Microsomal binding sites for antioestrogens in rat liver

Properties and detergent solubilization

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The properties of an antioestrogen binding site (AEBS), which has high affinity and specificity for nonsteroidal antioestrogens and structurally related compounds, have been studied in rat liver microsomes. When subcellular organelles were separated on Percoll density gradients the distribution of the AEBS paralleled that of NADPH-cytochrome *c* reductase, indicating that the AEBS is associated with the endoplasmic reticulum. Saturation analysis showed that [³H]tamoxifen was bound to a single class of saturable binding sites in liver microsomes with a K_D of 0.9 ± 0.1 nM at 0 °C. The equilibrium K_D was not significantly different at 22 °C. The K_D calculated from the association and dissociation rate constants for [³H]tamoxifen binding at 0 °C and 22 °C was compatible with the K_D measured at equilibrium. Ligand specificity studies using tamoxifen analogues showed qualitatively similar structure–affinity relationships for the AEBS from both rat liver and the MCF 7 breast cancer cell line. In general structural modifications caused correspondingly greater changes in affinity for rat liver AEBS than for MCF 7 AEBS. The AEBS was solubilized from microsomal membranes with sodium cholate. This was the only detergent of nine tested that solubilized the site in high yield without loss of activity. Solubilization using cholate was more effective in the presence of 1 M-NaCl. In the solubilized state there was an apparent loss of [³H]tamoxifen binding activity which could be restored by dilution of the detergent. Gel filtration indicated an M_r of 440 000–490 000 for the AEBS–cholate complex. These studies demonstrate that rat liver contains high concentrations of a microsomal AEBS which has similar properties and specificity to the AEBS previously described in human breast cancer cells. This site can be solubilized by sodium cholate to supply material suitable for further purification.

INTRODUCTION

The nonsteroidal antioestrogens, structurally related synthetic compounds, are competitive inhibitors of the binding of oestradiol to the intracellular oestrogen receptor. Numerous studies of the molecular interactions of these drugs and of their biological actions (which include oestrogen antagonist and agonist properties, antifertility effects and antitumour activities) indicate that many of their biological effects are mediated by the oestrogen receptor system (recent reviews include Sutherland & Jordan, 1981; Sutherland & Murphy, 1982; Katzenellenbogen *et al.*, 1983; Jordan, 1984, 1985).

Other data, however, suggest that some of the effects of antioestrogens, on growth and cellular proliferation in particular, are considerably more complex than would be anticipated if these effects were mediated solely by the oestrogen receptor (Martin, 1981; Faye *et al.*, 1983; Murphy & Sutherland, 1983, 1985; Reddel *et al.*, 1983, 1985; Sutherland *et al.*, 1983a,b).

Several recent investigations have shown that, in addition to binding to the oestrogen receptor, tamoxifen (TAM) and other nonsteroidal antioestrogens bind in a saturable manner and often with high affinity to a number of other cellular binding sites. These include: muscarinic receptors (Ben-Baruch *et al.*, 1982), dopamine receptors (Hiemke & Ghraf, 1984), histamine receptors (Brandes *et al.*, 1985), cytochromes *P*-450 (Ruenitz *et al.*, 1984), calmodulin (Lam, 1984), protein kinase C (O'Brian *et al.*,

1985) and a high affinity binding site of unknown function termed the antioestrogen binding site (AEBS) (Sutherland & Foo, 1979; Sutherland *et al.*, 1980). The roles, if any, of these binding sites in mediating any of the effects of nonsteroidal antioestrogens, in particular their apparently non-oestrogen-receptor-mediated effects, have not been directly established, although workers in this laboratory have shown that analogues with high affinity for AEBS have more potent antiproliferative effects on breast cancer cells *in vitro* than analogues with low affinity for AEBS (Murphy & Sutherland, 1983, 1985).

Several workers have studied the properties of the AEBS in an effort to define its function either in drug action or in the normal physiology of the cell. More recent studies include those of Kon (1983), Miller & Katzenellenbogen (1983), Sudo *et al.*, (1983), Winneker & Clark (1983), Murphy *et al.* (1984), Watts & Sutherland (1984) and Watts *et al.* (1984). The present paper extends these previous observations and reports on the biochemical properties and detergent solubilization of a rat liver microsomal AEBS.

EXPERIMENTAL

The buffer used in all experimental procedures was 10 mM-Tris/HCl/25 mM-KCl/0.25 M-sucrose, pH 7.4 at 22 °C, (TSK) unless otherwise indicated. Protein con-

Abbreviations used: AEBS, antioestrogen binding site; TAM, tamoxifen.

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centration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (Calbiochem-Behring Corp., La Jolla, CA, U.S.A.) as a standard. In the tissue distribution studies microsomal protein concentration was determined in the microsomal pellet produced by the addition of 10 mM-CaCl₂ to the 10000 *g* 20 min supernatant and centrifugation at 9000 *g* for 5 min (Schenkman & Cinti, 1978).

Tissue homogenization and fractionation

All procedures were carried out at 0–4 °C. Tissues were removed from 18 h starved male or female Sprague-Dawley or Fisher rats (200–250 g) and rapidly cooled on ice. The tissue was minced and homogenized (10% or 20%, w/v, in TSK) in a Teflon/glass Potter-S homogenizer (six strokes at 1000 rev./min). The homogenate was centrifuged at 10000 *g* for 20 min. The supernatant was the post-mitochondrial fraction.

To prepare the microsomal fraction, the post-mitochondrial fraction was centrifuged at 130000 *g* for 1 h in a Beckman 50 Ti rotor. The microsomal pellet was washed free of adsorbed protein by resuspension in 0.15 M KCl/TSK and centrifugation at 130000 *g*.

The washed microsomes were stored (as a suspension in TSK) for up to 3 months at –20 °C without significant loss of AEBS activity.

Percoll gradients. Rat liver post-mitochondrial fraction prepared from a 1:10 (w/v) homogenate was fractionated on a Percoll gradient self-generated *in situ*. PMF (0.5 ml) was mixed with 11 ml of 30% Percoll (Pharmacia) in 0.25 M-sucrose, pH 7.4, in 16 mm × 76.2 mm tubes. Parallel tubes containing Density Marker Beads (Pharmacia) were used to measure gradient density. Centrifugation was at 30000 rev./min (60000 *g*) in a 50 Ti Beckman rotor for 45 min. Fractions were collected from the bottom of the tube and assayed for protein, AEBS (single saturating dose technique), 5'-nucleotidase (EC

3.1.3.5) and NADPH-cytochrome *c* reductase (EC 1.6.2.4) activity (enzyme markers for plasma membrane and endoplasmic reticulum respectively) as described elsewhere (Watts *et al.*, 1984; Watts & Sutherland, 1984).

Binding of TAM to AEBS

[³H]TAM (76–87 Ci/mmol; Amersham Australia, Sydney, Australia), TAM and analogues (supplied through the courtesy of I.C.I. Pharmaceuticals Division, Macclesfield, Cheshire, U.K.) were stored at –20 °C as ethanol solutions. Aqueous solutions of [³H]TAM and unlabelled drugs were prepared in TSK containing 0.02% bovine serum albumin.

All binding assays were done in the presence of 1 μM-oestradiol to eliminate interaction of TAM with the oestrogen-receptor. Saturation analysis, single saturating dose and competitive binding assays were performed as previously described (Watts *et al.*, 1984; Watts & Sutherland, 1984) either overnight at 0–4 °C or at 22 °C for 1–2 h. Results obtained at these two temperatures were not significantly different.

Previous published data have shown that rat liver microsomes do not metabolize TAM during 24 h at 0 °C (Sudo *et al.*, 1983). However, concentrated microsomal preparations (2–88 mg of microsomal protein/ml) at 37 °C in the presence of NADPH show significant metabolism (Robertson *et al.*, 1982; Ruenitz *et al.*, 1984; Lyman & Jordan, 1985) but this is abolished in the absence of NADPH (Dr P. Ruenitz, personal communication). These findings indicate that the metabolism of TAM is unlikely to be a complicating factor in interpretation of the present kinetics data obtained in experiments at 0–22 °C, with very low concentrations of washed microsomes (0.01–0.04 mg of microsomal protein/ml) in the absence of NADPH.

In order to measure [³H]TAM binding to detergent-solubilized microsomes it was necessary to restore binding activity by dilution of solubilized material with

Table 1. Distribution of AEBS in microsomal fractions of rat tissues

Tissues from female rats were homogenized (1:10, w/v, in TSK) and post-mitochondrial fractions were prepared. The specific binding of [³H]TAM in dilutions of these fractions was measured by saturation analysis in the presence of 1 μM-oestradiol to eliminate binding to oestrogen receptors, and the AEBS concentration and dissociation constant (*K_D*) were calculated by Scatchard analysis.

Tissue	Concentration		<i>K_D</i> (nM)
	(fmol/mg of microsomal protein)	(pmol/g of tissue)	
Liver	30500 ± 4900*	780 ± 100	0.9 ± 0.1
Colon	1060 ± 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	0
Skeletal muscle	0	0	0
Small intestine	0	0	0

* Value for liver is for KCl-washed microsomes.

TSK buffer. Sodium cholate concentrations were generally diluted to between 0.01 and 0.1% (w/v) prior to assay of AEBS concentration.

Association rate. Microsomal suspensions were incubated at 0 °C and 22 °C with 5 nM-[³H]TAM in the absence (total binding) or presence (non-specific binding) of 1 μM-TAM. The reactions were stopped at various time points by addition of the reaction mixture to 0.5% charcoal/0.05% dextran in TSK at 0 °C and bound [³H]TAM was determined. The association rate constant (k_+) for the reaction over the first 60 min and 14 min at 0 °C and 22 °C respectively was calculated assuming second-order kinetics for the formation of the [³H]TAM-AEBS complex, using the method previously described (Watts *et al.*, 1984).

Dissociation rate. Using an isotopic dilution method to measure the dissociation rate constant (k_-) of the [³H]TAM-AEBS complex, microsomal suspensions were prelabelled with 5 nM-[³H]TAM either in the absence (total binding) or presence (non-specific binding) of 1 μM-TAM for 1 h at 22 °C. Unbound ligand was removed by the addition of charcoal/dextran. After removal of charcoal by centrifugation, TAM was added as a concentrated ethanol solution to a final concentration of 1 μM and bound [³H]TAM in the reaction mixtures at 0 °C or 22 °C was determined at various time points by charcoal/dextran adsorption. k_- was calculated, assuming first order kinetics, from the slope of the lines given by $\log(RL/RL_0)$ versus time (over the first 60 min and 30 min at 0 °C and 22 °C respectively), where RL_0 and RL were the concentrations of the [³H]TAM-AEBS complex at times 0 and t respectively.

Effect of pH, ionic strength and enzyme treatment on [³H]TAM binding. TSK buffers were adjusted to various pH values with HCl or NaOH or to various ionic strengths with KCl and then used to prepare dilute microsome suspensions for assay. The pH of the final assay incubation mixtures was measured at 22 °C. [³H]TAM binding was assessed by saturation analysis for 1 h at 22 °C.

To test the sensitivity of the AEBS to enzymes, microsomal suspensions were incubated at 30 °C for 1 h with trypsin (type I, in the presence of 2 mM-Ca²⁺), lipase (type XI) or RNAase (type IA in the presence of 0.5 mM-Mg²⁺) from Sigma Chemical Co. (St. Louis, MO, U.S.A.). After dilution AEBS concentration was measured by the single saturating dose technique.

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 130000 *g* for 1 h in a Beckman 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 [³H]TAM binding assay the supernatant and pellet suspensions were diluted with TSK, typically between 10- and 50-fold.

Fig. 1. Subcellular fractionation of rat liver post-mitochondrial fraction on Percoll gradient

Rat liver post-mitochondrial fraction was mixed with 30% Percoll in 0.25 M-sucrose and centrifuged at 60000 *g* for 45 min in a Beckman 50 Ti rotor. Fractions, collected from the bottom of the tubes, were assayed for AEBS (□) (single saturating dose technique), NADPH-cytochrome *c* reductase (×) (marker enzyme for endoplasmic reticulum) and 5'-nucleotidase (○) (marker enzyme for plasma membrane) activities as described in the Experimental section. Gradient density (●) was determined by Density Marker Beads.

Gel filtration chromatography

Microsomes were solubilized in TSK containing 1% (w/v) sodium cholate and 1 M NaCl. A 5 ml portion of the 130000 *g* supernatant was chromatographed in the presence of the same buffer on a Fractogel HW-65F column (84 cm × 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-[³H]TAM was added and specific [³H]TAM binding was measured as the difference between [³H]TAM bound in the absence and presence of 1 μM unlabelled TAM.

Calibration proteins, fractionated separately under the same conditions, were thyroglobulin, ferritin, bovine serum albumin and ovalbumin (M_r 669000, 440000, 67000 and 43000 respectively). The elution volumes of these proteins were not significantly altered by the presence of detergent.

Hydrophobic interaction chromatography

Rat liver microsomes were solubilized in TSK containing 1% (w/v) cholate and 1 M-NaCl and centrifuged at 130000 *g* 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[CH₂]_{*n*}CH₃ and phenyl-agarose, and agarose-NH[CH₂]_{*n*}NH₂ ($n = 0-12$). For the latter, alkylamino, series of agaroses the applied sample was adjusted to 4 M-NaCl to increase the strength of the hydrophobic

Fig. 2. Effect of pH on [^3H]TAM binding to AEBS

The specific [^3H]TAM binding capacity of rat liver microsomes at various pHs was measured by saturation analysis (see the Experimental section). 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.

interaction. The columns were washed with 1 ml of buffer and the combined eluates were assayed for AEBS (single saturating dose technique) and protein recoveries.

Statistics

Results are reported as mean $\pm$ S.E.M.

RESULTS

Tissue and subcellular distribution

In order to identify tissues containing high levels of AEBS suitable for further characterization and purification, saturable [^3H]TAM binding was measured in the post-mitochondrial fractions of a range of rat tissues (Table 1). High-affinity (K_D 1–2 nM) saturable binding sites for TAM were present in a number of tissues, e.g. liver, stomach, ovary and brain, 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.

We have previously reported the AEBS to be localized in the microsomal fraction of rat liver, apparently associated with the endoplasmic reticulum (Watts & Sutherland, 1984). Those findings, which used differential centrifugation and sucrose gradient techniques, were confirmed by experiments where liver post-mitochondrial fraction was fractionated on Percoll gradients (Fig. 1). In these separations AEBS activity more closely paralleled the distribution of the marker enzyme for

endoplasmic reticulum (NADPH–cytochrome *c* reductase) than that for the plasma membrane (5'-nucleotidase).

Kinetic studies

Saturation analysis of the binding of [^3H]TAM to rat liver AEBS determined under equilibrium conditions showed a high affinity interaction with a dissociation constant, K_D , of 0.9 ± 0.1 nM (range 0.6–1.6 nM), which was not significantly different when measured at 0 °C or 22 °C. Measurement of the kinetic rate constants of the reaction showed that the association (k_+) and dissociation (k_-) rates of the binding of [^3H]TAM to rat microsomal AEBS were compatible with second- and first-order reactions respectively. The rate constants measured at 0 °C and 22 °C were: k_+ , $(5.43 \pm 1.08) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $(3.35 \pm 0.95) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$; k_- , $(2.65 \pm 0.67) \times 10^{-5} \text{ s}^{-1}$ ($t_{1/2}$ 435 min at 0 °C) and $(2.39 \pm 0.08) \times 10^{-4} \text{ s}^{-1}$ ($t_{1/2}$ 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.

The effects of pH on [^3H]TAM binding on AEBS are shown in Fig. 2. 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 a decrease in the binding affinity of [^3H]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 (results not shown).

The activity of the AEBS was unaffected by treatment with RNase (400 $\mu\text{g}/\text{ml}$) but lipase at low concentrations (10 $\mu\text{g}/\text{ml}$) reduced activity by 55–85%. Trypsin reduced activity by 15% and 34% at 250 and 1000 $\mu\text{g}/\text{ml}$ 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 $\mu\text{g}/\text{ml}$.

Ligand specificity studies

In order to define the structural requirements for binding to AEBS in rat liver microsomes we studied the ability of TAM analogues to compete with the binding of [^3H]TAM (Table 2). Relative binding affinity was influenced by structural changes to both the hydrophobic triphenylethylene portion of the ligand and to the hydrophilic basic aminoether side chain. The effects of substitution on the aromatic portion of the ligand were evident when TAM, 79,280, 132,800 and 148,067 were compared. The introduction of F, OH or OCH_3 groups which are electron-donating through the resonance effect led to a 70–80% reduction in affinity.

The side chain terminal amino group played a major role in determining binding affinity. The TAM analogue carrying the terminal cyclic amino group $-\text{N}(\text{CH}_2\text{CH}_2)\text{O}$ (47,399) had increased affinity, whereas analogues with $-\text{N}(\text{C}_2\text{H}_5)_2$ (45,960) or $-\text{NHCH}_3$ (55,548) terminal groups had lower affinities than TAM [$-\text{N}(\text{CH}_3)_2$].

Changes in the length of the hydrocarbon portion of the side chain also produced changes in relative binding

Table 2. Relative binding affinities of tamoxifen analogues for AEBS

To investigate the influence of ligand structure on binding to the AEBS, rat liver microsomes were incubated with a saturating concentration of [³H]TAM (5 nM) and increasing concentrations of unlabelled TAM analogues. Binding affinities relative to TAM (100%) were calculated by the method of Korenman (1970). The substituent at R² is C₂H₅– for all the TAM analogues shown.

ICI code number	R ¹	R ³	R ⁴	Relative binding affinity (%) for AEBS from:	
				Rat liver	MCF 7*
Tamoxifen (<i>trans</i>)	H	H	O[CH ₂] ₂ N(CH ₃) ₂	100	100
55,548	H	H	O[CH ₂] ₂ NHCH ₃	12 ± 1	19 ± 2
79,280	OH	H	O[CH ₂] ₂ N(CH ₃) ₂	30 ± 5	80 ± 7
45,960	H	H	O[CH ₂] ₂ N(C ₂ H ₅) ₂	21 ± 5	46 ± 4
47,108	H	H	O[CH ₂] ₃ N(CH ₃) ₂	127 ± 15	36 ± 1
47,590	H	H	O[CH ₂] ₄ N(C ₂ H ₅) ₂	119 ± 26	54 ± 7
47,699 (<i>cis</i>)	H	H	O[CH ₂] ₂ N(CH ₃) ₂	73 ± 7	98 ± 9
94,230	H	H	OCH ₂ CH(OH)CH ₂ N(CH ₃) ₂	14 ± 3	2 ± 0.3
132,802	H	OCH ₃	O[CH ₂] ₂ (CH ₃) ₂	22 ± 0.2	35 ± 5
148,067	H	F	O[CH ₂] ₂ N(CH ₃) ₂	31 ± 3	97 ± 1
47,399	H	H	O[CH ₂] ₂ -c-N(CH ₂ CH ₂) ₂ O	1993 ± 924	219 ± 14

* From Watts *et al.* (1984).

affinity, the magnitude and direction of which were apparently dependent upon the terminal amino group and the tissue source of AEBS (TAM versus 47,108; 45,960 versus 47,590).

Other structural factors influencing binding affinity were *cis/trans* isomerism about the triphenylethylene double bond (TAM versus 47,699), which had only a small influence on relative binding affinity, and the presence of a side chain hydroxyl group (47,108 versus 94,230) which caused a large reduction in relative binding affinity.

Detergent solubilization of AEBS

As a first step towards the purification of AEBS we defined conditions for detergent solubilization and investigated the properties of the solubilized binding site.

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 [³H]TAM binding activity (Table 3). 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 [³H]TAM by preventing the charcoal adsorption of free [³H]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 3 shows that of the nine detergents tested only sodium cholate and 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. [³H]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 binding activity for the AEBS. At low cholate concentrations (≤ 0.5% w/v), binding was not significantly affected but at higher (i.e. solubilizing) concentrations affinity for [³H]TAM was rapidly lost, causing the apparent binding capacity to fall to zero at detergent concentrations > 1%. However binding activity could subsequently be fully restored by reducing cholate concentrations by dilution to less than 0.5% (w/v). The dissociation constant for [³H]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 presence of deoxycholate, versus 20% loss in the

Table 3. 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 130000 *g* for 1 h the supernatants and resuspended pellets were diluted and assayed for specific [³H]TAM binding by the single saturating dose assay (see the Experimental section). Results are expressed as a percentage of specific [³H]TAM binding in the original microsomes. Abbreviations: N.D., not determined; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid; Chapso, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonic acid.

Detergent	Specific binding (%) in:	
	Supernatant	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.

Fig. 3. Efficiency of cholate solubilization at different microsomal concentrations

Total protein (open symbols, broken lines) and AEBS (closed symbols, solid lines) were measured in 130000 *g* supernatants after solubilization of microsomes with increasing concentrations of cholate. In this experiment the microsomal protein concentrations were 6.1 mg/ml (Δ , $\blacktriangle$), 3.0 mg/ml ($\circ$, $\bullet$) and 0.6 mg/ml ($\square$, $\blacksquare$). Recoveries in the supernatant are expressed as a percentage of the initial quantities of microsomal protein or AEBS.

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).

Fig. 3 shows the ability of cholate to solubilize AEBS and protein at different concentrations of microsomes.

Fig. 4. Effect of salt on cholate solubilization of microsomes

Sodium cholate was added, to the final concentrations shown, to microsomes (1 mg of protein/ml) in the presence (closed symbols) or absence (open symbols) of 1 M-NaCl. Solubilized AEBS ($\circ$, $\bullet$) and protein ($\square$, $\blacksquare$) were assayed (see the Experimental section) in the supernatants after centrifugation at 130000 *g* for 1 h.

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

Fig. 5. 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 A_{280} ($\blacktriangle$) and, after dilution, for specific [3 H]TAM binding ($\circ$) as described in the Experimental section. V_0 represents the column void volume (approx. $M_r \times 10^6$) and the calibration proteins were thyroglobulin, ferritin, bovine serum albumin and ovalbumin (M_r 669000, 440000, 67000 and 43000 respectively).

other detergents, through a lowering of critical micellar concentration 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 (Fig. 4). 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 (approx. 70%) of total membrane protein but only a small amount (approx. 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.

Properties of the detergent-solubilized AEBS

Gel filtration. To investigate whether the cholate-solubilized AEBS was truly solubilized rather than merely associated with slowly sedimenting membrane fragments which did not sediment at 130000 g, 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 50000–500000 (Fig. 5). The majority of binding activity was eluted as a relatively broad peak slightly ahead of the main protein

Fig. 6. Hydrophobic chromatography of detergent-solubilized AEBS

Samples of rat liver microsomes solubilized by 1% cholate in the presence of 1 M-NaCl were passed through columns containing hydrophobic agaroses of increasing hydrocarbon chain length: agarose-NH[CH₂]_nCH₃ and phenyl-agarose (a) or agarose-NH[CH₂]_nNH₂ (b). For the latter series of agaroses the applied sample was adjusted to 4 M-NaCl. The eluates were assayed for AEBS ($\circ$) and protein ($\blacktriangle$) recovery.

peak. The apparent M_r of the mid-peak was 440000–490000. When whole microsomes were chromatographed on the same column in the absence of detergent, all AEBS activity was eluted at the void volume, as would be expected for a membrane-bound component (results not shown).

Hydrophobic interaction chromatography. Hydrophobic interaction chromatography of solubilized microsomes (Fig. 6) 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. Little or no activity remained in the eluate from hexyl-, octyl-, decyl-, phenyl- or dodecylamino-agarose columns. However, subsequent treatment with buffers containing low NaCl or high cholate or ethylene glycol concentrations eluted bound protein but no apparent AEBS activity from these agaroses (results not shown). Precipitation of such a hydrophobic membrane component subsequent to elution, particularly during the charcoal/dextran centrifugation step of the [3 H]TAM-

binding assay, was considered as a possible cause of the apparent loss of binding activity, but we found no evidence that this was the case.

DISCUSSION

Although the presence of AEBS has been reported in a number of tissues from different species (Faye *et al.*, 1980; Sutherland *et al.*, 1980; Murphy & Sutherland, 1981; Kon, 1983; Murphy *et al.*, 1984), detailed information on the properties of this molecule are only available for uterus (Gulino & Pasqualini, 1982; Sudo *et al.*, 1983) and breast cancer cells (Miller & Katzenellenbogen, 1983; Watts *et al.*, 1984). The present study defines the binding properties and ligand specificity of AEBS in rat liver, compares these with properties of AEBS from MCF 7 cells, and establishes conditions for detergent solubilization of the AEBS as a first step in its purification.

Distribution studies of AEBS in rat tissues showed that the liver was a concentrated and convenient source of AEBS for further study. The site was not restricted to classical oestrogen target tissues, even though other studies have shown that in some tissues (rat liver: Winneker & Clark, 1983; rat and guinea pig uterus: Faye *et al.*, 1980; Gulino & Pasqualini, 1983) levels of AEBS are under oestrogenic control. Qualitatively similar data on the tissue distribution of microsomal AEBS in the rat have been presented by Sudo *et al.* (1983). However, we found significantly higher AEBS concentrations in liver, perhaps because of variations in assay conditions, in particular our use of lower liver microsomal protein concentrations which reduces interference from low-affinity or non-specific binding components.

The tissue distribution of the AEBS and the finding that the AEBS is not concentrated in the plasma membrane but rather in the endoplasmic reticulum argues against the possibility that the AEBS may be one of the cell surface receptors which have been reported to bind TAM (see the Introduction).

At 0 °C rat liver AEBS association and dissociation rate constants have similar values to those of the MCF 7 AEBS. However at 22 °C these rate constants are 2–5-fold lower in rat liver. The values for MCF 7 AEBS were: k_+ , $5.62 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $1.59 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 0 °C and 22 °C respectively; k_- $2.95 \times 10^{-5} \text{ s}^{-1}$ ($t_{1/2}$ 392 min) and $1.32 \times 10^{-3} \text{ s}^{-1}$ ($t_{1/2}$ 8.75 min) at 0 °C and 22 °C respectively (calculated from Watts *et al.*, 1984, and C. K. W. Watts & R. L. Sutherland, unpublished work). The effects of pH and ionic strength on the binding of [³H]TAM to rat liver were similar to those previously observed for MCF 7 AEBS.

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 cofactors or in the maintenance of appropriate binding site structure.

When ligand specificity was compared between rat liver and MCF 7 AEBS both quantitative and some qualitative differences were apparent (Table 2). In general the rat liver AEBS has more rigorous requirements with regard to ligand structure, that is, alterations in the ligand, especially in the terminal amino group and the

hydrophobic triphenylethylene portion of the molecule, cause larger changes in binding affinity for liver AEBS than for MCF 7 AEBS (e.g. TAM versus 79,280, 47,399 and 148,067). In contrast, binding affinity for the MCF 7 AEBS is more sensitive to changes involving the alkyl portion of the alkylaminoether side chain (TAM versus 47,108 and 94,230) but not when the terminal group is $-\text{N}(\text{C}_2\text{H}_5)_2$ (45,960 versus 47,590). TAM analogues with nonbasic side chains had no affinity for either rat or MCF 7 AEBS. 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.

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 our findings, Brandes *et al.* (1985) have reported the solubilization of AEBS with 1% digitonin. Gel filtration of cholate-solubilized microsomes demonstrates that the AEBS is in a truly soluble form and not just associated with membrane fragments, which would cause binding activity to appear at the column void volume. Whether the peak of activity at an M_r of 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. Solubilization of AEBS was efficient only at high cholate and salt concentrations, demonstrating that the AEBS is probably a strongly hydrophobic membrane component. Although solubilized AEBS lost [³H]TAM binding activity, this activity could be reconstituted by detergent removal. This finding may indicate that binding is dependent upon the association of the AEBS with other membrane components which is disrupted in the presence of detergent. Detergent removal perhaps allows reassociation with these components. The observation that [³H]TAM binding activity was not recovered from hydrophobic agarose columns under conditions where it would be expected to be eluted could also be explained if the AEBS and other components necessary for binding activity had been separated during chromatography. Whether AEBS is irreversibly inactivated under these conditions or whether activity can be reconstituted requires further investigation.

Another factor that may contribute to the apparent loss of activity at > 0.5% cholate concentrations is the probable association of [³H]TAM under these conditions with detergent micelles, resulting in interference with ligand binding.

A consequence of the loss of AEBS binding activity in the solubilized state is that binding-site-directed affinity chromatography techniques are not feasible as a means of AEBS purification. In other experiments to test possible purification techniques we found that a variety of lectin–agaroses (concanavalin A–, castor bean–, lentil– and wheatgerm–agaroses) failed to bind significant amounts of solubilized AEBS. Because lectin–sugar interactions can take place in the presence of detergents, this finding indicates the probable lack of a carbohydrate component on the AEBS.

The findings of the present study provide a basis for further studies on the purification of the AEBS and therefore further understanding of its functional signifi-

cance both in drug action and in normal cellular physiology.

These studies were supported in part by the National Health and Medical Research Council of Australia. We thank Dr. A. Wakeling and Dr. B. Tait of I.C.I. Pharmaceuticals Divisions for supply of the tamoxifen analogues, Narelle Hobbs for technical assistance and Judy Hood for preparation of the manuscript.

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Received 7 November 1985/21 January 1986; accepted 21 February 1986