OH$
ICI 145680	$OCH_2CH(OH)CH(CH_3)OH$
CN 59,786	$O[CH_2]_2Cl$
CN 62,883	$O[CH_2]_2N_3$
CN 64,223	$O[CH_2]_2$-c-NC_4H_4
CN 65,122	$O[CH_2]_2$-c-C_5H_9
CN 65,581	$O[CH_2]_3CH(CH_3)_2$
TACE	OCH_3
2014	H
8753	OCH_3

[a] R_4 refer to Figure 6.1; [b] Metabolite E.

TABLE 6.3 RELATIVE BINDING AFFINITIES OF ENCLOMIPHENE ANALOGUES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS[1]

Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA AEBS (Rat Liver)	RBA AEBS[a] (MCF-7)
19	Enclomiphene (trans)	H	Cl	H	$O[CH_2]_2N(C_2H_5)_2$	186±25	142±5
20	Zuclomiphene (cis)	H	Cl	H	$O[CH_2]_2N(C_2H_5)_2$	43 ±8	71 ±7
21	8,650	H	Cl	H	$O[CH_2]_2N(C_3H_7)_2$	21 ±5	9 ±1
22	6,866	H	Cl	H	$O[CH_2]_3N(C_2H_5)_2$	45 ±5	44 ±5
23	10,222	H	Cl	H	$NH[CH_2]_2N(C_2H_5)_2$	131±13	15 ±1
24	9,599	H	Cl	H	$O[CH_2]_2NHC_2H_5$	52 ±5	37 ±2

[1] R_{1-4} refer to Figure 6.1; [a] from Murphy and Sutherland (1983a).

Cis-/trans-isomerism about the triphenylethylene double bond resulted in reduced affinity for the cis-isomer (19 vs 20). RBA of clomiphene analogues for ER are shown in Table 6.4.

TABLE 6.4 RELATIVE BINDING AFFINITIES OF TAM AND CLOMIPHENE ANALOGUES AND E_2 FOR MCF 7 AEBS AND ER

COMPOUND	TABLE	RBA (%) AEBS	RBA (%) ER
TAM	6.1	100	1.3 ± 0.3
cis-TAM	6.1	98	0.05
4-hydroxyTAM	6.1	80 ± 7	41 ± 3
N-desmethylTAM	6.1	19 ± 2	2 ± 1
Enclomiphene	6.2	142 ± 5	2
Zuclomiphene	6.2	71 ± 7	0.05
6866	6.2	44 ± 5	6
9599	6.2	37 ± 2	0.7
10222	6.2	15 ± 1	5
E_2	-	0	100
Metabolite E	6.8	0	0.8 ± 0.1
ICI 145680	6.8	0	1.4 ± 0.3

6.3.3. CI 628 Analogues

RBA of CI 628 analogues for rat liver and MCF 7 AEBS are shown in Tables 6.5A, B, C. The order of binding affinity as determined by the terminal amino group structure was: -c-$N(CH_2CH_2)_2CH_2$ (piperidino-) > -c-NC_4H_8 (pyrollidino) > $-N(C_2H_5)_2$ > $-N(CH_3)_2$ > $-NHCH_3$ > -c-NC_3NH_3 (imidazole) > -c-NC_2H_4 (aziridine) (28 vs 27 vs 26 vs 25 vs 29; 33 vs 38; 37 vs 30 vs 36; 44 vs 31; 55 vs 51 vs 46).

The effect of hydrocarbon side chain length on binding affinity appeared to depend upon the terminal amino group structure. For compounds with ether-linked side chains terminating in dimethylamino groups the order of binding affinity as determined by the indicated number of side chain methylene units was 3 > 4 > 5 = 6 > 2 in the case

TABLE 6.5A RELATIVE BINDING AFFINITIES OF CI 628 ANALOGUES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS[1]

Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
25	65,071	OCH_3	NO_2	H	$O[CH_2]_2N(CH_3)_2$	5 ±0·2	18 ±0·5
26	55,325	OCH_3	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	35 ±4	38 ±3
27	CI628	OCH_3	NO_2	H	$O[CH_2]_2$-pyrrolidine	51 ±4	82 ±9
28	56,295	OCH_3	NO_2	H	$O[CH_2]_2$-piperidine	95 ±13	140 ±7
29	63,356	OCH_3	NO_2	H	$O[CH_2]_2$-imidazole	5 ±0·3	
30	62,501	OCH_3	NO_2	H	$O[CH_2]_3N(CH_3)_2$	32 ±3	21 ±3
31	64,983	OCH_3	NO_2	H	$O[CH_2]_4N(CH_3)_2$	12 ±2	15 ±1
32	64,984	OCH_3	NO_2	H	$O[CH_2]_5N(CH_3)_2$	12 ±0·1	10 ±0·5
33	65,070	OCH_3	NO_2	H	$O[CH_2]_6N(CH_3)_2$	19 ±3	11 ±3
34	66,640	OCH_3	NO_2	H	$OCH(CH_3)CH_2N(CH_3)_2$	33 ±4	23 ±3
35	61,665	OCH_3	NO_2	H	$OCH_2C(CH_3)_2N(CH_3)_2$	107 ±2	134 ±12
36	63,557	OCH_3	NO_2	H	$O[CH_2]_3NHCH_3$	6 ±0·5	
37	57,788	OCH_3	NO_2	H	$O[CH_2]_3$-pyrrolidine	57 ±12	

[1] R_{1-4} refer to Figure 6.1.

TABLE 6.5B RELATIVE BINDING AFFINITIES OF CI 628 ANALOGUES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS[1]

Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
38	66,419	OCH_3	NO_2	H	$O[CH_2]_6N$⟨⟩	0·8 ±0·3	
39	65,470	OCH_3	NO_2	H	$S[CH_2]_2N(C_2H_5)_2$	27 ±2	
40	66,843	OCH_3	NO_2	H	$S[CH_2]_2N(C_2H_5)_2$	27 ±5	
41	70,117	OCH_3	NO_2	H	$[CH_2]_3N(CH_3)_2$	64 ±4	64 ±7
42	65,969	OCH_3	NO_2	H	$[CH_2]_4N(CH_3)_2$	31 ±0·9	21 ±2
43	66,113	OCH_3	NO_2	H	$[CH_2]_5N(CH_3)_2$	61 ±5	31 ±3
44	70,347	OCH_3	NO_2	H	$[CH_2]_3N$⟨⟩	99 ±8	133±14

[1] R_{1-4} refer to Figure 6.1.

TABLE 6.5C RELATIVE BINDING AFFINITIES OF CI 628 ANALOGUES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS[1]

Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
45	65,912	H	NO_2	H	$OCH(CH_3)CH_2N(CH_3)_2$	40 ±4	
46	67,013	Cl	NO_2	H	$O[CH_2]_3N(CH_3)_2$	49 ±4	
47	63,591	H	NO_2	Cl_2(a)	$O[CH_2]_2N(CH_3)_2$	14 ±1	
48	55,067	H	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	114 ±2	
49	55,169	CH_3	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	91 ±1	
50	55,220	CH_3	NO_2	CH_3	$O[CH_2]_2N(C_2H_5)_2$	80 ±11	
51	55,368	Cl	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	76 ±12	81 ±0·8
52	55,752	Cl	NO_2	Cl (b)	$O[CH_2]_2N(C_2H_5)_2$	55 ±3	
53	56,422	CF_3 (c)	NO_2	H	$O[CH_2]_2N(C_2H_5)_2$	175 ±20	205 ±17
54	56 252	OC_2H_5	NO_2	H	$O[CH_2]_2N$⬡	33 ±0·6	
55	63,052	Cl	NO_2	H	$O[CH_2]_2N$⬡	119 ±1	127 ±2
56	56,152	$O[CH_2]_2N$⬡	NO_2	Cl(b)	$O[CH_2]_2N$⬡	8 ±2	

[1] R_{1-4} refer to Figure 6.1; [a] 2,4 chloro-; [b] ortho-; [c] meta-;

of rat liver AEBS (30 vs 31 vs 32 vs 33 vs 25). This order was the same for MCF 7 AEBS except that compound 25 with 2 methylene units did not have decreased affinity. In fact this was the only CI 628 analogue to show major differences in RBA between MCF 7 and rat liver AEBS. A slightly different order was observed when no ether side chain linkage was present (41 vs 43 vs 42), in which case the binding order was 3 = 5 > 4 methylene units (rat liver) or 3 > 5 > 4 methylene units (MCF 7). For pyrollidino- or diethylamino-terminal groups the same binding affinity was found for compounds with either 2 or 3 methylene units (27 vs 37; and 39 vs 40, respectively). Side chain branching increased binding affinity (25 vs 34 and 35).

The nature of the bond linking the side chain to the remainder of the molecule influenced binding affinity. Thus a C-C bond instead of an ether linkage produced compounds of higher affinity (41 - 43 vs 25 and 30 - 32; 44 vs 27 or 37). A thioether bond caused no change in affinity (26 vs 39). The importance of side chain structure on RBA is also demonstrated by the several CI 628 analogues which had no affinity for AEBS (Table 6.2). The side chains of this group of compounds are non-basic, including the pyrrole derivative, CN 64,233 (approximate pKa = -3.8; Robertson et al., 1982a).

A comparison of CI 628 analogues also enabled the effects of aromatic substitution on binding affinity to be studied. The apparent order of influence of various substituents (from compounds of highest to lowest affinity) was : CF_3 > H > CH_3 > 2 x CH_3 = Cl > 2 x Cl > OCH_3 > OC_2H_5. Thus the presence of the electron-withdrawing trifluoromethane group resulted in high affinity, whereas substituents which are electron-donating relative to H (because of resonance or inductive effects) reduced affinity.

6.3.4 H Series of Compounds

The affinities of bibenzyl and stilbene derivatives for the rat liver and MCF 7 AEBS (Tables 6.6A, B) were generally low, perhaps not unexpectedly because of the presence of aromatic hydroxy and methoxy substituents which, as has been shown for triphenylethylenes, decrease RBA. In both tissues, compounds with aromatic methoxy substituents, had higher affinities than compounds with hydroxy substituents (57 vs 58; 59 vs 60; 62 vs 63). Side chain terminal amino group structure was the most important determinant of binding affinity and for rat liver the order was: $-c-N(CH_2CH_2)_2CH_2$ = $-c-N(C_4H_8)$ = $-N(C_2H_5)_2$ > $-c-N(CH_2CH_2)_2O$ = $-N(CH_3)_2$ (compounds 64 - 68 and 70 - 72). The similar order for MCF 7 AEBS was $-c-NC_4H_8$ > $-c-N(CH_2CH_2)_2CH_2$ > $-c-N(CH_2CH_2)_2O$ = $-N(C_2H_5)_2$ > $-N(CH_3)_2$. Dibasic aminoether derivatives had decreased affinity (68 vs 69; 72 vs 73).

Structural variations in that part of the molecule connecting the two benzene rings (i.e. presence or absence of unsaturation, number and positions of methyl substituents) had little or no significant effect on affinity for rat liver AEBS. The number of methyl substituents also had no effect on affinity for MCF 7 AEBS (60 vs 70; 63 vs 65) except that a decrease was observed in the case of compounds 58 vs 70. Position of the methyl substituents either influenced (58 vs 60; 61 vs 62) or had no effect (57 vs 59) on affinity. Unsaturation caused a general decrease in RBA for MCF 7 AEBS (57 vs 62; 65 vs 70; 58 vs 63) presumably because an optimal molecular conformation is no longer possible.

6.3.5 PR Series of Compounds

The PR series of hydroxylated clomiphene derivatives (Table 6.7) illustrated the effects on binding affinity of vinyl substituents

TABLE 6.6A RELATIVE BINDING AFFINITIES OF STILBENE AND BIBENZYL DERIVATIVES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS

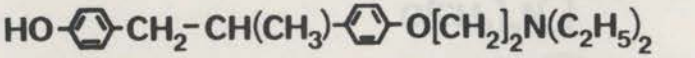
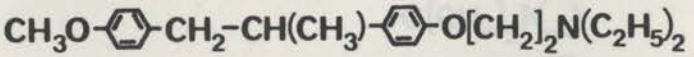
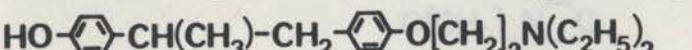
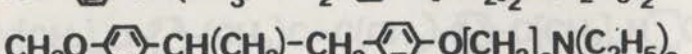
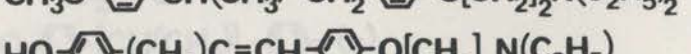
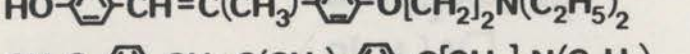
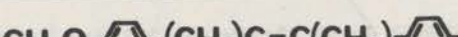
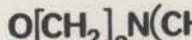
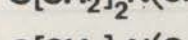
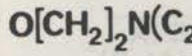
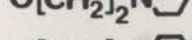
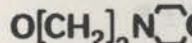
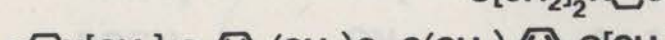
Compound No.	Code No.		RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
57	H233		0·7 ± 0·2	1·4 ± 0·2
58	H237		9 ± 3	33 ± 4
59	H223		1·4 ± 0·5	0·6 ± 0·2
60	H228		8 ± 1	12 ± 2
61	H279			1·1 ± 0·2
62	H288			0·3 ± 0·1
63	H166			3·3 ± 0·1

TABLE 6.6B RELATIVE BINDING AFFINITIES OF STILBENE AND BIBENZYL DERIVATIVES FOR MCF 7 RAT LIVER MICROSOMAL AEBS

Compound No.	Code No.		RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
		 (a)		
64	H369	$O[CH_2]_2N(CH_3)_2$	2·2 ± 0·2	3·3 ± 0·1
65	H246	$O[CH_2]_2N(C_2H_5)_2$	8 ± 1	7 ± 1
66	H315		12 ± 1	19 ± 2
67	H336		13 ± 1	12 ± 1
68	H385		4 ± 0·1	7 ± 0
69	H374		0·7 ± 0·2	
		(b)		
70	H310	$O[CH_2]_2N(C_2H_5)_2$	10 ± 1	15 ± 0
71	H730		20 ± 4	43 ± 1
72	H731		23 ± 7	30 ± 3
73	H725		1·9 ± 0·1	5 ± 0

[a] trans Compounds 64 - 68; [b] Compounds 70 - 73

TABLE 6.7 RELATIVE BINDING AFFINITIES OF PR COMPOUNDS FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS[1]

Compound No.	Code	R_2 [(a)]	RBA		
			AEBS (Rat Liver)	AEBS (MCF-7)	ER (MCF-7)
74	PRH	H	18 ± 1	41 ± 5	9 ± 1
75	$PRNO_2$	NO_2	8 ± 2	30 ± 3	18 ± 4
76	PRCN	CN	6 ± 1	38 ± 5	9 ± 3
77	PRBr	Br	23 ± 6	60 ± 6	28 ± 4
78	PREt	CH_2CH_3	34 ± 8	68 ± 6	35 ± 5
79	PRCl	Cl	50 ± 11	119 ± 20	89 ± 12

[1] R_{1-4} refer to Figure 6.1; [a] R_1 = OH, R_3 = H, R_4 = $O[CH_2]_2(C_2H_5)_2$ in all cases.

(position R_2, Figure 6.1) on the triphenylethylene molecule. For rat liver AEBS the order of binding affinities was Cl > C_2H_5 = Br = H > CN = NO_2, while for MCF 7 AEBS a similar order was observed Cl > C_2H_5 = Br > H = CN = NO_2.

The PR series of compounds showed a good correlation between affinity for AEBS and ER especially in MCF 7 cells (Table 6.7, Figure 6.4). This suggests that substituents at the R_2 position (and/or the structural features of the ligand that they influence) interact with a similar environment in both binding sites.

6.3.6 Cyclofenil Series

Cyclofenil derivatives (Table 6.8) had markedly lower affinities for rat liver AEBS than for MCF 7 AEBS, with the exception of compound 81 which carries a terminal diethylamino group in the side chain. However, the effects on RBA of differing terminal groups was qualitatively the same for both tissues. The order of binding was $-c-N(CH_2CH_2)_2O$ > $-c-N(CH_2CH_2)_2CH_2$ = $-N(CH_3)_2$ > $-N(C_2H_5)_2$.

In the related diphenylethylene series (Table 6.8, compounds 85 - 87) the effect of diethylamino and dimethylamino groups was reversed. Removal of the basic side chain abolished binding affinity (80; 85).

6.3.7 SKF-525A Series

Of all the classes of compounds in this study the SKF-525A series (Table 6.9) was the one in which binding affinity for rat liver AEBS bore qualitatively the least relationship to affinity for MCF 7 AEBS. Quantitatively affinities were generally higher for the former. For both sites aromatic substitution at R_1 or R_3 with Cl (89) or NO_2 (88) groups resulted in low affinity. In the remainder of the compounds the effects

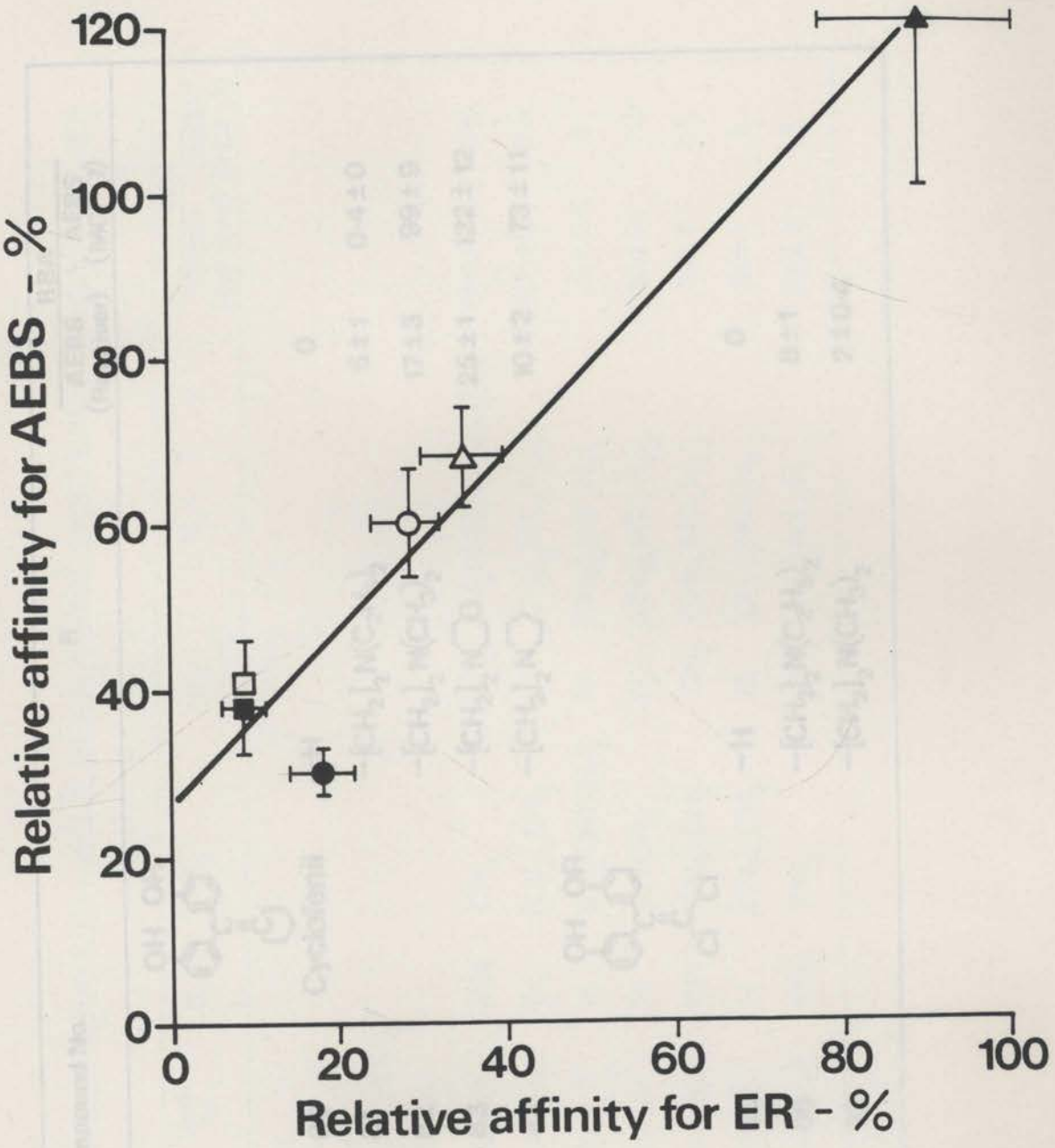


Figure 6.4: Relationship between relative binding affinities of the PR series of hydroxylated triphenylethylenes for the ER and AEBS of MCF 7 cells. RBA were measured in competitive binding assays as described in Section 2.4.5. The least-squares linear regression line is shown. Compounds are represented by the following symbols: 74 (□), 75 (●), 76 (■), 77 (○), 78 (△), 79 (▲).

TABLE 6.8 RELATIVE BINDING AFFINITIES OF CYCLOFENIL ANALOGUES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS

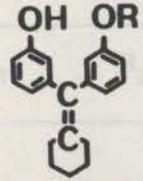
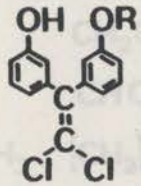
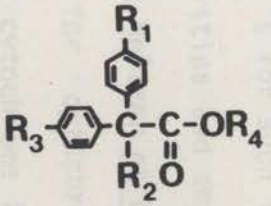
Compound No.	R	RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
 Cyclofenil			
80	–H	0	
81	$-[CH_2]_2N(C_2H_5)_2$	6±1	0·4±0
82	$-[CH_2]_2N(CH_3)_2$	17±3	99±9
83	$-[CH_2]_2N$	25±1	122±12
84	$-[CH_2]_2N$	10±2	73±11
			
85	–H	0	
86	$-[CH_2]_2N(C_2H_5)_2$	8±1	
87	$-[CH_2]_2N(CH_3)_2$	2±0·4	

TABLE 6.9 RELATIVE BINDING AFFINITIES OF SKF-525A ANALOGUES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS

Compound No.	Code No.	R_1	R_2	R_3	R_4	RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
							
88	80533	NO_2	CH_3	NO_2	$CH_2CH(CH_3)N(C_2H_5)_2$	0·6 ± 0·1	0·5
89	19086A	Cl	$[CH_2]_2CH_3$	Cl	$[CH_2]_2N(C_2H_5)_2$	2·5 ± 0·2	0·5
90	490	H	$CH_2CH(CH_3)_2$	H	$[CH_2]_2N(C_2H_5)_2$	5 ± 0·5	4
91	485A	H	$CHCH_2$	H	$[CH_2]_2N(C_2H_5)_2$	10 ± 2	1·2
92	19500A	CH_3	$[CH_2]_2CH_3$	CH_3	$[CH_2]_2N(C_2H_5)_2$	11 ± 1	2·6
93	525A	H	$[CH_2]_2CH_3$	H	$[CH_2]_2N(C_2H_5)_2$	12 ± 1	2
94	10108A	CH_3	CH_3	CH_3	$[CH_2]_2N(C_2H_5)_2$	33 ± 3	3

of aromatic substitution depended upon the nature of the substituent at R_2 and upon the tissue source of AEBS. For rat liver AEBS, CH_3 substitution had little effect on RBA (92 vs 93) and affinity tended to increase with decreasing size of the substituent at R_2 (90 vs 92; 93 vs 94). Compound 91 with an unsaturated R_2 substituent had a lower affinity than would be predicted from its size. In the case of the MCF 7 AEBS, aromatic substitution at R_1 and R_3 with a CH_3 group also had little effect on RBA and, in contrast to rat liver, the influence of the R_2 substituent was minimal. Other reported values for the RBA of SKF-525A (compound 93) are 18% (rat liver microsomes, Ruenitz and Bagley 1985b) and 1.1% (mouse liver microsomes, Lyman and Jordan 1985c).

SKF-525A is a potent inhibitor of cytochrome P-450 and several other inhibitors structurally related to TAM (including Type I and Type II binders) also bind to the AEBS (Lyman and Jordan, 1985c). However, other cytochrome P-450 inhibitors (metyrapone and ellipticine, Table 6.14) have no significant affinity. Other evidence supports the conclusion that the AEBS is not an inhibitory or other binding site on cytochrome P-450. Although TAM and other antioestrogens are known to interact with cytochrome P-450 and competitively inhibit oxidative metabolism of Type I substrates (Ruenitz and Toledo, 1980; Meltzer et al. 1984), the k_i's for such inhibition (6.8 - 22.9 μM for ethylmorphine demethylase activities and aminopyrine demethylase by TAM, desmethylTAM and 4-hydroxyTAM, Meltzer et al., 1984) suggest a much lower affinity of these ligands for cytochrome P-450 than for AEBS. The total concentration of cytochrome P-450 in the rat liver microsomes used in the present study (0.5 - 0.6 nmol/mg protein) is considerably higher than that of the AEBS (30.5 pmol/mg protein).In rat liver, cytochrome P-450 and AEBS have different submicrosomal distributions (Section

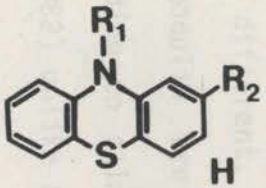
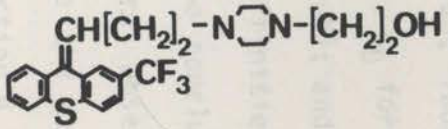
4.3.2) and preliminary experiments have shown that these two sites are differentially extracted from rat liver microsomes by sodium cholate; that fractions containing partially purified cytochrome P-450 have decreased levels of AEBS; and that unlike cytochrome P-450, AEBS is not significantly induced or depleted by treating rats with phenobarbital or cobalt-haem. However, such evidence cannot rule out the possibility that the AEBS is a cytochrome P-450 isoenzyme with unique properties. Although metabolism of TAM in rabbit liver microsomes occurs through the selective involvement of such isoenzymes (Ruenitz et al., 1984), the absence of TAM metabolism in MCF 7 cells despite high levels of AEBS also argues against identity with cytochrome P-450.

6.3.8 Binding of Phenothiazines to AEBS

A series of phenothiazine derivatives (Table 6.10) had relatively high affinity for the AEBS. A basic side chain was necessary for binding and affinity was determined both by the side chain structure (perphenazine vs prochlorperazine vs chlorpromazine) and by aromatic substitution (trifluoperazine vs chlorpromazine, rat liver; perphenazine vs fluphenazine, MCF 7). Tissue differences were also evident (fluphenazine). The structurally related thioxanthene derivative flupenthixol also had a high affinity for AEBS.

The binding of these compounds to AEBS appears to be unrelated to their activity as calmodulin antagonists. Such a conclusion also holds for TAM, which Lam (1984) has shown is a potent antagonist of calmodulin activation of cAMP phosphodiesterase (k_i = 0.96 μM). Thus the RBA values of phenothiazines predict apparent K_D values for the AEBS of approximately 1 - 70 nM, which are quite different from the corresponding K_D values for calmodulin (e.g. trifluoperazine 1 - 1.5 μM;

TABLE 6.10 RELATIVE BINDING AFFINITIES OF PHENOTHIAZINE AND THIOXANTHENE DERIVATIVES FOR MCF 7 AND RAT LIVER MICROSOMAL AEBS

Compound	R_1	R_2	RBA AEBS (Rat Liver)	RBA AEBS (MCF-7)
Phenothiazine		H	0	0
Trifluoperazine	$[CH_2]_3$-$N(CH_3)_2$	CF_3	31±6	18±3
Perphenazine	$[CH_2]_3$-N⌒N-$[CH_2]_2OH$	Cl	39±4	33±5
Prochlorperazine	$[CH_2]_3$-N⌒N-CH_3	Cl	21±2	12±1
Chlorpromazine	$[CH_2]_3$-$N(CH_3)_2$	Cl	1·5±0·2	–
Fluphenazine	$[CH_2]_3$-N⌒N-$[CH_2]_2OH$	CF_3	41±1	11
Flupenthixol			100[a]	

[a] Calculated from K_D, Table 6.11

chlorpromazine 5 μM; Roufogalis, 1982). Secondly, the IC_{50} values for inhibition of cAMP phosphodiesterase (e.g. TAM 2 μM; chlorpromazine 6 - 47 μM; fluphenazine, 5 μM; trifluoperazine 5 - 10 μM; Norman et al., 1979; Roufogalis, 1982; Lam, 1984) are in agreement with the affinities of these compounds for calmodulin rather than for AEBS. Several other compounds which are weak calmodulin antagonists also have low affinity for AEBS e.g. SKF-525A (Table 6.9), amitryptyline and imipramine (Table 6.12), desmethylimipramine and propranalol (Table 6.13) (IC_{50} for cAMP phosphodiesterase inhibition 130, 130 - 250, 125, 125 and 180 μM respectively, Roufogalis, 1982). However, many potent calmodulin antagonists (Roufogalis, 1982) have no affinity for AEBS. These include the very potent antagonist R24571, (calmidazolium, Table 6.14); the naphthalene-sulfonamide derivative W-7 (Table 6.14, K_D for calmodulin 11 μM); haloperidol (Table 6.12, K_D for calmodulin 9 μM); and (+)butaclamol (Table 6.12). Thus the binding specificities and affinities of these two sites are very different. Further support for the conclusion that the AEBS and calmodulin are distinct entities are observations that antagonist binding to calmodulin is inhibited in the absence of Ca^{2+} (Roufogalis, 1982) unlike binding to the AEBS (Section 4.3.2), and that the effects of pH on ligand binding are dissimilar, [^{3}H]trifluoperazine binding to calmodulin decreasing below pH 4.2 and above pH 7.5 whereas AEBS showed optimal binding between pH 7 and pH 8 (Sections 3.3.4, 4.3.4 and Roufogalis, 1982).

6.3.9 Binding of [^{3}H]Fluphenazine and [^{3}H]Flupenthixol to Rat Liver Microsomes

The observation that phenothiazine derivatives bind with apparent high affinity to AEBS was potentially significant given that these

compounds are also known to interact with a variety of cellular binding sites in addition to calmodulin including: dopamine D_1 and D_2, serotonin ($5\text{-}HT_2$), α_1- and α_2-adrenergic, muscarinic cholinergic and histamine receptors (Seeman, 1981; Christensen et al., 1984). To determine whether the AEBS was in fact one of these receptors the binding of a variety of ligands to the AEBS was investigated. First, the binding properties of [^{3}H]fluphenazine and [^{3}H]flupenthixol were directly studied in tissue microsomal fractions (Table 6.11). Both [^{3}H]fluphenazine and [^{3}H]flupenthixol bound with high affinity to rat liver microsomes, the K_D of the former compound being used to calculate an apparent RBA of 44%, in agreement with the value determined by competition assay (Table 6.10). [^{3}H]TAM and [^{3}H]flupenthixol bound to an equal number of sites whereas [^{3}H]fluphenazine bound to additional sites from which it was displaced to a greater extent by 100 nM fluphenazine than by 100 nM TAM. Similar observations were also made in rat brain microsomes (not shown).

TABLE 6.11 SPECIFIC BINDING OF [^{3}H]TAM, [^{3}H]FLUPHENAZINE AND [^{3}H]FLUPENTHIXOL TO RAT LIVER MICROSOMES

LIGAND	K_D (nM)	C (pmol/mg protein)
[^{3}H]TAM[a]	1.1 ± 0.2	18.54 ± 1.71
[^{3}H]Fluphenazine[a]	2.5 ± 0.6[c]	25.85 ± 3.66
[^{3}H]Fluphenazine[b]	3.1 ± 0.5	30.49 ± 2.68
[^{3}H]Flupenthixol[a]	1.1 ± 0.3[d]	17.32 ± 0.24
[^{3}H]Flupenthixol[b]	1.4 ± 0.1	17.08 ± 1.71

[a] Non-specific binding determined in the presence of 100 nM TAM; [b] Non-specific binding determined in the presence of 100 nM fluphenazine; [c] Apparent RBA = 44%; [d] Apparent RBA = 100%.

The lack of affinity of TAM for the population of apparently fluphenazine-specific sites was confirmed in competition studies which showed that fluphenazine fully competed for [^{3}H]TAM binding at high concentrations but displaced [^{3}H]fluphenazine to a greater extent than TAM at all unlabelled ligand concentrations > 40 nM. The identity of these fluphenazine-specific binding sites, which are not competed for by (+)butaclamol (see below), is unknown.

6.3.10 Binding of Ligands with Miscellaneous Pharmacological Activity to AEBS

Both fluphenazine and flupenthixol are unselective antagonists for dopamine D_1 and D_2 receptors (k_i for inhibition of [^{3}H]piflutixol binding to D_1 receptor 1.4 - 14 nM, and k_i for inhibition of [^{3}H]spiperone and [^{3}H]haloperidol binding to D_2 receptors 0.7 - 3.4 nM, Seeman, 1981; Christensen et al., 1984). To investigate whether the AEBS could be either of these receptors a series of dopamine receptor ligands was tested for competitive binding to the AEBS (Table 6.12). (+)Butaclamol, which has high affinity for D_1 and D_2 receptors (Seeman, 1981; Leff et al., 1985), was unable to displace significant amounts of either [^{3}H]TAM or [^{3}H]fluphenazine binding in rat liver microsomes. The butyrophenones, haloperidol and spiperone (spiroperidol), which are selective D_2 antagonists (k_i for inhibition of [^{3}H]spiperone binding to D_2 receptors 1.9 and 0.17 nM respectively, k_i for inhibition of [^{3}H]piflutixol binding to D_1 receptors 430 and 2300 nM respectively, Christensen et al., 1984), also had no affinity for AEBS. Dopamine itself has been shown to have no affinity for AEBS (Brandes et al., 1985). Although Hiemke and Ghraf (1984) have shown that TAM, CI 628, enclomiphene and zuclomiphene are able to bind to D_2 receptor with

relatively low affinity (k_i for inhibition of [^{3}H]spiperone and [^{3}H]domperidone binding 5 - 12 μM), no specific binding of [^{3}H] spiperone to rat liver microsomes was detected in the present studies. Together, these data show that the AEBS is not the D_1 or D_2 dopamine receptor.

Similarly, although fluphenazine and flupenthixol bind to the serotonin 5-HT_2 receptor (k_i for inhibition of [^{3}H]spiperone binding to cortical membranes 5.8 and 16 nM respectively, Christensen et al., 1984), the failure of (+)butaclamol, spiperone or haloperidol (which are more potent serotonin antagonists) to bind to AEBS effectively rules out the possibility of equivalence between AEBS and the serotonin receptor. Furthermore, Hiemke and Grahf (1984) found that TAM and other antioestrogens are unable to inhibit either [^{3}H]hydroxytryptamine or [^{3}H]spiperone binding in rat frontal cerebral cortex showing that these antioestrogens do not bind to $5HT_1$ or $5HT_2$ receptors. Table 6.12 shows that several other serotonin antagonists and uptake (amine pump) inhibitors and serotonin itself (5-hydroxytryptamine) have little or no affinity for AEBS.

As noted above fluphenazine and flupenthixol have α_1-adrenergic antagonist activity (k_i for inhibition of [^{3}H]prazosin binding 3.3 and 15 nM respectively; Christensen et al., 1984). However, haloperidol and spiperone share this property (k_i 14 and 8.3 nM respectively) and Table 6.13 shows that a series of other α- (and β-)adrenergic ligands also have little or no affinity for the AEBS. Hiemke and Grahf (1984) found that antioestrogens did not inhibit the binding of the adrenergic receptor antagonists [^{3}H]dihydroalprenolol (α_1-, α_2-adrenergic) or [^{3}H]dihydroergocryptine (β-adrenergic).

Binding of clomiphene to muscarinic cholinergic receptors has been

reported (Ben-Baruch et al., 1982) with an apparent K_D of 10^{-6} M determined for both cis- and trans-isomers. Fluphenazine and flupenthixol also have limited affinity for the muscarinic receptor (k_i for inhibition of acetylcholine binding 2.5 and 0.6 μM respectively, Christensen et al., 1984) but other muscarinic and nicotinic cholinergic receptor ligands have no affinity for the AEBS (Tables 6.12, 6.13).

Fluphenazine and flupenthixol act as histamine receptor antagonists and this was also investigated as a possible clue to the identity of the AEBS. Reported k_i values for inhibition of histamine binding to its receptor are 65 and 15 nM for fluphenazine and flupenthixol respectively (Christensen et al., 1984). Table 6.14 shows that of several histamine H_1 antagonists only hydroxyzine had high affinity for AEBS. Histamine agonists and H_2 antagonists had no affinity. These results are in good

TABLE 6.12 RBA OF DOPAMINE AND SEROTONIN RECEPTOR LIGANDS FOR THE AEBS

COMPOUND	RECEPTOR SELECTIVITY	RBA (%) RAT LIVER	RBA (%) MCF 7
(+)Butaclamol	D_1, D_2 antagonist[a]	0	0.3
Spiperone	D_2 antagonist[a]	0	-
Haloperidol	D_2 antagonist[a]	0	0
Dopamine	endogenous ligand	0[b]	-
Amitryptyline	serotonin antagonist[c]	0.2	0.4 ± 0.2
Imipramine	serotonin antagonist[c]	0.5	-
Trimipramine	serotonin antagonist[c]	0.4 ± 0.2	0.4 ± 0.2
5-Hydroxy-tryptamine	endogenous ligand	0[b]	-

[a] Also has serotonin and α_1-adrenergic antagonist activity; [b] Brandes et al. (1985); [c] Also has serotonin uptake inhibitory, antihistamine, anticholinergic and antiadrenergic properties (Bowman and Rand, 1980)

agreement with those presented by Brandes et al. (1985).

The possible relationship between AEBS and histamine-receptor was further investigated by studying the binding of the H_1 ligand [^{3}H]pyrilamine to the rat liver microsomes. Linear Scatchard plots of [^{3}H]pyrilamine binding were obtained after correction for non-specific binding. The concentration of binding sites (i.e. H_1 receptor; 28.95 ± 0.88 pmol/mg microsomal protein) was similar to AEBS concentration determined by [^{3}H]TAM binding (32.17 ± 2.93 pmol/mg protein). [^{3}H]pyrilamine binding was of slightly lower affinity than that of [^{3}H]TAM (K_D 2.17 ± 0.07 and 1.6 ± 0.1 nM respectively). Competitive binding studies showed that TAM had an RBA for the [^{3}H]pyrilamine binding site of 0.3% (compared to the RBA of pyrilamine for AEBS of 0.2 ± 0.1%). Hence despite similar concentrations and affinities for their respective binding sites, TAM and pyrilamine show only limited cross-reactivity, demonstrating the separate nature of the two sites. In support of this conclusion no [^{3}H]pyrilamine binding was detected in MCF 7 microsomes stored at -20°C for 1 - 4 months despite normal levels of AEBS as determined by [^{3}H]TAM binding. Furthermore, histamine receptors have been detected in high concentrations in purified rat liver plasma membrane (Imoto et al., 1985), whereas the present study has shown that the AEBS is most likely located in the endoplasmic reticulum (Section 4.3.2).

Brandes (Brandes et al., 1985; Kroeger and Brandes, 1985; Brandes and Bogdanovic, 1986) has proposed that the AEBS is a histamine- or 'histamine-like' receptor. This conclusion was reached on the basis that H_1 antagonists including DPPE (Section 6.4.1.; Brandes, 1984; Brandes and Hermonat, 1984) show cross-reactivity with AEBS, and that TAM acts as a histamine (H_1) antagonist in histamine-induced smooth muscle

TABLE 6.13 RBA OF ADRENERGIC AND CHOLINERGIC RECEPTOR LIGANDS FOR THE AEBS

COMPOUND	RECEPTOR SELECTIVITY	RBA (%) RAT LIVER	MCF 7
Desmethyl-imipramine	α-adrenergic antagonist[a]	0.05	-
Epinephrine	endogenous α-adrenergic ligand	0[b]	-
Norepinephrine	endogenous α-adrenergic ligand	0[c]	-
Propranalol	β-adrenergic antagonist	1.4[c]	-
(-)Isopro-teranol	β-adrenergic antagonist	0.07[c]	-
Atropine	muscarinic antagonist	0	0
Hyoscamine	muscarinic antagonist	0	-
Quinuclidonyl benzoate	muscarinic antagonist	0	-
Benactyzine	muscarinic antagonist[d]	0	0
Isopropamide	muscarinic antagonist	0[e]	-
Adiphenine	muscarinic antagonist	0.5[f]	-
Acetylcholine	endogenous muscarinic ligand	0	-
Tetracaine	nicotinic antagonist	< 0.1	-

[a] Also norepinephrine uptake (amine pump) inhibitor; [b] Brandes et al. (1985); [c] Sudo et al. (1983), rat uterus; [d] Also monoamine oxidase inhibitor; [e] Ruenitz and Bagley (1985b); [f] Lyman and Jordan (1985c), mouse liver.

TABLE 6.14 RBA OF HISTAMINE RECEPTOR LIGANDS AND MISCELLANEOUS COMPOUNDS FOR THE AEBS

COMPOUND	RECEPTOR SELECTIVITY	RBA (%) RAT LIVER	RBA (%) MCF 7
Hydroxyzine	H_1 receptor antagonist	25 ± 3	25
Pyrilamine	H_1 receptor antagonist	0.2 ± 0.1	-
Phenyl-toloxamine	H_1 receptor antagonist	0.8 ± 0.3	0.7
Diphen-hydramine	H_1 receptor antagonist	$\leq$ 0.05	-
Histamine phosphate	endogenous ligand	0	-
β Aminomethyl-pyrazole	endogenous ligand	0[a]	-
Cimetidine	H_2 receptor antagonist	0	-
Ranitidine	H_2 receptor antagonist	0[a]	-
Aminophylline	cAMP phosphodiesterase inhibitor	0	-
Perhexillene	membrane-active agent	-	0
Triparanol	antilipemic	14[b]	-
R24571	calmodulin inhibitor	0	0
W-7	calmodulin inhibitor	0	-
Metyrapone	cytochrome P-450 inhibitor	0[c]	-
Ellipticine	cytochrome P-450 inhibitor	< 0.4[b]	-

[a] Brandes et al. (1985); [b] Lyman and Jordan (1985c), mouse liver; [c] Ruenitz and Bagley (1985b) found a RBA of approximately 0.5% for this compound.

contraction at very high concentrations (30 μM optimal concentration). Because their studies also showed that the AEBS was unlikely to be the H_1 receptor it was concluded the AEBS may be an unknown "H_3" receptor. These observations are at variance with those of Morris (1985; and Section 1.6.3) who found that such effects of TAM are non-specific and non-competitive. Moreover, it is extremely unlikely that the AEBS mediates this activity given that the concentrations of TAM required are several orders of magnitude greater than necessary for binding site saturation. To propose that the AEBS has identity with a receptor or binding site merely because of ligand cross-reactivity is clearly fallacious and thus there is in fact no convincing evidence to show that the AEBS is some unknown H_3 receptor.

6.3.11 Endogenous Inhibitors of [^{3}H]TAM Binding to the AEBS

The existence of endogenous inhibitors of [^{3}H]TAM binding was suggested by the observation that pre-treatment of rat liver microsomes with charcoal (2.5% w/v for 2 hours at 0°C) resulted in a 40% increase in [^{3}H]TAM binding. Inhibitory activity was also found to be present in solvent extracts of tissues and Figure 6.5 shows the ability of ethanol extracts of rat liver and skeletal muscle to inhibit [^{3}H]TAM binding to rat liver microsomal AEBS. Greater concentrations of inhibitory activity were present in liver, the tissue which also has the higher AEBS concentration (Table 4.1).

Clark et al. (1983) and Murphy et al. (1984b, 1985) have partially characterized the inhibitory activity in solvent extracts of serum and tissues. The latter study identified at least two major classes of ethanol/ether-soluble compounds in human serum that interact with chicken liver cytosol AEBS, including a non-sterol fraction consisting

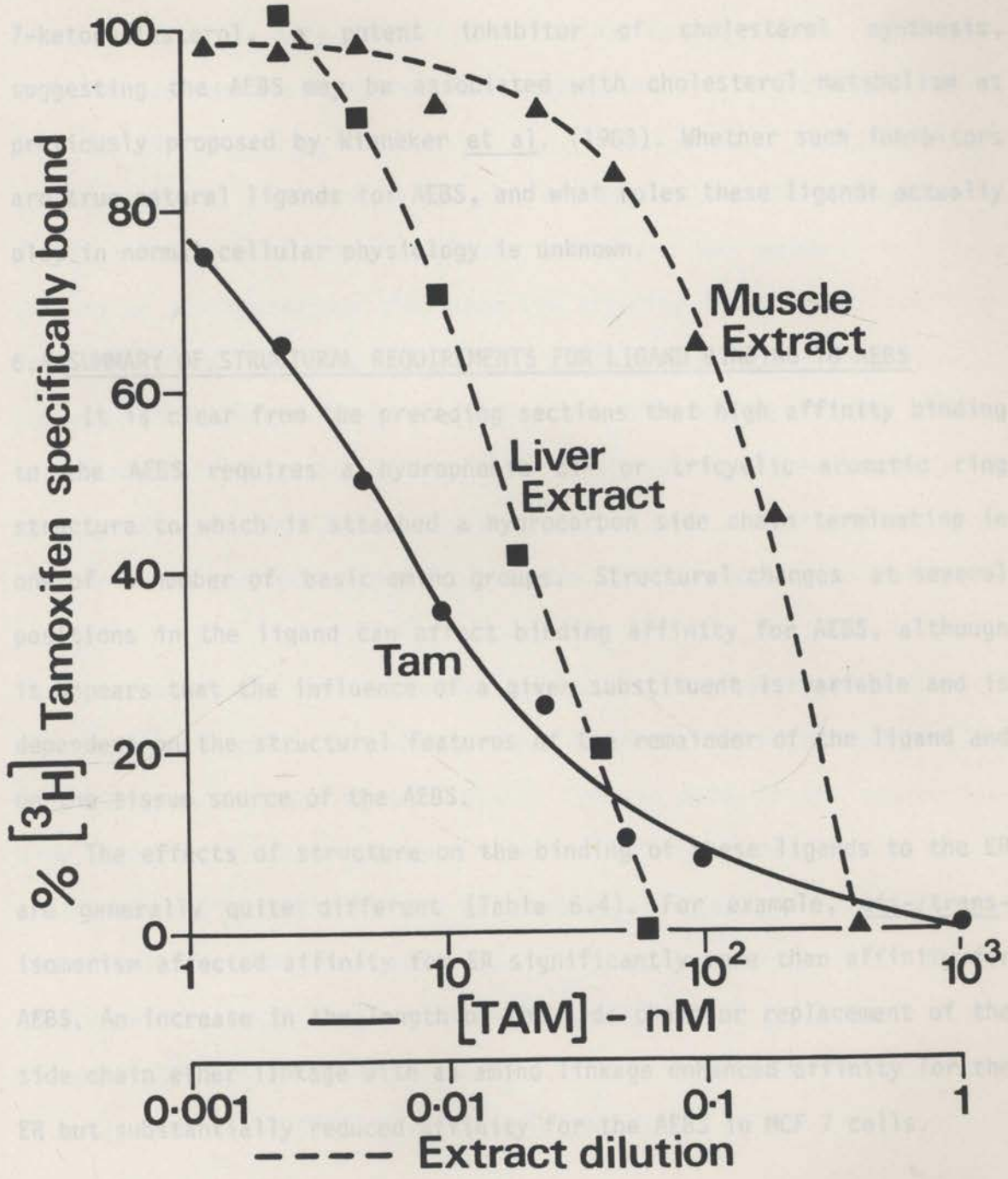


Figure 6.5: Inhibition of [^{3}H]TAM binding to AEBS by ethanol extracts of rat tissues. Equal wet weights of liver and muscle from mature male rats were extracted with boiling ethanol for 1 h. The extracts were filtered and diluted, and used in competitive binding assays with [^{3}H]TAM and rat liver microsomal AEBS.

of fatty acid esters and a sterol fraction containing two distinct inhibitory activities (Section 6.4.5). One of these appears to be 7-ketocholesterol, a potent inhibitor of cholesterol synthesis, suggesting the AEBS may be associated with cholesterol metabolism as previously proposed by Winneker et al. (1983). Whether such inhibitors are true natural ligands for AEBS, and what roles these ligands actually play in normal cellular physiology is unknown.

6.4 SUMMARY OF STRUCTURAL REQUIREMENTS FOR LIGAND BINDING TO AEBS

It is clear from the preceding sections that high affinity binding to the AEBS requires a hydrophobic di- or tricyclic aromatic ring structure to which is attached a hydrocarbon side chain terminating in one of a number of basic amino groups. Structural changes at several positions in the ligand can affect binding affinity for AEBS, although it appears that the influence of a given substituent is variable and is dependent on the structural features of the remainder of the ligand and on the tissue source of the AEBS.

The effects of structure on the binding of these ligands to the ER are generally quite different (Table 6.4). For example, cis-/trans-isomerism affected affinity for ER significantly more than affinity for AEBS. An increase in the length of the side chain or replacement of the side chain ether linkage with an amino linkage enhanced affinity for the ER but substantially reduced affinity for the AEBS in MCF 7 cells.

6.4.1. Aromatic ring structure

In general triphenylethylene derivatives have higher RBA than compounds with fewer aromatic rings. Compounds with a single benzene ring invariably have low affinity. Two such compounds are tert-butyl-

phenoxyethyl diethylamine (BPEA; Sheen et al., 1985b), which had a RBA of 6% for MCF 7 AEBS, and bromophenoxyethyl dimethylamine, which had a RBA for rat liver AEBS of 0.7% (Ruenitz and Bagley, 1985b). The local anaesthetics tetracaine, procaine, benzocaine and lignocaine have no affinity (Watts et al., 1984).

As well as the H compounds (Table 6.6A, B) and SKF-525A analogues (Table 6.9), several other compounds containing two benzene rings in a variety of configurations bind with low affinity to the AEBS including: propranalol (RBA = 1.4%; Table 6.13); N,N-diethyl-2-[(4-phenylmethyl)-phenoxy]-ethanamine. HCl (DPPE, RBA = 8 - 35%; Brandes, 1984; Brandes and Hermonat, 1984; Brandes et al., 1985); the histamine antagonists hydroxyzine and pyrilamine (Table 6.14); and a miscellany of other compounds as reported by Lyman and Jordan (1985c) and Ruenitz and Bagley (1985b).

As well as triphenylethylene-related antioestrogens, several classes of compounds containing three aromatic or heterocyclic rings (including condensed ring structures) bind to AEBS if the appropriate side chain is also present. These include: the benzothiophene antioestrogens LY 117018 (RBA = 2%; Sudo et al. 1983), and keoxifene (LY 156758, RBA = 10%, mouse liver AEBS, Lyman and Jordan, 1985c); dibenzazepines (e.g. imipramine and derivatives, RBA $\leq$ 1%, Table 6.12); phenothiazines (e.g. trifluoperazine, Table 6.10) and thioxanthenes (e.g. flupenthixol, Table 6.10).

6.4.2 Aromatic substitution

The effects of aromatic substitution are dependent not only upon the substituents themselves but also on other structural features of the molecule, in particular the vinyl substituents of triphenylethylenes and

the basic side chain. Thus for CI 628 analogues (48 vs 75) hydroxylation resulted in a large decrease in affinity. Hydroxylation of clomiphene (19 vs 20 vs 79) slightly decreased affinity for rat liver but had little effect on MCF 7 AEBS (taking into account that the PR compounds are 50/50 mixtures of the cis- and trans-isomers). For TAM analogues with diethylamino side chain terminal groups (4 vs 78) aromatic hydroxylation produced a small increase in binding affinity. This is in contrast to hydroxylation of TAM analogues with dimethylamino side chain terminal groups (1 vs 3; 14 vs 15) which resulted in decreased affinity, especially for rat liver AEBS.

In general, aromatic substituents which are electron- donating because of resonance effects or high electron density (e.g. OH, OCH_3, Cl) or inductive effects (CH_3) result in reduced binding affinity, whereas an electron-withdrawing group has the opposite effect (e.g. CF_3). Although hydrogen-bonding effects involving oxygen-containing substituents at this position might also contribute to decreased binding affinity, especially if this region of the ligand is required to interact with hydrophobic regions of the AEBS, the high affinity CF_3 group would also form hydrogen bonds. These effects are not restricted to the triphenylethylenes as is evident from the effects of substitution in the H series (Section 6.3.4), the SKF-525A series (Section 6.3.7), and phenothiazines (Section 6.3.8).

Generally, the effects on binding affinity of aromatic substitution are qualitatively similar for MCF 7 and rat liver AEBS, but are quantitatively greater in the latter tissue.

6.4.3 Geometric isomerism

Cis-/trans-isomerism about the triphenylethylene double bond either has no effect (1 vs 7, MCF 7 AEBS) or results in moderately reduced affinity of the cis form (1 vs 7, rat liver AEBS; 19 vs 20).

6.4.4 Vinyl substitution

The substituent on the triphenylethylene vinyl bond (R_2) influences RBA as demonstrated by the PR series (Section 6.3.5). Similar effects are observed in non-hydroxylated triphenylethylenes, and comparisons of compounds 4, 19 and 48 show that both C_2H_5 and NO_2 substituents cause reduction in affinity relative to Cl. The effects of substituents on RBA did not appear to correlate with molecular size (e.g. groups with large size e.g. CN, NO_2 and C_2H_5 had both low and high affinities), although steric factors might play a role in some circumstances as shown by the reduced affinity of a propyl-substituted TAM analogue (1 vs 13). A correlation was observed, however, with another property of these groups, that is their respective directive effects on substitution in the benzene nucleus, i.e. their ortho-, para- or meta-directing properties. NO_2 and CN groups which have low affinity for AEBS, are meta-directing and are strongly electron withdrawing, whereas the C_2H_5 group has intermediate RBA, is electron donating, and is moderately ortho-, para-directing. The halogens Br and Cl are strongly electronegative and hence electron withdrawing but, unlike NO_2 and CN, unshared pairs of electrons in these atoms are able to interact with the unsaturation electrons elsewhere in the substituted molecule. This resonance effect results in Br and Cl groups being strongly ortho-, para-directing, and presumable also results in chloro-substituted triphenylethylenes in particular, having high affinity for the AEBS by

allowing optimal interaction with the binding site. However, it is uncertain whether these effects on RBA result from local interactions (i.e. between the substituent and its binding site environment) or whether they are due to the transmission of electronic effects through the extended conjugated system of the triphenylethylene molecule, as is the case in benzene substitution. Such an explanation has been proposed to account for differences in binding affinity for ER of another series of hydroxylated triphenylethylenes through changes in phenolic hydroxyl group acidity (Ruenitz et al. 1983a). Interestingly, the influence of substituents at R_2 on binding affinity is the opposite to that observed for aromatic substitution at R_1 (Section 6.4.2).

6.4.5 Side chain

An alkyl-substituted amino terminal side chain group appears to be an absolute requirement for binding to the AEBS, with the possible exception of certain cholesterol metabolites. Table 6.2 shows the side chain structure of several TAM and CI 628 analogues which have no affinity. Although compounds which bind to the AEBS have side chains capable of hydrogen bonding (because of the availability of the nitrogen lone pair of electrons), Table 6.2 shows that side chains of several non-binding compounds also have this property because of the presence of hydroxyl groups (e.g. ICI 145680). Basicity is another property which side chains of compounds binding to AEBS have in common, whereas the side chains of non-binding compounds are all non-basic. This suggests that an ionic interaction, perhaps with acidic amino acid or phospholipid residues, is important for the formation of the AEBS-ligand complex. Although Sudo et al. (1983) concluded that base strength was not correlated with affinity for AEBS among a series of TAM analogues,

additional data suggest that such a relationship may exist. The pKa values used to reach this conclusion are approximate only and are in fact those of the corresponding secondary amines used in the synthesis of these compounds (Robertson et al., 1982a). Thus TAM analogues with terminal amino groups with lower basicity relative to the dimethylamino group (pKa = 10.8) of TAM (e.g. morpholino-, pKa = 8.7, compound 11; and methyl-piperazino-, pKa = 9.8, Sudo et al., 1983) had higher RBA, whereas those with more basic groups (secondary amines e.g. desmethyl-, compound 2; and guanidino-, pKa = 13.7, Sudo et al., 1983) had lower affinity. Furthermore, the presence in other compounds of a highly basic quarternary amino group abolishes affinity (Ruenitz and Bagley, 1985b), as does the very weakly basic pyrollido- group, pKa = -3.8 (CN 64,233, Table 6.2; Sudo et al., 1983). Weak bases e.g. aziridine- (pKa = 8, compound 38; Monsma et al., 1984) and imidazole- (pKa = 7.0, compound 29) groups produced very low affinity analogues. These observations suggest that there may be an optimal ligand basicity, with lower or higher base strengths resulting in decreased affinity. This is perhaps analogous to the pH optimum seen for the binding of [^{3}H]TAM to AEBS (Sections 3.3.4, 4.3.4).

Steric and other factors must also determine the influence of the terminal group, as groups with similar base strengths can produce widely differing RBA e.g. dimethylamino- vs diethylamino- (pKa = 10.5) vs pyrollidino- (pKa = 11.3) vs piperidino (pKa = 11.2), compounds 25 vs 26 vs 27 vs 28. In addition, the structure of the remainder of the ligand must be taken into account. Thus dimethylamino derivatives of TAM and cyclofenil have greater affinity than the corresponding diethylamino derivatives (1 vs 4; 81 vs 82), whereas the reverse is true of CI 628 (25 vs 26) and diphenylethylene (86 vs 87) analogues. A morpholino-

containing side chain results in a high affinity TAM analogue (compound 11), but has little effect on the RBA of the corresponding stilbene compound (compound 68) or cyclofenil derivative (compound 83). In addition, the presence of a pyrollidino-group, which generally results in high RBA (compounds 16, 27, 55), has little influence on RBA of analogues with aromatic hydroxyl substituents (3 vs 16).

In summary, for all classes of compounds tertiary basic heterocyclic amino terminal groups (e.g. pyrrolidino-, piperidino-, methylpiperazino- and morpholino-), result a in generally higher affinity than tertiary basic alkyl amino groups (e.g. dimethylamino-, diethylamino- and dipropylamino-) which in turn have higher affinity than secondary amino groups (methylamino-, ethylamino-).

Two compounds which contribute to the AEBS inhibitory activity of ethanol extracts of human serum (Section 6.3.11) have been tentatively identified as 5-cholestene-3β-ol-7-one (7-ketocholesterol), and 4-cholesten-3-one (RBA approximately 1% and 0.01% respectively; Murphy et al., 1985). These cholesterol metabolites are the only known compounds without basic side chains which bind, albeit with low affinity, to the AEBS. It may be that such compounds are not true competitive inhibitors of TAM binding, and act through non-competitive mechanisms, e.g. by disruption of the binding site lipid environment. It has been shown that high concentrations of crude serum extracts inhibit TAM binding non-competitively (Murphy et al., 1984b). Metryapone, a 1,2-dipyridyl derivative without a basic side chain, has also been reported by Ruenitz and Bagley (1985b) to bind to rat liver AEBS (RBA approximately 0.1%), although the present study found this compound to have no affinity.

As well as the terminal amino group, the structure of the remainder

of the side chain influences binding affinity. Changes in the length of the hydrocarbon chain produce effects on RBA, the magnitude and direction of which are dependent in a complicated manner on the structural features of the compound (in particular the terminal amino group structure) and on the tissue source of the AEBS. In general, chain length influences RBA most when the terminal group is $-N(CH_3)_2$, and with regard to tissue differences, MCF 7 AEBS has a preferred chain length of two methylene units, whereas for rat liver AEBS compounds with side chains of three or more methylene units have RBA greater than or equal to compounds with two methylene units. Compounds with hydrocarbon chains up to 5 and 6 methylene units may still have relatively high affinity for AEBS (compounds 32, 33, 43), presumably because such chains are able to adopt a conformation which maintains optimal interaction of the terminal amino group with the AEBS. Other permissible side chain configurations are illustrated in the miscellaneous series of diphenyl derivatives identified by Lyman and Jordan (1985c) as binding to the AEBS. These are: side chains containing a benzene ring (i.e. a p-diethylaminophenylethoxy side chain); and compounds with no hydrocarbon side chain in which the amino groups are directly substituted on the diphenyl moiety (e.g. 4-methyl-1,4-diazepine- and piperazine-groups).

Binding affinity is also influenced by substitution or branching of the side chain and by the nature of the chemical bond between the side chain and the aromatic portion of the ligand, which presumably not only has local effects but also determines side chain orientation.

6.4.6 Species/Tissue Differences

The above observations indicate both quantitative and some qualitative differences between rat liver and MCF 7 AEBS. It seems that the rat liver AEBS has more rigorous requirements for ligand structure, that is, perturbations in ligand structure often cause larger changes in RBA compared to MCF 7 AEBS. Whether these differences are due to the structures of the respective binding sites or to the influence of other microsomal components, in particular those which bind ligands with low affinity and high capacity (e.g. cytochrome P-450, calmodulin and other unidentified protein and lipid sites), has yet to be determined.

In summary, the data presented in this Chapter show the AEBS to be a unique intracellular binding site, distinct from a number of other receptor sites with which ligands binding to AEBS cross-react. The study has provided the most detailed information yet available on the AEBS structure-affinity relationship.

CHAPTER SEVEN

MCF 7 BREAST CANCER CELL PROLIFERATION AND EFFECTS OF ANTIOESTROGENS AND OTHER COMPOUNDS WITH AFFINITY FOR THE ANTIOESTROGEN BINDING SITE

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7.1 INTRODUCTION

The mechanistic basis for many features of antioestrogen action *in vivo* and *in vitro*, in particular that of oestrogen-irreversible and apparently non-ER-mediated effects, is poorly understood as detailed in Chapter 1. The major aims of the experimental work described in the present chapter were to define in detail the effects of antioestrogens on oestrogen-responsive breast cancer cells in culture, and to investigate whether non-ER binding sites, in particular the AEBS, are involved in ER-reversible and -irreversible effects on growth and cell cycle progression.

Previous studies from this and other laboratories (see Section 1.4.4) which have investigated the effects of TAM and its analogues on MCF 7 breast cancer cell proliferation can be summarized as follows:

1. these compounds have direct growth inhibitory effects on MCF 7 cells *in vitro*,
2. dose-response curves can be separated into an oestrogen-reversible, and presumably ER-mediated, component and at least one oestrogen-irreversible component,
3. potency among metabolites of TAM is related to affinity for ER but when analogues with equal affinity for ER but markedly different affinities for AEBS were tested the compounds with low or no affinity for AEBS were significantly less potent, and
4. decreased cell proliferation rate is associated with the accumulation of cells in G_1-phase of the cell cycle due to a TAM-induced shift of cells from a "rapidly cycling" pool to a "slowly cycling" pool thus causing a net increase in the G_1 transit time and inhibition of entry of cells into the DNA synthetic phase (S-phase) of the cell cycle.

To extend these studies, the PR series of vinyl-substituted hydroxylated clomiphene derivatives, synthesized by Dr Peter Ruenitz, provided compounds with a 10-fold range of affinities for ER and a 4-fold range of affinities for AEBS. The effects of these compounds were then compared with those of others known to bind to the AEBS but having lower affinity for ER. The compounds tested were a range of CI 628 analogues (triphenylethylene antioestrogens), H compounds (bibenzyl antioestrogens), SKF-525A analogues and phenothiazine derivatives. The latter compounds have calmodulin-inhibitory activities which might contribute to their growth inhibitory and cell cycle effects and so the properties of the potent calmodulin inhibitor, R24571, which has no affinity for AEBS, were also tested.

7.2 METHODS

All methods are as described in Section 2.6 and 2.7.

7.3 RESULTS AND DISCUSSION

7.3.1 PR Series

The effects of the PR series of compounds on MCF 7 cell growth are recorded in Figure 7.1. With the exception of PR H, all compounds had distinct biphasic dose-response curves resulting in 60 - 70% growth inhibition in the nanomolar concentration range, no further effect between 10^{-8} and 10^{-6} M, and a further concentration-dependent decrease in cell proliferation at micromolar concentrations of all drugs. Further experiments demonstrated that the effects of a 10^{-8} M concentration of these compounds on cell growth could be largely reversed by the simultaneous addition of equimolar concentrations of E_2 to the culture medium (Table 7.1). Effects on cell growth at 2.5 μM were only partially

reversed by the simultaneous addition of E_2 (Table 7.1). These data are compatible with these triarylethylene antioestrogens affecting MCF 7 cell proliferation rates by two distinct mechanisms, one of which involves the ER and is oestrogen-reversible and the other which is only

TABLE 7.1 OESTROGEN REVERSIBILITY OF EFFECTS OF PR COMPOUNDS ON MCF 7 CELL PROLIFERATION

COMPOUND NUMBER[a]	COMPOUND	Cell Number (% Control)[b] Drug Concentration 10 nM ± 10 nM E_2	Cell Number (% Control)[b] Drug Concentration 2.5 μM ± 1 μM E_2	S-Phase (% Control)[c] Drug Concentration 10 nM ± 10 nM E_2	S-Phase (% Control)[c] Drug Concentration 2.5 μM ± 1 μM E_2
79	PR Cl	31.3 ± 4.3	27.0 ± 1.7	26	36
	+ E_2	84.7 ± 0.3	56.2 ± 3.9	89	102
78	PR Et	33.7 ± 0.9	31.3 ± 1.9	25	35
	+ E_2	92.7 ± 11.8	50.1 ± 0.3	88	66
77	PR Br	33.3 ± 1.2	28.0 ± 2.0	23	35
	+ E_2	94.0 ± 5.5	66.4 ± 6.3	96	79
75	PR NO_2	44.0 ± 3.8	33.7 ± 0.9	38	39
	+ E_2	97.3 ± 2.3	80.1 ± 4.3	93	100
76	PR CN	39.3 ± 1.5	28.0 ± 1.0	34	30
	+ E_2	80.7 ± 2.3	85.0 ± 2.2	84	108
74	PR H	68.7 ± 2.0	26.3 ± 1.9	85	30
	+ E_2	90.0 ± 1.0	64.5 ± 2.6	89	90

[a] Structures given in Table 6.7; [b] Mean ± SEM, n = 3; [c] Single observation.

apparent at concentrations of > 10^{-6} M, being mediated by an as yet undefined mechanism. The PR series of hydroxylated triarylethylenes, which have high affinities for ER, has allowed clear distinction of these two mechanisms and confirmed earlier observations with 4-hydroxyTAM (Reddel et al., 1983). Previous studies (Murphy and Sutherland, 1983a, 1985; Reddel et al., 1985; Sutherland et al., 1983b)

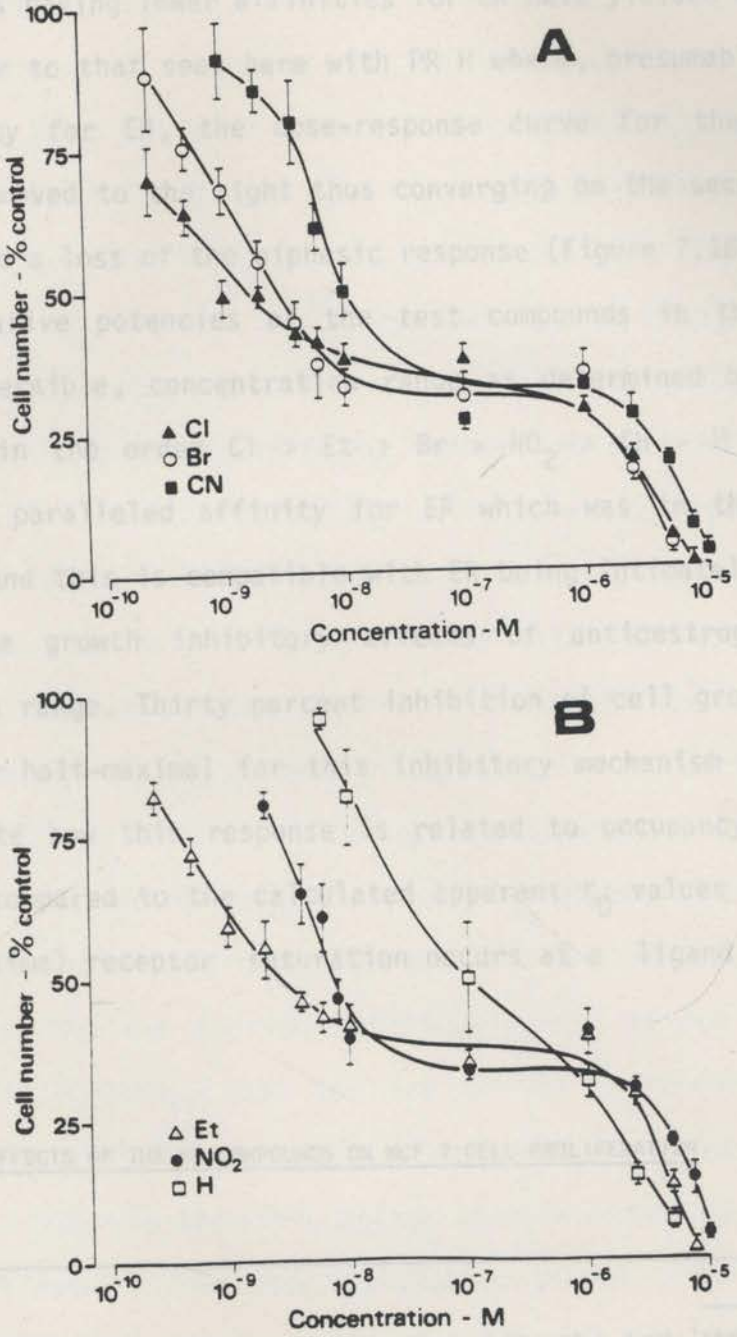

Figure 7.1: Effects of PR compounds on the growth of MCF 7 cells. Cells were grown and treated with drugs as described in Section 2.6. Since cell numbers at the end of experiments were 15.6-fold greater than at drug inoculation, complete arrest of cell proliferation occurred when cell numbers were 6.4% of control. Data points are the mean ± S.E.M. of 6 - 12 estimates from 2 - 4 separate experiments. (A) and (B) represent the dose-response curves for PR Cl (▲), PR Br (○) and PR CN (■); and PR Et (△), PR H (□) and PR NO_2 (●), respectively.

with compounds having lower affinities for ER have yielded dose-response curves similar to that seen here with PR H where, presumably because of lower affinity for ER, the dose-response curve for the ER-mediated mechanism is moved to the right thus converging on the second mechanism and leading to a loss of the biphasic response (Figure 7.1B).

The relative potencies of the test compounds in the nanomolar, oestrogen-reversible, concentration range as determined by their IC_{30} values were in the order Cl > Et > Br > NO_2 > CH > H (Table 7.2). Potency thus paralleled affinity for ER which was in the same order (Table 7.2) and this is compatible with ER being intimately involved in mediating the growth inhibitory effects of antioestrogens in this concentration range. Thirty percent inhibition of cell growth (IC_{30}) is approximately half-maximal for this inhibitory mechanism (Figure 7.1). To investigate how this response is related to occupancy of ER, IC_{30} values were compared to the calculated apparent K_D values for ER, given that half-maximal receptor saturation occurs at a ligand concentration

TABLE 7.2 EFFECTS OF THE PR COMPOUNDS ON MCF 7 CELL PROLIFERATION

COMPOUND NUMBER[a]	COMPOUND	IC_{30} (nM)	IC_{90} (μM)	IC_{DD} (μM)	IC_{CX} (μM)	RBA AEBS	RBA ER
79	PR Cl	0.25	4.2	5.5	6.1	119 ± 20	89 ± 12
78	PR Et	0.58	5.4	6.1	7.1	68 ± 6	35 ± 5
77	PR Br	0.85	3.6	4.3	5.3	60 ± 6	28 ± 4
75	PR NO_2	3.4	8.5	10.3	11.6	30 ± 3	18 ± 4
76	PR CN^2	5.4	7.1	7.4	8.7	38 ± 5	9 ± 3
74	PR H	20	3.8	5.5	6.5	41 ± 5	9 ± 1

[a] Structures given in Table 6.7.

equal to K_D. The K_D values for ER were determined from the RBA for ER (Table 7.2) and a K_D value of 0.1 nM previously measured for the interaction of E_2 with the ER of MCF 7 cells (Reddel et al., 1983) and were in the order: E_2 (0.10 nM) $\leq$ Cl (0.11 nM) < Et (0.29 nM) < Br (0.36 nM) < NO_2 (0.56 nM) < CN (1.11 nM) = H (1.11 nM). The relationship between K_D and IC_{30} was not linear and low affinity ligands, i.e. those with NO_2, CN and H substituents, had potencies 6 - 8-fold lower than predicted from the corresponding K_D values, while the high affinity ligands (Cl, Et and Br) were only 2 - 2.4-fold less potent. The non-linear coupling between binding and response may indicate differences in intrinsic activity of the ligand-ER complex or that properties of these molecules other than their affinities for ER also play a role in determining overall antioestrogenic and antiproliferative activity. A further examination of the relationship between potency at low concentrations and RBA suggested that IC_{30} might be linearly related to log-transformed values for AEBS and ER, and although the linear correlation coefficient for log AEBS IC_{30} vs RBA is not statistically significant ($r = 0.45$), that for log ER RBA approaches statistical significance ($r = 0.708$, $p = 0.1$ approximately). Potency in the low concentration range is therefore better correlated with affinity for ER than for AEBS despite the close correlation between affinity of the PR compounds for these two sites (Figure 6.4), and this is confirmed by multiple linear regression analysis which shows only affinity for ER makes a significant contribution to potency.

The order of potencies in the micromolar range was significantly different from that seen at lower concentrations supporting a different mechanism of growth inhibition (Figure 7.1). In particular PR H became markedly more potent at these concentrations while PR NO_2 was relatively

less potent. As a measure of potency in this concentration range, IC_{90} values (ninety percent inhibition of cell growth; Table 7.2) were in the order Br $\leq$ H $\leq$ Cl < Et < CN < NO_2. These values were not significantly correlated with affinity for either ER or AEBS but were more closely related to the latter. Thus linear correlation between IC_{90} and log AEBS RBA or log ER RBA gave r values of 0.63 (n.s.) or 0.32 (n.s.) respectively. Concentrations of drug that prevented any increase in cell number above drugging density (IC_{DD}, Table 7.2) were in the order Br < H = Cl < Et < CN < NO_2 (r values for log AEBS RBA and log ER RBA *vs* IC_{DD}: 0.65 (n.s.) and 0.30 (n.s.) respectively). Similarly, the concentrations of drugs that were cytotoxic and reduced cell number to 50% of the drugging density (IC_{CX}, Table 7.2) were in the order Br < Cl < H < Et < CN < NO_2 (r values for IC_{CX} *vs* log AEBS RBA and log ER RBA: 0.70, p = 0.1; and 0.37, n.s., respectively). Multiple regression analysis confirmed that at all oestrogen-irreversible concentrations the potency of inhibition of proliferation was more closely correlated with affinity for AEBS than for ER, although correlations with both affinities were statistically significant and were greatest when non-log transformed data were used.

Cell cycle phase distribution data were obtained in the same experiments and are summarized in Figure 7.2. All compounds induced a dose-dependent decrease in the proportion of cells in the S-phase of the cell cycle and this was accompanied by a concomitant accumulation of cells in the G_1-phase (data not shown), with the proportion of cells in the G_2 + M phase remaining relatively constant. Such data are consistent with the previous finding from this laboratory that TAM and its analogues inhibit cell growth primarily by increasing the net G_1 transit time and thus inhibiting the entry of cells into the DNA synthetic phase

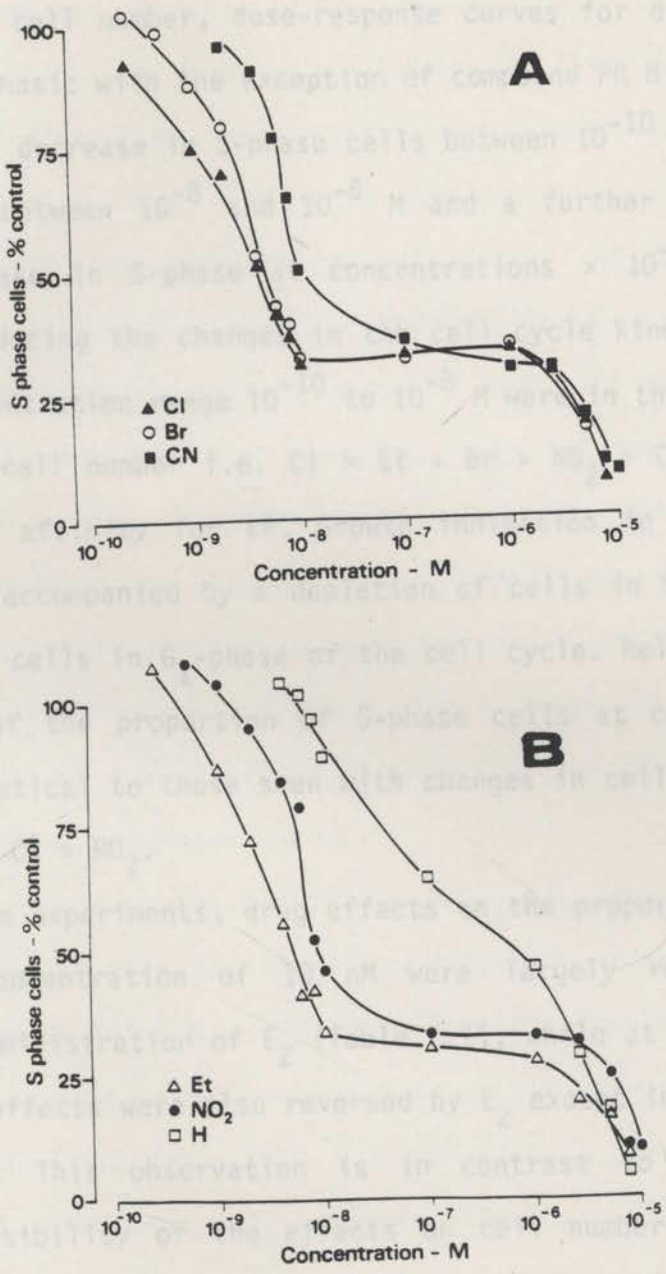

Figure 7.2: Effect of treatment of MCF 7 cells with PR compounds on the percentage of cells in the S-phase of the cell cycle. Cells from the experiments described in Figure 7.1 were stained for analytical DNA flow cytometry, analysed, and the proportion of cells in various phases of the cell cycle calculated as detailed in Section 2.7. Data are expressed as a percentage of the S-phase cells measured in control cultures i.e. 36.1 ± 1.0% and are the means of 2 - 4 observations from 2 - 4 separate experiments. Symbols are as presented in Figure 7.1.

of the cell cycle (Sutherland et al., 1983a,b, 1984). As was observed with effects on cell number, dose-response curves for drug effects on S-phase were biphasic with the exception of compound PR H i.e. there was a dose-dependent decrease in S-phase cells between 10^{-10} and 10^{-8} M, no further change between 10^{-8} and 10^{-6} M and a further concentration-dependent decrease in S-phase at concentrations > 10^{-6} M. Relative potencies in inducing the changes in the cell cycle kinetic parameters within the concentration range 10^{-10} to 10^{-8} M were in the same order as the effects on cell number i.e. Cl > Et > Br > NO_2 > CN > H and were correlated with affinity for ER. Growth inhibition in the micromolar range was also accompanied by a depletion of cells in S-phase and an accumulation of cells in G_1-phase of the cell cycle. Relative potencies for depletion of the proportion of S-phase cells at concentrations > 10^{-6} M were identical to those seen with changes in cell number i.e. Br $\geq$ H $\geq$ Cl > Et > CN > NO_2.

In separate experiments, drug effects on the proportion of S-phase cells at a concentration of 10 nM were largely reversed by the simultaneous administration of E_2 (Table 7.1), while at a concentration of 2.5 μM the effects were also reversed by E_2 except in the case of PR Br and PR Et. This observation is in contrast to the incomplete oestrogen-reversibility of the effects on cell number which occurred with all the PR compounds at concentrations of 2.5 μM, as noted previously. Thus in the presence of E_2 cell numbers remained depressed, despite a relative restoration in the proportion of S-phase cells. This suggests that these compounds have inhibited cell proliferation by an increase in cell death rate. This phenomenon is illustrated in Figure 7.3A, where a line has been fitted to pooled data relating cell number to cells in S-phase expressed as a percentage of control, at all drug

concentrations. In the absence of E_2 the slope of this line is close to unity showing that any decrease in cell number can be largely accounted for by the decrease in the S-phase population. A slight discrepancy does exist, however, with the S-phase population always being relatively greater than expected for a given cell number, showing the presence of an additional inhibitory component on cell growth. At the highest drug concentrations, where cell number is reduced to below 10% of control values, this additional component accounts for more than 50% of the decrease in cell number, and is presumed to be cytotoxic activity. In the presence of E_2 the relationship between cell number and S-phase undergoes a transformation (Figure 7.3A). Although an effect on S-phase remains, this is insufficient to account for more than approximately 50% inhibition of cell growth, indicating that a large proportion of cell growth inhibition is now occurring through the non-cell cycle specific cytotoxic mechanism postulated above.

Although potency in the micromolar range was more closely related to affinity for AEBS than ER, the large disparity between affinity for AEBS, which was in the 0.8 - 3.3 nM range, and the concentrations of drug required for half-maximal responsiveness for this second mechanism of growth inhibition i.e. 2 - 7.5 μM argues against the direct involvement of AEBS in this process. Perhaps a more plausible explanation is that those properties of the triarylethylene molecule which favour strong interactions with AEBS also facilitate interactions with regulatory molecules controlling this pathway.

7.3.2 CI 628 Analogues

All CI 628 analogues tested, with the exception of compound 25, were potent inhibitors of cell proliferation in the micromolar

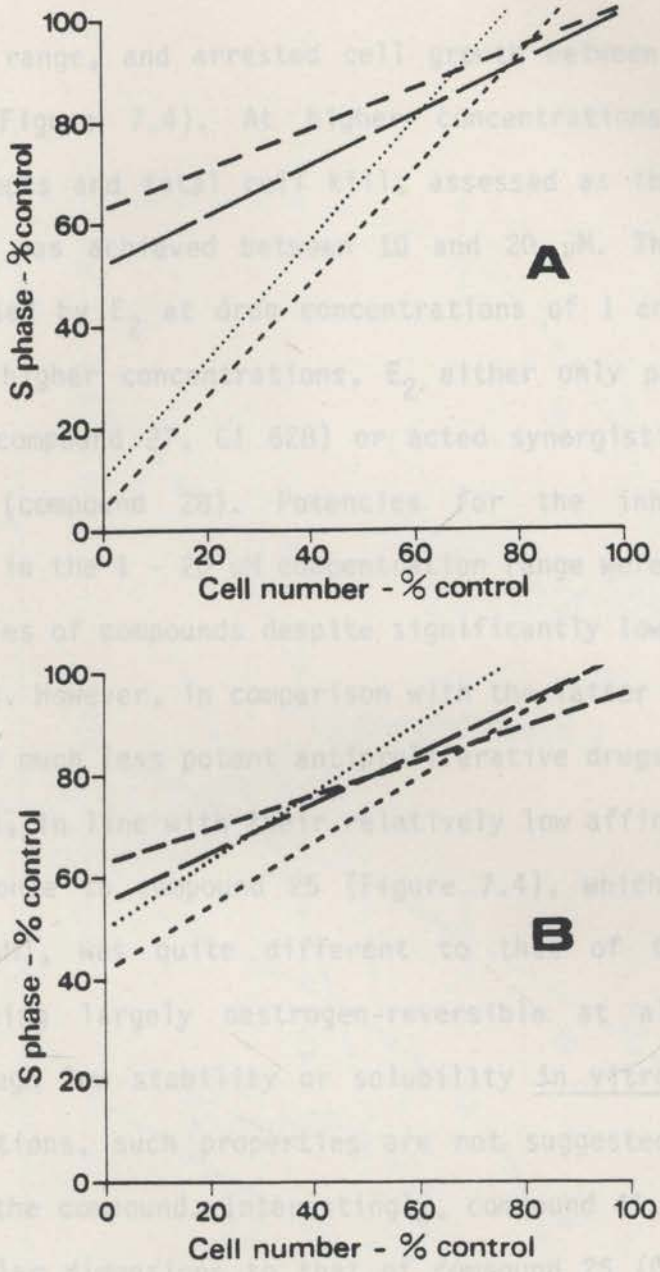

Figure 7.3: Relationship between cell number and cells in S-phase after drug treatment. At all concentrations of a given series of compounds either in the presence or absence of E_2, cell number and the corresponding proportion of cells in S-phase were determined, and expressed as a percentage of untreated control values. The lines, calculated by linear regression analysis, represent: (A) data from the PR series in the absence (-----) or presence (— — —) of E_2 and CI 628 analogues in the absence (.....) or presence (——) of E_2; (B) bibenzyl compounds in the absence (-----) or presence (.....) of E_2, SKF 525A analogues (——) and phenothiazine derivatives (— — —).

concentration range, and arrested cell growth between 4.4 and 10.5 μM (Table 7.3, Figure 7.4). At higher concentrations the drugs had cytotoxic effects and total cell kill, assessed as the absence of any viable cells, was achieved between 10 and 20 μM. These effects were largely reversed by E_2 at drug concentrations of 1 and 2.5 μM (Figure 7.4) but, at higher concentrations, E_2 either only partially reversed the effects (compound 27, CI 628) or acted synergistically to inhibit cell growth (compound 28). Potencies for the inhibition of cell proliferation in the 1 - 20 μM concentration range were similar to those of the PR series of compounds despite significantly lower affinities for ER (Table 7.3). However, in comparison with the latter series the CI 628 analogues were much less potent antiproliferative drugs at submicromolar concentrations, in line with their relatively low affinities for ER.

The response to compound 25 (Figure 7.4), which had low potency (IC_{90} > 20 μM), was quite different to that of the other CI 628 analogues, being largely oestrogen-reversible at all concentrations tested. Although low stability or solubility in vitro may account for these observations, such properties are not suggested by the chemical structure of the compound. Interestingly, compound 41, which has a side chain of similar dimensions to that of compound 25 ($O[CH_2]_2N(CH_3)_2$ and $[CH_2]_3N(CH_3)_2$, compounds 25 and 41 respectively) is also of low potency. Compound 35, which has a methyl-substituted side chain otherwise identical to that of compound 25, has relatively low cytotoxic potency, and TAM, which has a side chain identical to compound 25, also has relatively low potency in the micromolar range (IC_{DD} 8 μM, Murphy and Sutherland, 1985). Such side chains, extending only a relatively short distance from the aromatic portion of the molecule and terminating in a dimethylamino group, appear suboptimal for growth inhibitory activity in

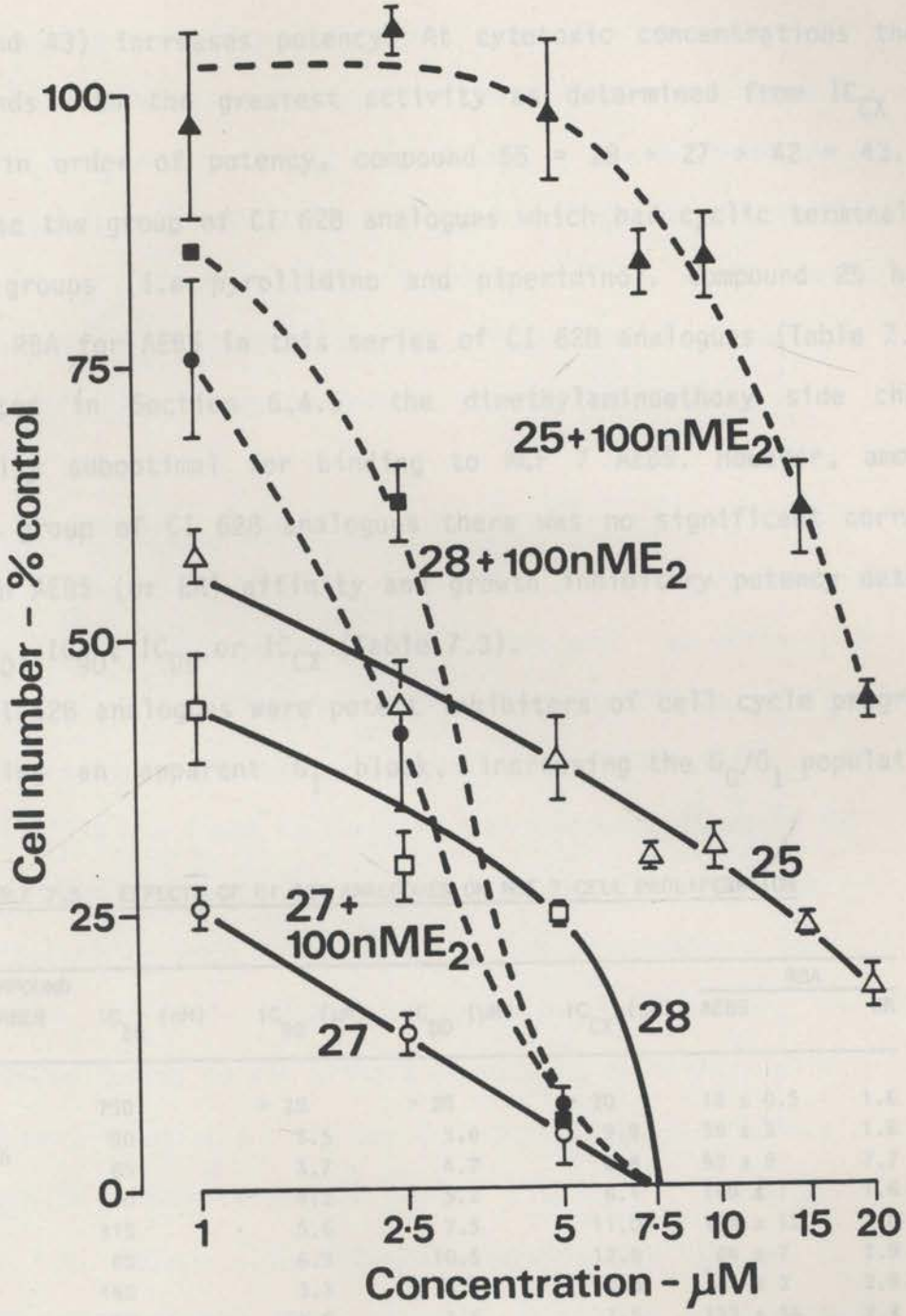

Figure 7.4: Effect of CI 628 analogues on the growth of MCF 7 cells. Cells were grown and treated with drugs as described in Section 2.6. Representative dose-response curves are shown for compounds 25 (△), 27 (○) and 28 (□) in the absence (open symbols, solid lines) or presence (solid symbols, dashed lines) of 100 nM E_2.

the micromolar range. Increasing side chain dimensions by increasing the size of the amino substituents (compound 26 and 28; compound 41 vs compound 43) increases potency. At cytotoxic concentrations the five compounds with the greatest activity as determined from IC_{CX} values were, in order of potency, compound 55 = 28 > 27 > 42 = 43. These comprise the group of CI 628 analogues which had cyclic terminal alkyl amino groups (i.e pyrollidino and piperidino). Compound 25 has the lowest RBA for AEBS in this series of CI 628 analogues (Table 7.3) and as noted in Section 6.4.5 the dimethylaminoethoxy side chain is generally suboptimal for binding to MCF 7 AEBS. However, among the entire group of CI 628 analogues there was no significant correlation between AEBS (or ER) affinity and growth inhibitory potency determined as IC_{30}, IC_{90}, IC_{DD} or IC_{CX} (Table 7.3).

CI 628 analogues were potent inhibitors of cell cycle progression, producing an apparent G_1 block, increasing the G_0/G_1 population and

TABLE 7.3 EFFECTS OF CI 628 ANALOGUES ON MCF 7 CELL PROLIFERATION

COMPOUND NUMBER	IC_{30} (nM)	IC_{90} (μM)	IC_{DD} (μM)	IC_{CX} (μM)	RBA AEBS	RBA ER
25	750	> 20	> 20	> 20	18 ± 0.5	1.6
26	90	3.5	5.6	9.9	38 ± 3	1.6
27[b]	65	3.7	4.7	6.8	82 ± 9	7.7
28	50	4.2	5.2	6.1	140 ± 7	1.6
35	315	5.6	7.5	11.0	134 ± 12	7.0
41	65	6.9	10.5	12.6	64 ± 7	2.9
42	140	3.3	4.9	7.5	21 ± 2	2.9
43	50	4.0	5.6	7.5	133 ± 14	9.2
53	280	6.6	7.3	8.5	205 ± 17	4.7
55	185	4.1	5.1	6.1	119 ± 1	4.7

[a] Structures given in Tables 6.5 A,B,C; [b] CI 628.

hence decreasing the proportion of cells in both the S and G_2 + M phases, as illustrated by the series of DNA histograms obtained at increasing concentrations of CI 628 (compound 27, Figure 7.5). Linear correlations between cell number and cells in S-phase for all analogues over the concentration range 1 - 20 μM, in the presence or absence of E_2, are shown in Figure 7.3A. The relationships between cell number and S-phase are similar to those observed for PR series, with clearly distinct oestrogen-reversible and -irreversible mechanisms of cell growth inhibition being apparent.

7.3.3 Bibenzyl Derivatives

The H series of bibenzyl derivatives allowed the growth inhibitory effects of weak antioestrogenic compounds with low affinity for ER but relatively high affinity for AEBS to be studied. These compounds were synthesized by Emmens' group as potential antifertility agents (Emmens et al., 1969) who found that all of the compounds shown in Table 7.4 had local antioestrogenic effects in the mouse vagina, but only H 237 had this activity when administered subcutaneously. None displayed oestrogenic effects. With the exception of H 233, the bibenzyl derivatives tested in the present study were effective inhibitors of MCF 7 cell proliferation at micromolar concentrations, completely arresting cell growth between 5 and 10 μM and killing all cells at 15 - 20 μM (Table 7.4, Figure 7.6). The simultaneous addition of E_2 failed to significantly reverse these effects. Among the six compounds studied there was no significant correlation between binding affinity for AEBS and potency, and at all concentrations there was a tendency for potency to parallel RBA for ER.

H 233 was a weak inhibitor of growth and failed to achieve 90%

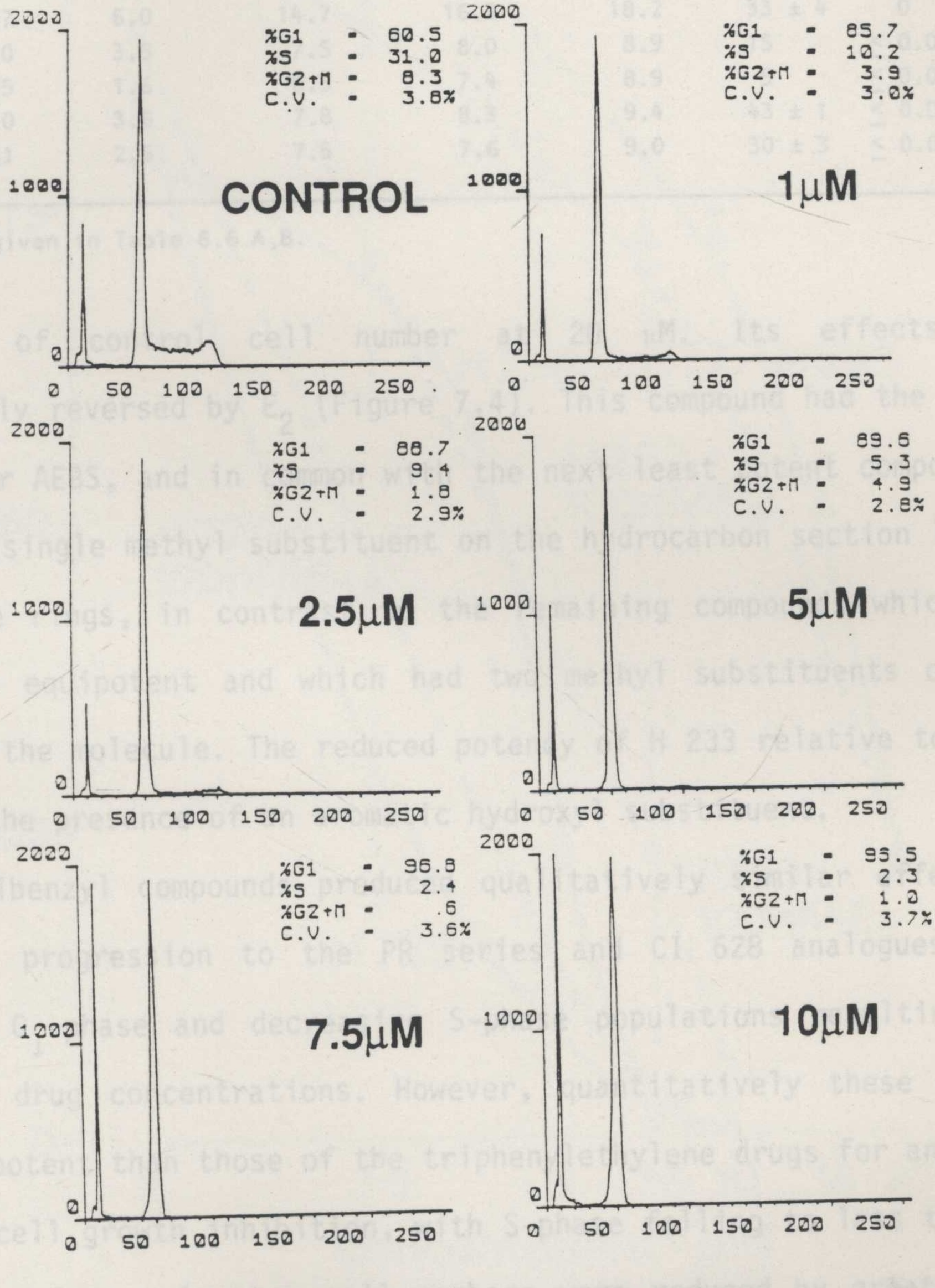

Figure 7.5: Effect of CI 628 (Compound 27) on cell cycle phase distribution. Cells from the experiment described in Figure 7.4 were stained for DNA flow cytometry, analysed and the proportion of cells in various phases of the cell cycle calculated as detailed in Section 2.7.

TABLE 7.4 EFFECTS OF BIBENZYL COMPOUNDS ON MCF 7 CELL PROLIFERATION

COMPOUND NUMBER[a]	COMPOUND	IC_{30} (μM)	IC_{90} (μM)	IC_{DD} (μM)	IC_{CX} (μM)	RBA AEBS	RBA ER
57	H 233	6.5	> 20	> 20	> 20	1	0
58	H 237	6.0	14.7	16.2	18.2	33 ± 4	0
70	H 310	3.8	7.5	8.0	8.9	15	≤ 0.04
73	H 725	1.6	6.5	7.4	8.9	5	≤ 0.08
71	H 730	3.6	7.8	8.3	9.4	43 ± 1	≤ 0.02
72	H 731	2.5	7.5	7.6	9.0	30 ± 3	≤ 0.02

[a] Structures given in Table 6.6 A,B.

inhibition of control cell number at 20 μM. Its effects were significantly reversed by E_2 (Figure 7.4). This compound had the lowest affinity for AEBS, and in common with the next least potent compound, H 237, has a single methyl substituent on the hydrocarbon section linking its benzene rings, in contrast to the remaining compounds which were essentially equipotent and which had two methyl substituents on this section of the molecule. The reduced potency of H 233 relative to H 237 is due to the presence of an aromatic hydroxyl substituent.

The bibenzyl compounds produced qualitatively similar effects on cell cycle progression to the PR series and CI 628 analogues, with increasing G_1-phase and decreasing S-phase populations resulting from increasing drug concentrations. However, quantitatively these effects were less potent than those of the triphenylethylene drugs for any given degree of cell growth inhibition, with S-phase falling to less than 50% of control values only when cell numbers were reduced by greater than 90% (Figure 7.3B). The cell cycle effects were only marginally reversed by E_2 (Figure 7.6), in line with the low ER affinity of these compounds. Instead, the response to these compounds was quantitatively similar to

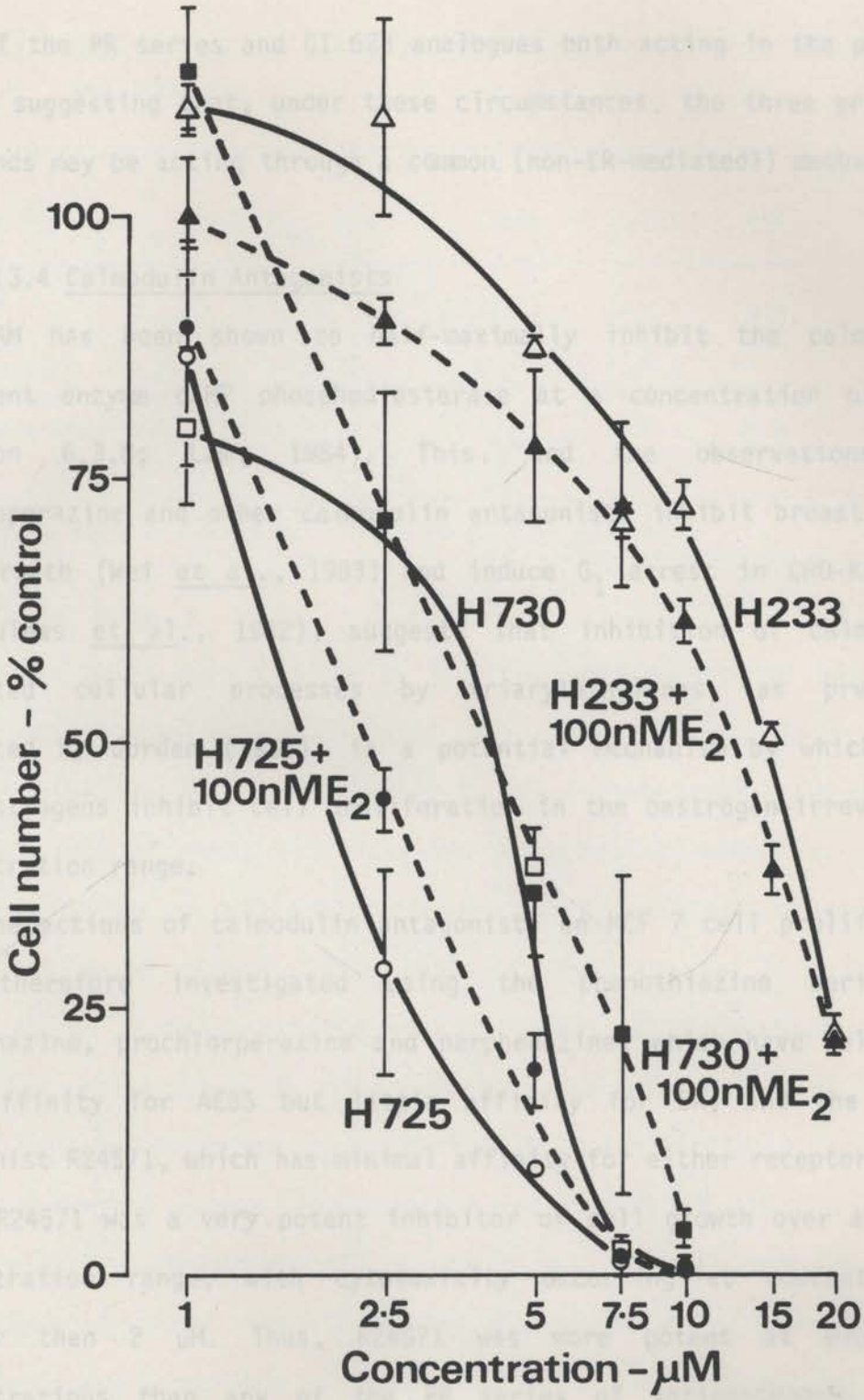

Figure 7.6: Effects of bibenzyl derivatives on the growth of MCF 7 cells. Cells were grown and treated with drugs as described in Section 2.6. Representative dose-response curves for H 233 (△) H 725 (○) and H 730 (□) in the absence (open symbols, solid lines) and presence (closed symbols, dashed lines) of 100 nM E_2.

that of the PR series and CI 628 analogues both acting in the presence of E_2, suggesting that, under these circumstances, the three groups of compounds may be acting through a common (non-ER-mediated?) mechanism.

7.3.4 Calmodulin Antagonists

TAM has been shown to half-maximally inhibit the calmodulin-dependent enzyme cAMP phosphodiesterase at a concentration of 2 μM (Section 6.3.8; Lam, 1984). This, and the observations that trifluoperazine and other calmodulin antagonists inhibit breast cancer cell growth (Wei et al., 1983) and induce G_1 arrest in CHO-K1 cells (Chafouleas et al., 1982), suggests that inhibition of calmodulin-activated cellular processes by triarylethylenes, as previously suggested by Jordan (1984), is a potential mechanism by which these antioestrogens inhibit cell proliferation in the oestrogen-irreversible concentration range.

The actions of calmodulin antagonists on MCF 7 cell proliferation were therefore investigated using the phenothiazine derivatives fluphenazine, prochlorperazine and perphenazine, which have relatively high affinity for AEBS but little affinity for ER, and the potent antagonist R24571, which has minimal affinity for either receptor (Table 7.5). R24571 was a very potent inhibitor of cell growth over a narrow concentration range, with cytotoxicity occurring at concentrations greater than 2 μM. Thus, R24571 was more potent at micromolar concentrations than any of the PR series of antioestrogens. These effects were not significantly reversed by E_2 (Figure 7.7). The phenothiazines also displayed growth inhibitory and cytotoxic properties, but at relatively high concentrations. These effects were oestrogen-irreversible (Figure 7.7) and potencies were not correlated

TABLE 7.5 EFFECTS OF SKF-525A ANALOGUES, PHENOTHIAZINES AND R24571 ON MCF 7 CELL PROLIFERATION

COMPOUND	IC_{30} (μM)	IC_{90} (μM)	IC_{DD} (μM)	IC_{CX} (μM)	RBA AEBS	RBA ER
19086A[a]	6.2	13.8	13.6	14.4	0.5	-
19500A[a]	3.4	15	14.4	19	2.6	-
Fluphenazine[b]	5.0	10.5	10.9	11.7	11	0.05
Perphenazine[b]	5.9	11.6	12.7	14.5	33 ± 5	0.03
Prochlorperazine[b]	5.9	13.3	15.2	16.5	12 ± 1	0.004
R24571	0.3	1.8	2.1	2.3	0	$\leq$ 0.01

[a] Structures given in Table 6.9; [b] Structures given in Table 6.10.

with RBA for AEBS.

Cell numbers were reduced to less than 50% of control before the phenothiazines induced significant cell cycle changes. With further growth inhibition, a gradual decline in the S-phase population occurred (Figure 7.3B). At prochlorperazine and perphenazine concentrations of $\geq$ 10 μM, inhibition of cell proliferation could be partially attributed to an inhibition of mitosis, with cells accumulating in G_2 + M phase. Hence, at 15 μM cells treated with perphenazine had a 15.9% G_2 + M population, while those treated with prochlorperazine had 31.6% of cells in G_2 + M phase, representing approximately 2- and 4-fold increases over untreated control values. Fluphenazine and R24571 did not display this activity at any concentration. In common with other compounds tested, R24571-induced growth inhibition was associated with accumulation of cells in G_1 and a decrease in the S-phase population. Although the data are somewhat limited, growth inhibition and cytotoxicity appear to occur in the concentration range where these compounds maximally inhibit calmodulin (Section 6.3.8). This relationship requires further study, although the range of effects these compounds have on cell cycle

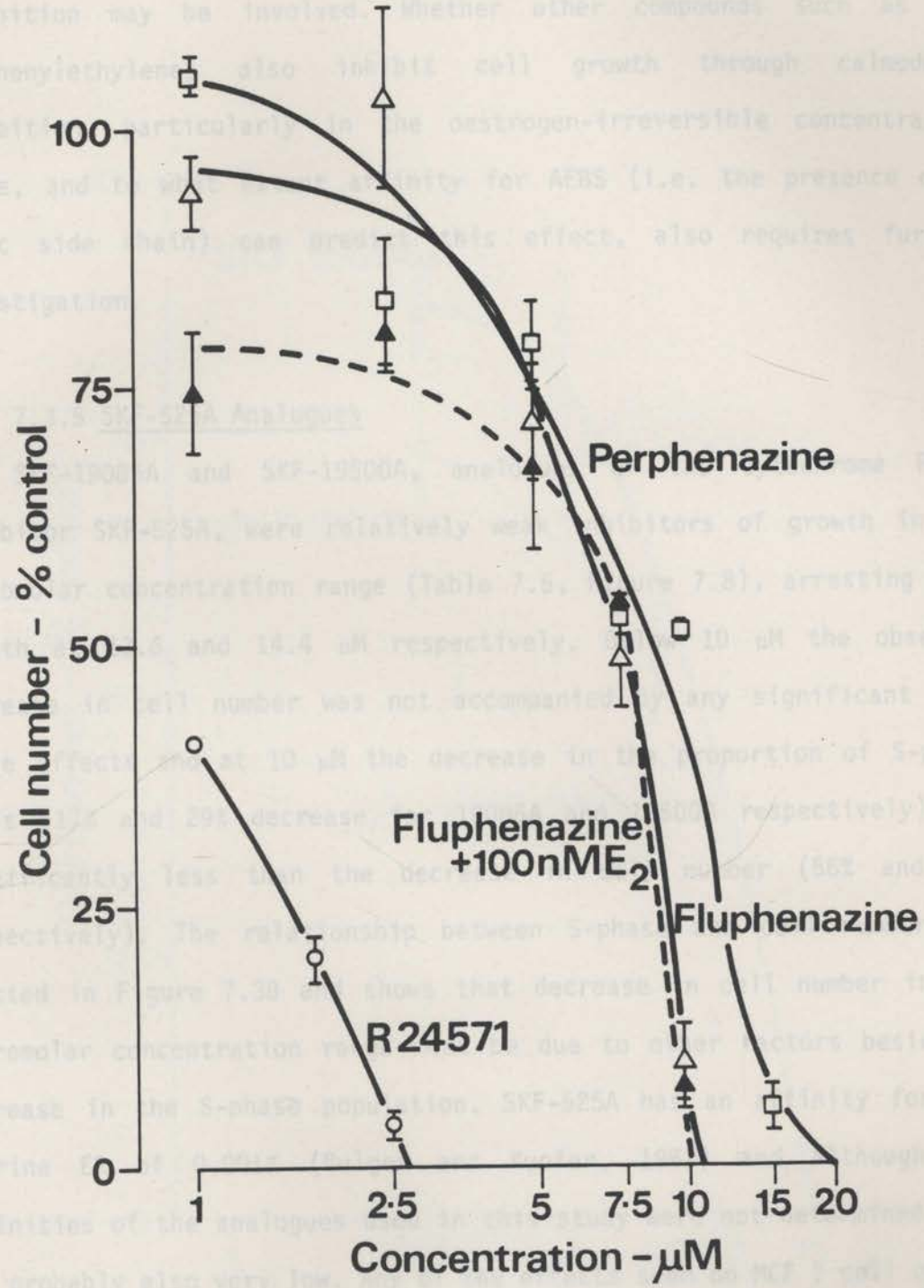

Figure 7.7: Effect of calmodulin antagonists on the growth of MCF 7 cells. Cells were grown and treated with drugs as described in Section 2.6. Representative dose-response curves are shown for R24571 (○) perphenazine (□), fluphenazine (△), and fluphenazine + 100 nM E_2 (▲).

parameters suggests that other mechanisms apart from calmodulin inhibition may be involved. Whether other compounds such as the triphenylethylenes also inhibit cell growth through calmodulin inhibition, particularly in the oestrogen-irreversible concentration range, and to what extent affinity for AEBS (i.e. the presence of a basic side chain) can predict this effect, also requires further investigation.

7.3.5 SKF-525A Analogues

SKF-19086A and SKF-19500A, analogues of the cytochrome P-450 inhibitor SKF-525A, were relatively weak inhibitors of growth in the micromolar concentration range (Table 7.5, Figure 7.8), arresting cell growth at 13.6 and 14.4 μM respectively. Below 10 μM the observed decrease in cell number was not accompanied by any significant cell cycle effects and at 10 μM the decrease in the proportion of S-phase cells (17% and 29% decrease for 19086A and 19500A respectively) was significantly less than the decrease in cell number (56% and 74% respectively). The relationship between S-phase and cell numbers is plotted in Figure 7.3B and shows that decrease in cell number in the micromolar concentration range must be due to other factors besides a decrease in the S-phase population. SKF-525A has an affinity for rat uterine ER of 0.001% (Bulger and Kupfer, 1982) and although the affinities of the analogues used in this study were not determined they are probably also very low. Any of the effects seen on MCF 7 cell growth are thus unlikely to be ER-mediated.

The results of this study have extended previous observations made in this laboratory (Murphy and Sutherland, 1983a, 1985; Reddel et al., 1983), and allow several conclusions to be drawn regarding the

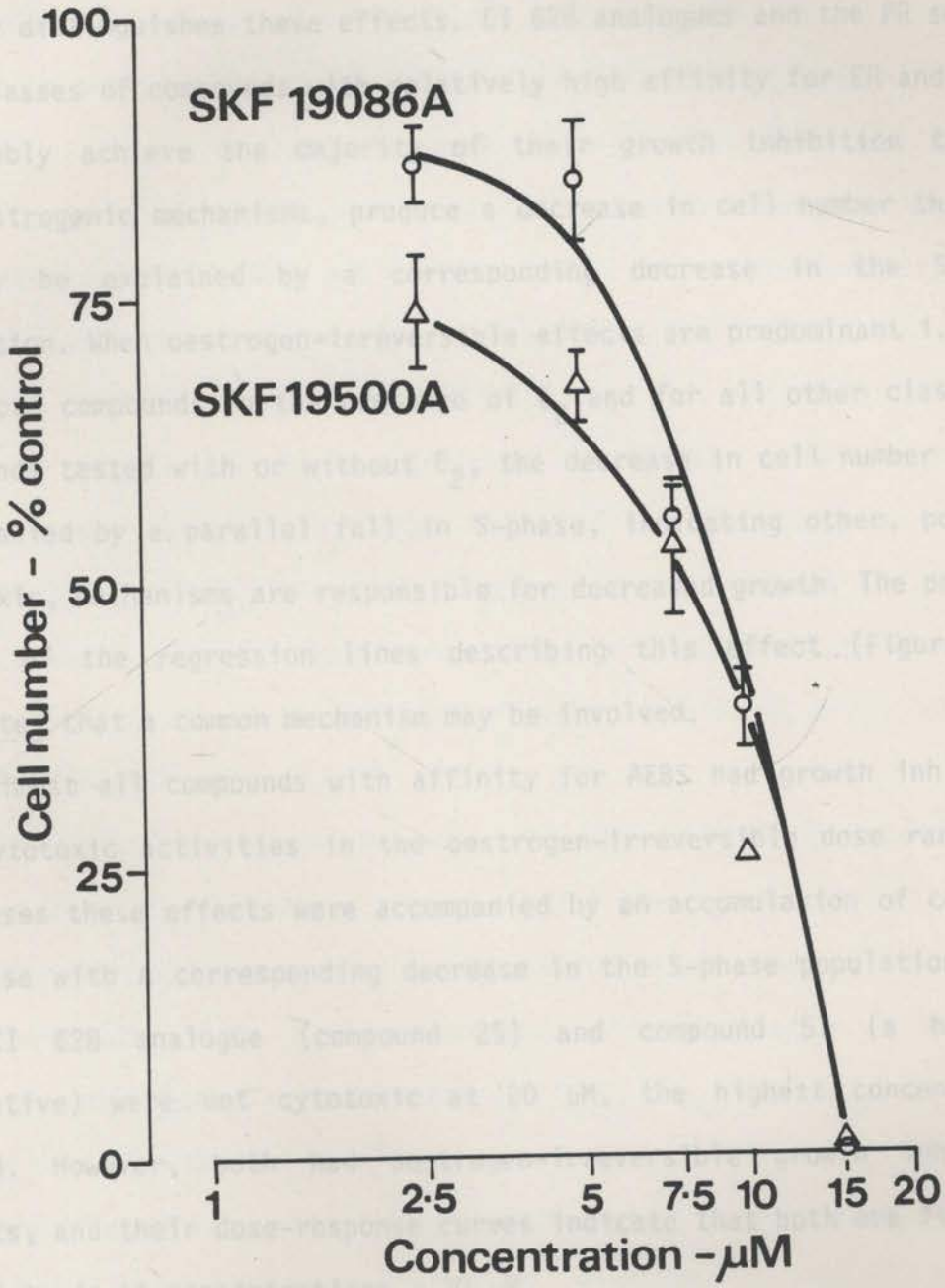

Figure 7.8: Effects of SKF-525A analogues on the growth of MCF 7 cells. Cells were grown and treated with drugs as described in Section 2.6. Representative dose-response curves are shown for SKF-19086A (○) and SKF-19500A (△).

relationship between ligand binding to ER and AEBS, and inhibition of MCF 7 breast cancer cell proliferation. Firstly, for several classes of compounds, this study has confirmed the existence of oestrogen-reversible and -irreversible effects on cell proliferation. Figure 7.3 clearly distinguishes these effects. CI 628 analogues and the PR series, both classes of compounds with relatively high affinity for ER and which presumably achieve the majority of their growth inhibition through antioestrogenic mechanisms, produce a decrease in cell number that can largely be explained by a corresponding decrease in the S-phase population. When oestrogen-irreversible effects are predominant i.e. for the above compounds in the presence of E_2 and for all other classes of compounds tested with or without E_2, the decrease in cell number is not accompanied by a parallel fall in S-phase, indicating other, possibly cytotoxic, mechanisms are responsible for decreased growth. The parallel nature of the regression lines describing this effect (Figure 7.3) indicates that a common mechanism may be involved.

Almost all compounds with affinity for AEBS had growth inhibitory and cytotoxic activities in the oestrogen-irreversible dose range. In all cases these effects were accompanied by an accumulation of cells in G_1-phase with a corresponding decrease in the S-phase population. Only one CI 628 analogue (compound 25) and compound 57 (a bibenzyl derivative) were not cytotoxic at 20 μM, the highest concentration tested. However, both had oestrogen-irreversible growth inhibitory effects, and their dose-response curves indicate that both are likely to be cytotoxic at concentrations > 20 μM.

Other studies from this laboratory (Murphy and Sutherland, 1985; and unpublished observations) have shown that compounds structurally related to those used in the present study but without basic side chains

(and hence without affinity for AEBS) have reduced potency in the oestrogen-irreversible growth inhibitory concentration range and do not express cytotoxic activity at concentrations < 20 μM. However, despite the obvious functional importance of a basic side chain, the present study does not support the hypothesis that the AEBS directly mediates biological activity. When the pooled data for all compounds is subject to linear regression analysis both affinity for AEBS and ER are generally correlated with potency at all concentrations, particularly when log transformed data are used. However, the affinities for ER and AEBS of these series of compounds are related (log AEBS = 1.63 + 0.212 log ER, $r = 0.644$, $p < 0.001$, $n = 22$) and when this factor is taken into account, using multiple regression analysis, potency at all concentrations remains correlated with RBA for ER (IC_{30}, $p < 0.005$; IC_{90}, $p < 0.005$; IC_{DD}, $p < 0.01$; IC_{CX}, $p < 0.025$) whereas affinity for AEBS proves to have no significance. Only in the case of the PR series, where affinities for both sites are closely correlated, are potency and affinity for AEBS related.

The good correlation between affinity for ER and potency as determined by IC_{30} values is in accord with the hypothesis that effects on cell proliferation at low, oestrogen-reversible drug concentrations are ER-mediated i.e. anti-oestrogenic. The correlation between ER affinity and potency as determined by IC_{90}, IC_{DD} and IC_{CX} may simply reflect the contribution made by antioestrogenic activity.

These studies, then, fail to support the hypothesis that interactions with AEBS are directly involved in control of cell proliferation. The differences in cell cycle changes induced by TAM in ER-positive and ER-negative cell lines, despite the presence of AEBS in both (Reddel et al., 1983), supports this conclusion. Further studies

are required to determine the biochemical basis of oestrogen-reversible and -irreversible growth inhibition.

CHAPTER 8

GENERAL DISCUSSION

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Knowledge of the intracellular binding of antioestrogens is critical to an understanding of the mechanisms involved in their antioestrogenic and antitumour activity. Numerous studies have described the generally good correlation between affinity for ER, potency, and the reversal of many effects of these drugs by the simultaneous or subsequent administration of oestrogen. There is, therefore, strong evidence that direct interaction with ER is of central importance. The present study, however, was concerned with the characterization of other high affinity intracellular interactions of antioestrogens, in particular those with the AEBS, and assessing their direct or indirect involvement in the expression of both ER-mediated, and apparently non-ER-mediated biological activities. Unexplained features of drug action in which such sites were considered to have a potential role included the molecular mechanisms determining the expression of antagonist or agonist activities, including species and tissue differences and several apparently non-ER-mediated actions observed both *in vivo* and *in vitro*. Among these were: cytotoxic effects on uterine glandular epithelium (Martin, 1981) and on breast cancer cells in culture (Sutherland *et al.*, 1984); inhibition of smooth muscle contractility (Lipton *et al.*, 1984; Brandes *et al.*, 1985; Morris, 1985); side effects observed during antioestrogen therapy including those apparently involving the autonomic nervous system (Ben-Baruch, 1982; Morris, 1985; Nolvadex Adjuvant Trial Organisation, 1985); inhibition of cytochrome P-450-mediated drug metabolism (Ruenitz and Toledo, 1980; Meltzer *et al.*, 1984); and inhibition of calmodulin dependent enzymes (Lam, 1984; Tattant and Wallace, 1985).

8.1 SUBCELLULAR LOCALIZATION AND BIOCHEMICAL CHARACTERIZATION OF THE AEBS

Although first identified as a cytosolic entity, the majority of AEBS can be recovered in all tissues from the microsomal fraction. Analytical subcellular distribution studies in MCF 7 cells, rat liver and rat uterus (Miller and Katzenellenbogen, 1983; Sudo et al., 1983; Watts et al., 1984; Watts and Sutherland, 1984) have shown that AEBS is most likely associated with the endoplasmic reticulum (Figure 3.1, 4.2). These and other studies have noted the presence of low concentrations of this site in other subcellular compartments, particularly within the nuclear fraction (Gulino and Pasqualini, 1982; Murphy et al., 1984b; Chouvet and Saez, 1984; Kon, 1985a,b). It is highly likely microsomal contamination can account for these findings, a conclusion reinforced by the close similarity in properties of the nuclear and microsomal AEBS. There is also no evidence for translocation of the AEBS from extranuclear to nuclear compartments (Murphy and Sutherland, 1981b).

The ultimate subcellular localization of the AEBS remains to be determined. Association with rough endoplasmic reticulum is suggested by one submicrosomal fractionation technique (Figures 3.2, 4.4), but although similar observations have been made by others (Katzenellenbogen et al., 1985a), another technique showed a more even distribution between rough and smooth microsomal fractions (Figure 4.5). A clear demonstration of localization to rough endoplasmic reticulum would be of great interest given that the presence of ribosomes and the associated membrane components involved in protein synthesis are the major features distinguishing this organelle from smooth endoplasmic reticulum. To clarify the localization of the AEBS electron microscopy could be utilized to characterize more precisely the organelle composition of the

subcellular fractions generated by different techniques. Such studies would be greatly enhanced by the use of autoradiographic techniques using [^{3}H]TAM or [^{125}I]TAM, or by the availability of AEBS-directed antibodies.

Biochemical characterization of the AEBS reveals a molecular species with receptor-like properties, that is with high affinity, present in low concentrations, and with specificity for a relatively narrow range of structurally-related compounds. The interaction of [^{3}H]TAM is reversible, and kinetic binding data are compatible with association and dissociation being bi- and mono-molecular processes respectively, with no evidence of ligand transformation occurring as the result of interaction with AEBS. The kinetic and equilibrium binding properties of the AEBS are similar to those of the ER, as determined by interaction with [^{3}H]TAM. However, other properties (including cellular concentration, subcellular distribution, thermal stability, enzyme sensitivity and ligand specificity) clearly demonstrate the separate nature of these two sites.

Although the AEBS is at least part protein, as shown by its sensitivity to proteolytic enzymes, particularly in the presence of detergent, lipid is also apparently required for activity. Solubilized from microsomal material only by high concentrations of the detergent sodium cholate, the AEBS has the properties of an intrinsic membrane protein, most likely of highly hydrophobic character. The successful detergent solubilization of the site without irreversible loss of binding activity, confirmed by the identification of a high molecular weight form (M_r 440,000 - 490,000, Figure 5.4), allows possible purification strategies to be considered. A major obstacle to progress in this direction is that ligand binding is, as yet, the only means of

following AEBS through any purification procedure. The loss of binding activity in the presence of detergent necessitates dilution to allow detection, reducing sensitivity and precluding the use of pre-labelled material during purification. Furthermore, procedures such as hydrophobic interaction chromatography and PEG-6000 precipitation apparently separate AEBS from other microsomal components, resulting in loss of activity as determined by charcoal/dextran-based assay procedures. This loss of activity can possibly be explained by the sedimentation of labelled aggregated material. However, the AEBS may be directly dependent upon other microsomal components for binding activity. Reconstitution techniques may be able to restore activity or maintain AEBS in a non-aggregated state. The lipid requirement of the AEBS as demonstrated by susceptibility to phospholipase and lipase also implies that reconstitution will be necessary to restore the binding activity of purified AEBS protein.

Experiments which have demonstrated the solubilization of PEG-precipitated material in sodium cholate in the absence of high salt concentrations may allow the use of ion-exchange techniques in the purification of AEBS. It may also be worthwhile investigating whether such material could be solubilized at concentrations of sodium cholate or other detergents low enough to allow [^{3}H]TAM binding. This would enable the purification of prelabelled material and also the use of highly selective binding site-directed affinity chromatography techniques. Photoaffinity labelling of microsomes with either TAM or phenothiazines needs further investigation to determine whether a true or pseudo-photolabelling process can be used to selectively covalently label the AEBS. Optimal conditions for labelling may require the use of radiation at different wavelengths or the use of scavenger molecules

other than BSA to prevent non-specific protein or lipid labelling. The unique observation that the tritium label of [^{3}H]TAM becomes covalently bound following U.V.-irradiation also requires investigation to determine whether the whole molecule or only a photolysed side chain fragment becomes bound. The phenomenon could have implications for the *in vivo* action of this and other antioestrogens, and might explain, for example, the phototoxicity (and retinal damage?) sometimes observed as side effects during antioestrogen therapy.

In summary, the purification of AEBS would be greatly facilitated by the availability of a non-dissociating label that could be monitored during chromatographic or other procedures. Alternatively, detergent solubilized material could be used in the production of antibodies against the AEBS. These might then allow detection of AEBS despite loss of binding activity during purification, and purification by immuno-affinity chromatography. Antibodies could also be used in the screening of expression libraries generated from MCF 7 mRNA, with the aim of isolating cDNA clones coding for the AEBS protein. Expression of AEBS cDNA in appropriate cell lines might facilitate purification and identification of the protein. Such an approach may only be justified if there is evidence that the AEBS has an important role in normal cellular physiology, or in the pharmacology of drug action.

8.2 ANTIOESTROGENIC ACTIVITY AND THE AEBS

8.2.1 Structure-Activity Studies

The structural requirements of ligand binding to AEBS have been fully discussed in Chapter 6 and it is not proposed to reiterate them here in any detail. In summary, binding to AEBS requires the presence of a side chain terminating in any one of a number of basic alkyl amino

groups, connected to a hydrophobic aromatic ring structure of sufficient size. Substituents on this ring structure can influence affinity by local (steric) or distant (electronic) effects, but the main determinant of binding affinity is side chain terminal group structure. Optimum binding is shown by tertiary-, in particular cyclic-, alkyl amino groups.

Several lines of evidence support the conclusion that the AEBS is unlikely to be directly involved in mediating antioestrogenic activity: a) antioestrogenic activity is expressed by compounds which have no affinity for AEBS; b) compounds which bind to AEBS do not necessarily display antioestrogenic activity and may in fact be predominantly oestrogenic (or else have neither of these activities) and c) among compounds that do bind to AEBS there is little correlation of RBA for this site with antioestrogenic activity.

It was earlier believed that an alkylaminoethoxy side chain was essential for the antioestrogenic activity of triphenylethylenes and related compounds (Jordan et al., 1981a). Analogues from which these side chains have been removed (e.g. of TAM, 4-hydroxyTAM and trioxifene, Abbott et al., 1976; Jordan and Gosden, 1982; Jordan et al., 1984; of nafoxidine, Lednicer et al., 1965, 1966, 1967 and Jordan et al., 1981a; and of diphenylethylene, Leclerq et al., 1983) have no antioestrogenic activity and generally act as full oestrogen agonists. These compounds have no affinity for the AEBS. However, it has become apparent that a variety of derivatives which carry non-basic side chains, and which also have no affinity for AEBS, may have antioestrogenic activity although usually of reduced potency. Thus, U 23,469 is antioestrogenic in rat uterus (Hayes et al., 1981) and weakly so in the chick oviduct (Sutherland, 1981), and has antitumour activity in oestrogen-dependent

DMBA-induced rat mammary tumours (Tsai et al., 1979). This compound has low affinity for ER (Ferguson and Katzenellenbogen, 1977; Sutherland, 1981) but no affinity for AEBS (Sudo et al., 1983). Similarly, several TAM analogues without basic side chains have been shown to possess antioestrogenic activity. 1,2-diphenyl-1-[4-(2-hydroxyethoxy)phenyl]but-1-ene, in which the dimethylamino group of TAM has been replaced by a hydroxyl group, has no affinity for AEBS (Sudo et al., 1983) and yet has comparable antioestrogenicity and ER binding affinity to TAM (Robertson et al., 1982a). ICI 145680 (Table 6.2) also has no affinity for AEBS, but has an affinity for ER equal to that of TAM, is a partial oestrogen antagonist - partial agonist in the rat uterotrophic assay, and has E_2-reversible growth inhibitory effects on MCF 7 cells in vitro, although it is less potent than TAM in this regard (Murphy and Sutherland, 1985). Other antioestrogenic compounds which lack basic side chains are referred to in Section 6.2.

The above examples demonstrate that expression of antioestrogenic activity is not dependent upon binding to AEBS. Neither does binding to AEBS necessarily result in antioestrogenic activity. Thus, the cis-isomers of TAM and enclomiphene i.e. ICI 47699 (compound 7) and zuclomiphene (compound 20) have relatively high affinity for AEBS, but are regarded as being weak oestrogens rather than antioestrogens, at least in the rat (Harper and Walpole, 1967; Jordan et al., 1981a,b). Both compounds are weakly antiuterotrophic only at very high dose.

A limited number of studies have investigated the antioestrogenic activities of compounds which bind to AEBS, but which have little or no affinity for ER. The most extensive study has been that of Lyman and Jordan (1985c), in which a series of five compounds was selected with structural resemblances to triphenylethylenes, particularly with regard

to side chain structure. The compounds had RBA for AEBS of 4 - 9.4% in mouse liver microsomes, similar to that of MER-25, (which, unlike TAM, is antioestrogenic in the mouse uterus), and all had low affinity for ER (< 0.06%). None of the compounds had uterotrophic activity. Three of the compounds were antioestrogenic as assessed by inhibition of E_2-induced increase in uterine weight, but mice treated with these compounds weighed less, suggesting these compounds were acting through toxic mechanisms. In addition, unlike MER-25, none of these compounds was able to inhibit the uterotrophic response to TAM. The authors concluded that none of these compounds were truly antioestrogenic, and that the AEBS does not mediate antioestrogenic activity. Affinity for ER appeared to be the main determinant of biological activity. It is perhaps unfortunate that the mouse was chosen as a model of antioestrogen action given that most antioestrogens (with the exception of MER-25) do not express this activity in this system.

Similar observations were made in a separate study by Lyman and Jordan (1985a) on the antioestrogenic effects of trifluoperazine in mice. Trifluoperazine, which has a relatively high affinity for AEBS (Table 6.10), has little or no affinity for ER *in vitro*, although *in vivo* it partially inhibited the uterine uptake of $[^3H]E_2$, suggesting the possibility of metabolism to a compound with higher affinity for ER. Although the drug appeared to be antiuterotrophic, this activity could be accounted for by diet restriction resulting from the tranquilizing activity of trifluoperazine.

The antioestrogenic properties of BPEA (Section 6.4.1.1) have been investigated by Sheen *et al*. (1985b). This compound, which has affinity for AEBS but none for ER (RBA 6% and < 0.0003% respectively), had neither uterotrophic nor antiuterotrophic activity in immature rats up

to 1 mg daily. The observations that concentrations of BPEA which should fully occupy the AEBS did not influence the partial agonist-antagonist properties of TAM in the uterus, or the growth inhibitory activities of TAM (dose range 10^{-11} - 10^{-6} M) in MCF 7 cells support the conclusion that binding to the AEBS does not directly mediate the effects of antioestrogens. It also argues against the earlier suggestion (Sutherland and Foo, 1979; Sudo et al., 1983) that the AEBS may indirectly modulate antioestrogen action by setting up concentration gradients into the cell, making high affinity compounds more readily available for interaction with the ER.

Brandes (1984), however, reached opposite conclusions in a study of the effects of DPPE (Section 6.4.1.1) on MCF 7 cells, finding that this compound inhibited cell growth and also enhanced the potency of TAM as a growth inhibitor. The effects of DPPE did not appear to be E_2-reversible. Similar E_2-irreversible effects on MCF 7 growth are observed for a wide range of compounds binding to the AEBS (Chapter 7).

In a further study, Brandes and Bogdanovic (1986) concluded that DPPE, acting through the AEBS, had antioestrogenic activity in the rat uterotrophic assay. Although the compound had antiuterotrophic activity when administered alone, the reduction of uterine weight even below that of untreated, immature ovariectomized rats is a phenomenon observed not even with the most potent triphenylethylene antioestrogens, suggesting that this is not an antioestrogenic effect per se. Such apparent antioestrogenic activity is also observed when DPPE and E_2 are administered together and may be mediated through the histamine antagonist activity of this compound (Kroeger and Brandes, 1985), perhaps on the smooth muscle of the myometrium. There is little evidence that the AEBS is involved in the biological activity of DPPE and studies

with [^{3}H]DPPE show that this compound also binds in rat liver microsomes to a population of sites distinct from the AEBS, (and H_1 receptor) with an affinity of 64 ± 13 nM and a binding site concentration of 12.5 pmol/mg protein (Brandes and Bogdanovic, 1986). The RBA of TAM for this site is approximately 25%. These results can be contrasted to those obtained by the same group for the rat liver microsomal AEBS for which DPPE had a binding affinity of 8 - 35% relative to TAM (predicting a K_D of approximately 3 - 12 nM). The AEBS concentration was 4 pmol/mg protein (Brandes and Hermonat, 1984; Brandes et al., 1985). Whether the biological effects of DPPE arise as the result of interaction with AEBS and/or the DPPE-specific site is unknown, but neither appears likely given that super-saturating concentrations of this compound (> 10^{-6} M) are required for activity.

Given the conflicting result obtained regarding the influence of DPPE and BPEA on the activity of TAM in MCF 7 cells it would be interesting to extend these studies with a larger range of compounds, preferably having higher affinity for AEBS, and similar low affinity for ER. The H series of compounds would be ideally suited to this purpose.

Among compounds with antioestrogenic activity which also bind to AEBS, activity is better correlated with affinity for ER. Thus, relative to TAM, 4-hydroxyTAM and LY 117018 have moderate and low affinities for AEBS respectively but both are more potent antioestrogens both in vivo (Jordan et al., 1977a; Black and Goode, 1980) and in vitro (Scholl et al., 1983), in line with their high affinities for ER. Both the present study and others with clomiphene analogues (Murphy and Sutherland, 1983a), and TAM metabolites (Reddel et al., 1983) TAM analogues (Murphy and Sutherland, 1985) and other antioestrogens (Wakeling et al., 1984) show MCF 7 cell growth-inhibitory potency in the oestrogen-reversible

concentration range that parallels RBA for ER but not AEBS. However, TAM analogues and other triphenylethylenes without basic side chains, such as ICI 145680, have significantly lower oestrogen-reversible growth inhibitory potency than TAM, despite similar affinity for ER. This lack of potency almost certainly arises because, without basic side chains, such compounds only express weak oestrogenic or antioestrogenic activity (i.e. they have low intrinsic antioestrogenic activity). These compounds are also less potent in the oestrogen-irreversible concentration range, and do not appear to express oestrogen-irreversible cytotoxicity, for which a basic side chain appears essential (Murphy and Sutherland, 1985). That such side chains are also required for binding to AEBS is most probably coincidental rather than of mechanistic significance as the potencies of the oestrogen-irreversible effects of compounds which bind to AEBS are not correlated with their affinities. Further, the concentrations at which such effects are apparent (> 5 μM) are approximately three orders of magnitude greater than are necessary to saturate a binding site with a K_D of 1 nM.

The majority of structure-activity data therefore suggests that AEBS is not directly involved in antioestrogenic activity.

8.2.2. Tissue Distribution Studies

The ubiquitous tissue distribution of the AEBS supports the above conclusion. In the rat (Table 4.1) the site is present in non-oestrogen target tissues and there is no overall correlation with ER concentration. While some studies have shown oestrogenic control of AEBS levels (Faye, et al., 1980; Winneker and Clark, 1983), these are difficult to interpret because the techniques used measured only a fraction of total cellular AEBS. The site is also found in low

concentrations in cytosols prepared from breast tumour biopsies from both ER-positive and ER-negative tumours (Sutherland and Murphy, 1981; Chouvet and Saez, 1984; Kon, 1985b) and in higher concentrations in the microsomal fraction of the majority of ER-positive and -negative tumours (Fernö and Borg, 1985). Only very weak correlations were observed between AEBS and ER levels. In an unpublished study from this laboratory on the microsomal AEBS content of a series of 97 breast tumour biopsies, no correlation was found between AEBS levels (expressed either on a microsomal protein, microsomal RNA or tissue weight basis) and ER or PgR levels, either for the entire series or for ER-positive tumours alone (> 10 fmol ER/mg cytosol protein). On a tissue weight basis ER-negative tumours had a higher mean level of AEBS (52.5 ± 11.0 vs 32.5 ± 3.6 fmol/mg tissue, $p = 0.095$). There was also a significant trend for AEBS content to be higher in cases where there was no malignant lymph node involvement (122 ± 31 vs 66 ± 15 or vs 49 ± 10 pmol/mg microsomal RNA for 0, 1 - 3 or ≥ 4 positive nodes respectively, $p = 0.11$, or $p = 0.034$). Otherwise, there was little or no significant correlation between AEBS tumour content and age, histological type or tumour size.

All breast cancer cell lines appear to contain microsomal AEBS. Apart from MCF 7 cells (Chapter 3) AEBS has been reported in T47-D (ER-positive) and MDA-MB-21 (ER-negative) lines (Miller and Katzenellenbogen, 1983) and in each of a series of eight ER-positive and -negative cell lines (Reddel et al., 1985). The latter study showed the concentration of AEBS was significantly greater in ER-positive lines (236,00 ± 29,900 vs 66,600 ± 16,800 sites/cell), compatible with the AEBS being an oestrogen-induced protein, but alternatively may merely be a reflection of the more highly differentiated state of ER-positive cell lines. A low concentration of a cytosolic AEBS has been detected in

ER-negative MDA-MB-231 and BT-20 lines (Chouvet and Saez, 1984). This site is thermolabile, sediments at 6.25 S on sucrose gradients, shifting to 4.5 S in the presence of 0.6 M KCl, and appears to be translocated to the nucleus in the presence of 4-hydroxyTAM, and is therefore clearly a distinct entity from the microsomal AEBS.

The significance of the presence of AEBS in tumours or cell lines in antioestrogen action remains speculative. There are, as yet, no data relating responsiveness to antioestrogen therapy and tumour AEBS status. Interestingly, in one recent trial the response to TAM as an adjuvant agent following mastectomy shows no correlation with ER status, contrary to the expected finding and is suggestive of non-ER mediated mechanisms of drug action (Nolvadex Adjuvant Trial Organisation, 1985). Among ER-positive cell lines there is also no correlation between ER levels and drug sensitivity (Reddel et al., 1985). Neither of these variables were correlated with AEBS content.

However, Faye et al. (1983) have suggested that the AEBS may have functional significance and have described a TAM-resistant MCF 7-derived cell line, RTx6, which has similar ER levels to the wild-type MCF 7 cells but almost undetectable AEBS. RTx6 cells were also resistant to the effects of 4-hydroxyTAM and nafoxidine. The mechanism of resistance has not been established and these observations remain to be confirmed.

It has been suggested that the AEBS could influence expression of antioestrogenic activity by altering the distribution and pharmacokinetics of antioestrogens (Sutherland et al., 1980). A concentration gradient arising from high concentrations of AEBS might account for the high tumour concentrations of TAM that have been observed (Daniel et al., 1981) and might also retard clearance and excretion. High intracellular concentrations of TAM bound to AEBS could

either result in optimal ER saturation by a ligand exchange process, or have the opposite effect and prevent TAM from blocking E_2 binding to ER. Experiments using BPEA previously referred to (Sheen et al., 1985) do not support either of these possibilities as saturation of AEBS with this drug did not influence the response of MCF 7 cells to TAM.

No study has yet been able to establish the identity and functional significance, if any, of the AEBS. If ligand binding induces a response, we do not yet known what to look for. Further definition of tissue and subcellular distribution may offer a clue.

The high concentration of AEBS in liver and in the endoplasmic reticulum strongly suggests that the site could be involved in drug metabolism, although there are high concentrations in MCF 7 cells which do not metabolise TAM. Identity with cytochrome P-450 is unlikely as discussed in Section 6.3.7, although other components of the drug metabolising system have not yet been ruled out. One could speculate for example that the AEBS is a 'recognition' site necessary for induction of specific cytochrome P-450 isoenzymes, comparable to the Ah receptor which has high affinity for polycyclic aromatic compounds and mediates induction of arylhydrocarbon hydroxylase activity through increased transcriptional activity. However unlike the AEBS, the Ah receptor is apparently cytosolic, translocates to the nucleus, and is not detectable in human breast cancer cell lines (Jaiswal et al., 1985).

Of course it is entirely possible that the AEBS has no functional significance at all, with binding merely occurring at some intracellular site which, coincidentally, has the appropriate conformation. The high affinity of some ligands coupled with the tentative identification of endogenous ligands (Section 6.3.11) argues against such a non-specific interaction.

8.3 OESTROGEN-IRREVERSIBLE ACTIVITY AND INTRACELLULAR BINDING OF ANTIOESTROGENS

Although the AEBS has not been shown to have identity with any previously characterized cellular receptors or binding sites, compounds which bind to AEBS also have cross-reactivity with several such sites (K_D = 10^{-6} M or greater) and further investigation is required to establish whether these interactions mediate oestrogen-irreversible biological activities of antioestrogenic compounds. Calmodulin is a protein centrally involved in the control of cell growth (Means and Chafouleas, 1982; Chafouleas et al., 1984) and its inhibition may be an important mechanism in the cell cycle and cytotoxic effects of antioestrogens and other compounds which bind to AEBS. It should prove relatively simple to correlate calmodulin inhibition with potency in the oestrogen-irreversible concentration range. Care would have to be taken, however, in selecting an appropriate calmodulin-dependent assay system. A particularly interesting enzyme that could be investigated in this regard is the cytosolic kinase which has been reported to phosphorylate the cytosolic form of ER (Section 1.5; Aurrichio et al., 1984a). This enzyme is stimulated by Ca^{2+} but completely inhibited by 15 μM trifluoperazine (Aurrichio et al., 1984b; Migliaccio et al., 1984) and is most likely calmodulin-dependent. If antioestrogens and related compounds can inhibit this enzyme at micromolar concentrations the resultant effects on cell growth would ultimately be mediated through ER but also be oestrogen-irreversible. This would then explain the similarities of cell cycle effects, at oestrogen-reversible and irreversible concentrations in ER-positive cell lines, of both antioestrogenic and non-antioestrogenic compounds (i.e. those with high and low affinities for ER). Such an effect could also result in the

different cell cycle effects observed in ER-negative cell lines at micromolar antioestrogen concentrations (Sutherland et al., 1984; Reddel et al., 1985). Whether the cytotoxic activity seen at higher drug concentrations is calmodulin-dependent or occurs through other mechanisms remains to be determined. Future experiments could compare the effects of calmodulin antagonists in ER-positive and -negative cell lines. The availability of cloned ER genes will allow the transfer of ER cDNA into ER-negative cell lines and this should greatly aid efforts to define the involvement of ER in oestrogen-reversible and -irreversible effects on cell proliferation.

8.4 A MODEL OF ANTIOESTROGEN ACTION

The known features of antioestrogen action have been incorporated into the model shown in Figure 8.1. Potentially, antioestrogens could exert their effects through any combination of the following mechanisms:

1a. Interaction with plasma membrane or synaptic receptors including histamine H_1, dopamine D_2 (and most probably D_1), and muscarinic cholinergic receptors (Section 6.3.10), occurring at submicromolar to low micromolar concentrations, and probably producing antagonist effects.

b. Non-specific (local anaesthetic- or detergent-like) interactions with plasma or intracellular membranes, occurring at micromolar concentrations, resulting in membrane disruption and changes in permeability. Such effects may be responsible for certain side effects of antioestrogen therapy, inhibition of smooth muscle contractility and reversal of doxorubicin resistance.

2. Following diffusion into the cell, interaction with the endoplasmic reticulum component cytochrome P-450 (Section 6.3.7) occurring at

low micromolar concentrations and resulting in antioestrogen metabolism, and both enzyme inhibition and induction.

3. Interaction with the AEBS, at low nanomolar concentrations. The consequences of ligand binding are unknown. Location on endoplasmic reticulum suggests potential involvement in drug metabolism or metabolism of endogenous substrates, Ca^{2+} mobilization or protein synthesis or secretion.

4. Inhibition of protein kinase C (PKC; O'Brian et al., 1985), with consequent effects on intracellular pH, and hence cell proliferation (Figure 8.2).

5. Interaction with calmodulin, which is distributed throughout all subcellular compartments, producing possibly general inhibition of calmodulin-dependent processes at low micromolar concentrations (Section 6.3.8). These could include cyclic nucleotide metabolism via cAMP phosphodiesterase (cAMP PDE; Lam, 1984) and adenylate cyclase; glycogen metabolism; Ca^{2+}-transport; and protein phosphorylation. Effects of antioestrogens on cell proliferation (e.g. G_1-phase block and possibly cytotoxicity) might be mediated through such effects, particularly through the tyrosine kinase involved in ER activation (Migliaccio et al., 1984). Interactions with calmodulin-dependent kinases and/or phosphomonoesterases (Tallant and Wallace, 1985) are thought to be responsible for TAM-induced accumulation of PtdIns(4)P and PtdIns(4,5)P_2 (Figure 8.2) in human platelets. This effect would be expected to facilitate formation of $InsP_3$ through the action of phospholipase C, leading to Ca^{2+} mobilization from endoplasmic reticulum with possible stimulation of cell proliferation (Figure 8.2). Experiments with trifluoperazine show that the overall effect of

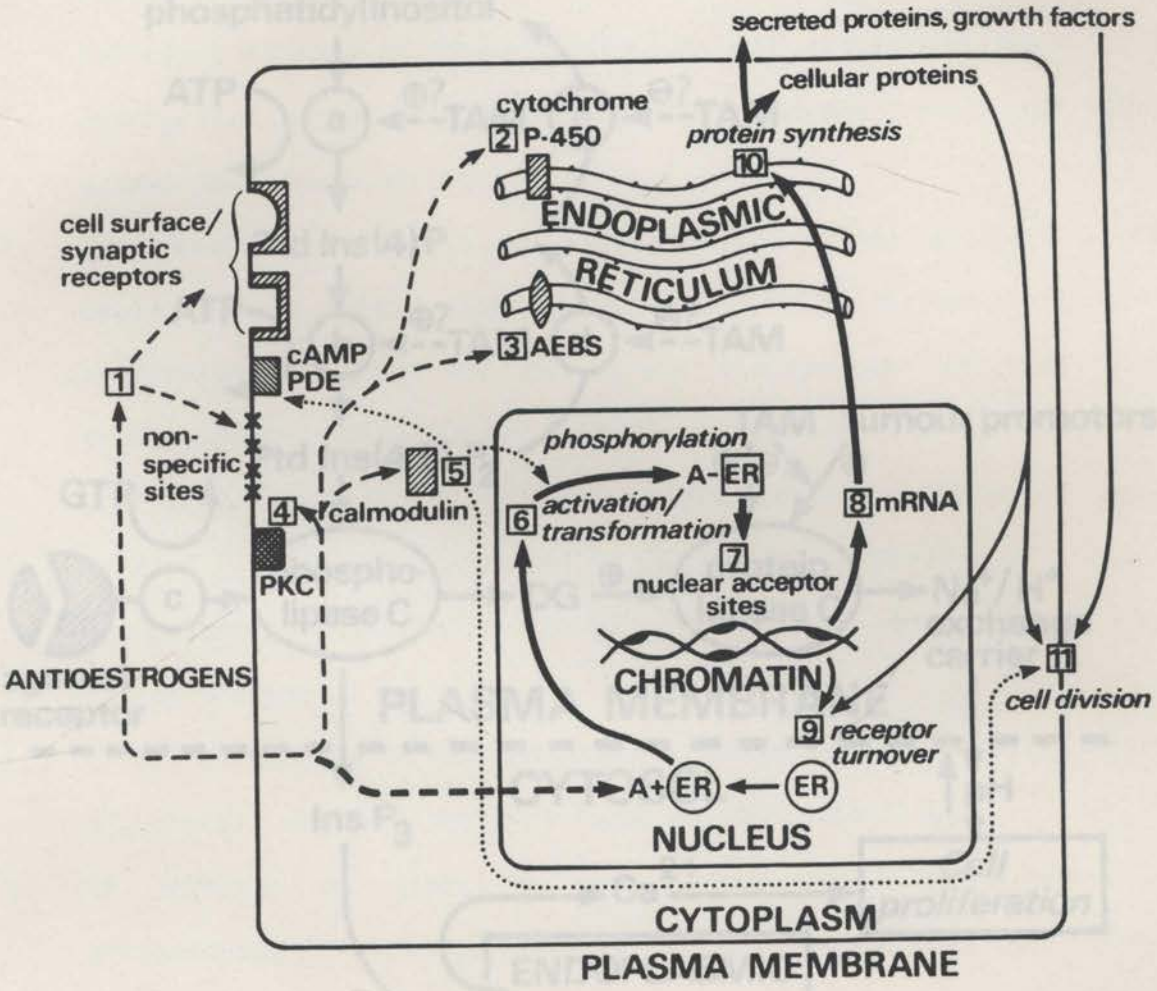

Figure 8.1: Cellular sites of action of antioestrogens.

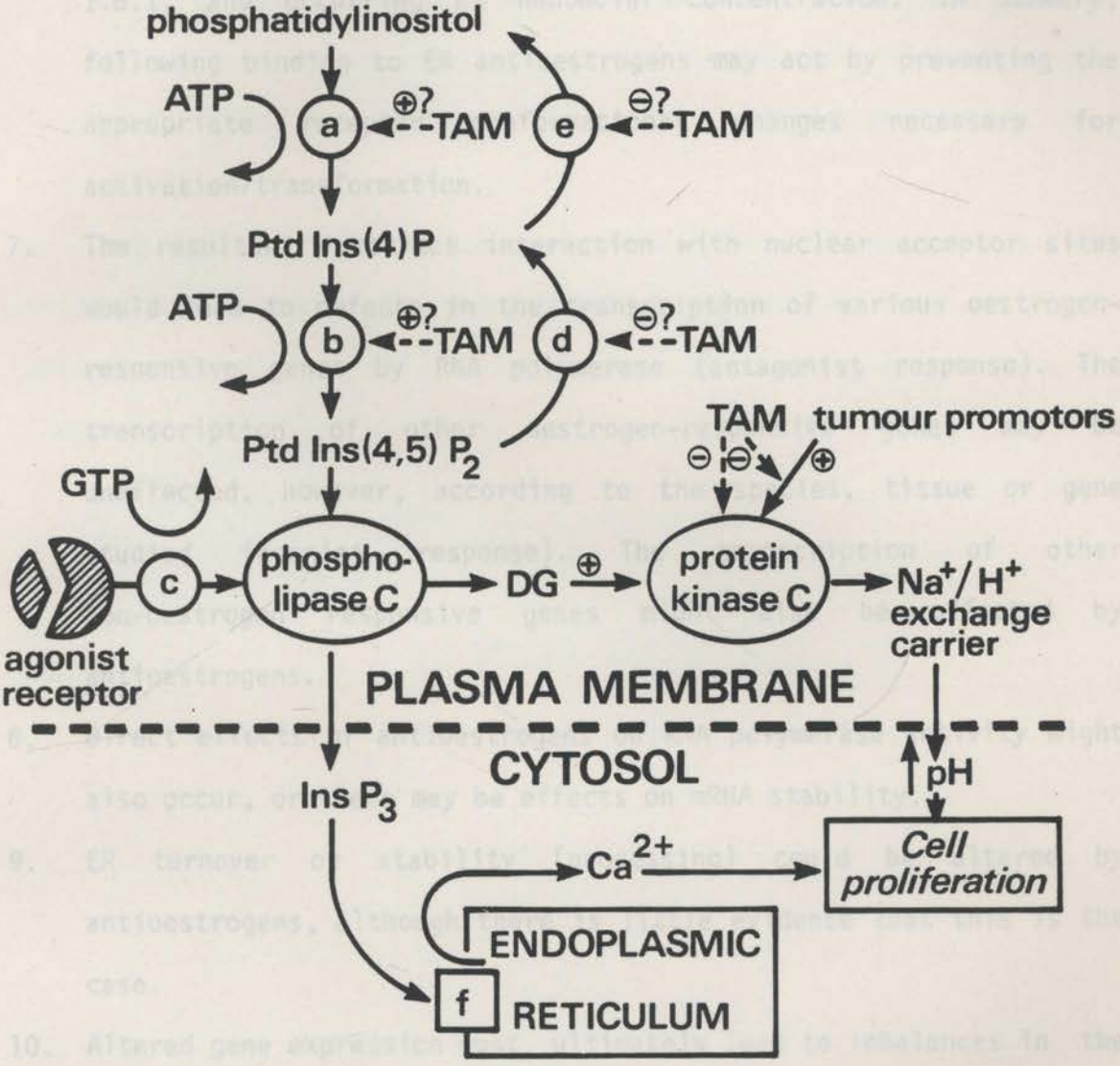

Figure 8.2: Involvement of TAM in inositol lipid pathways which lead to cell proliferation: PtdIns(4)P, phosphatidylinositol 4 phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; InsP_3, inositol trisphosphate; DG, diacylglycerol; a, phosphatidylinositol kinase; b, PtdIns(4)P kinase; c, GTP-binding protein(s); d, PtdIns(4,5)P_2 phosphomonoesterase; e, PtdIns(4)P phosphomonoesterase; f, InsP_3 receptor.

calmodulin antagonists in platelets is, however, inhibitory, due to blockage of Ca^{2+}-dependent mechanisms distal to Ca^{2+} mobilization (Tallant and Wallace, 1985).

6. Interaction with the nuclear ER system, as detailed in Section 1.6.1, and occurring at nanomolar concentration. In summary, following binding to ER antioestrogens may act by preventing the appropriate receptor conformational changes necessary for activation/transformation.
7. The resultant incorrect interaction with nuclear acceptor sites would lead to defects in the transcription of various oestrogen-responsive genes by RNA polymerase (antagonist response). The transcription of other oestrogen-responsive genes may be unaffected, however, according to the species, tissue or gene studied (agonist response). The transcription of other non-oestrogen responsive genes might also be affected by antioestrogens.
8. Direct effects of antioestrogens on RNA polymerase activity might also occur, or there may be effects on mRNA stability.
9. ER turnover or stability (processing) could be altered by antioestrogens, although there is little evidence that this is the case.
10. Altered gene expression must ultimately lead to imbalances in the supply of proteins required to support proper cellular function, including synthesis of secreted growth factors (e.g. EGF and IGF) and their corresponding cellular receptors. Direct effects of antioestrogens on ribosomal protein synthesis could occur, although there is, admittedly, no evidence to support this possibility.

11. The inhibition of cell proliferation produced by antioestrogens could therefore occur through a number of mechanisms: inhibition of growth factor secretion or down-regulation of the corresponding receptors; inadequate synthesis of intracellular proteins required for proliferation e.g. DNA polymerase; and interference with a variety of intracellular control mechanisms including those involving cAMP, inositol lipids, protein kinase C and calmodulin.

Complete resolution of the mechanisms of antioestrogen action will increase our understanding of the complex processes involved in the control of proliferation of both normal cells and oestrogen-dependent cancers.

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