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The Binding of Tamoxifen to Oestrogen Receptor Proteins under Equilibrium and Non-Equilibrium Conditions

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Abstract—*Data have been presented from sucrose density gradient analysis, protamine sulphate precipitation and specificity studies to show that oestradiol-17β and tamoxifen share a common binding protein. In addition, the binding of [^{3}H]-tamoxifen to cytosol preparations from human and rat mammary adenocarcinomas and rat uteri was measured under equilibrium conditions using Scatchard analysis. The relative affinity values obtained were, however, greater than those calculated from competitive binding studies. In addition the competition between tamoxifen and oestradiol-17β for oestrogen binding sites at 4°C decreased with increasing incubation time. Based on competitive binding experiments, the principal metabolite of tamoxifen, metabolite B, associated with oestrogen receptor proteins with a higher affinity than tamoxifen. Kinetic studies indicate that these variations probably result from the relative dissociation rates of metabolite B and tamoxifen from the oestrogen receptor, tamoxifen dissociating much more rapidly than metabolite B. It is also proposed that kinetic studies may have important implications with respect to the mechanism of action of tamoxifen.*

INTRODUCTION

THE ANTIOESTROGENIC properties of tamoxifen [1-(4-β-dimethylaminoethoxyphenyl) 1,2-diphenylbut-1-ene] have led to its clinical use in metastatic breast cancer in both pre- and postmenopausal women [1, 2]. Tamoxifen initiates tumour regression principally in those tumours containing oestrogen receptors [3, 4]. This applies equally to the experimental situation where the oestrogen receptor content of DMBA-induced mammary carcinomas correlates with the response of the tumours to tamoxifen treatment [5, 6]. It seems likely, that, in order to understand the mechanism by which tamoxifen influences tumour growth, the detailed nature of any interaction between the antihormone and the cytoplasmic oestrogen receptor protein should be considered. A number of metabolites of tamoxifen have also been identified in man [7] and in laboratory animals [8]. The principal compound, metabolite B [1-(4-β-dimethylaminoethoxyphenyl) 1-*p*-hydroxyphenyl, 2-phenylbut-1-ene], has potent anti-oestrogenic activity in the rat [9] and might, therefore, provide a supportive role during tamoxifen therapy of breast cancer.

The present study examines the binding characteristics of oestradiol-17β, tamoxifen and metabolite B to cytosol preparations from human and rat mammary tumours and rat uteri. Variations in binding properties may ultimately relate to the oestrogenicity/antioestrogenecity of the molecules.

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MATERIALS AND METHODS

Preparation of cytosol fractions

Tissue samples of human and rat mammary tumours and rat uteri were frozen in liquid nitrogen and pulverized in a spring-loaded plunger gun [10]. The pulverized tissue was quickly transferred to a Potter–Elvehjem homogenizer and homogenized in 10 mmole/l Tris–HCl buffer, pH 7.4, containing 5 mmole/l EDTA and 1 mmole/l dithiothreitol at 4°C (medium A: 1 g original tissue in 5 ml buffer). A high speed supernatant (cytosol) preparation was obtained by centrifugation of the homogenate in a Beckman L2-65B ultra-

centrifuge (T50 rotor, rav. 5.9 cm) for 45 min at 4°C. All animals (approx 120 days of age) were ovariectomised one day prior to removal of tissue.

Determination of the number and affinity of specific oestradiol-17β and tamoxifen binding sites

Aliquots of cytosol (200 μl, containing approximately 20 mg protein/ml) were incubated for 16 hr at 4°C with various concentrations of either: (1) [6,7-^{3}H]oestradiol-17β (0.5–5 nmole/l, specific radioactivity 45 Ci/mmole; Radiochemical Centre, Amersham, Bucks., U.K.) or (2) [^{3}H]tamoxifen (0.4–30 nmole/l, specific radioactivity 19.5 Ci/mmole; ICI Pharmaceuticals, Alderley Park, Cheshire, U.K.) in the presence or absence of a 1000-fold excess of unlabelled non-radioactive ligand (200 μl). After incubation, tubes were maintained at 4°C and an equal volume (400 μl) of charcoal suspension [0.5% (w/v) Norit A, 0.05% (w/v) Dextran T-70, 0.1% (w/v) gelatin) in medium A added. The resultant mixture was shaken for 30 min at 4°C and charcoal removed by centrifugation at 800 *g* for 10 min. Aliquots (500 μl) of the supernatant containing protein-bound [^{3}H]oestradiol-17β or [^{3}H]tamoxifen were added to 5 ml of scintillation fluid (5 g 2,5-diphenyloxazole, 500 ml Triton X-100, 1 l toluene) and radioactivity measured in a Nuclear Chicago (Mark II) liquid scintillation spectrophotometer using external standardisation. Specifically bound counts were determined by subtraction of the counts obtained in the presence of non-radioactive ligand from those obtained in its absence. Results were analysed by Scatchard analysis [11].

The purity of [^{3}H]tamoxifen was checked by thin layer chromatography on polygram silica gel/u.v. 254 using solvent system toluene: triethylamine, 90:10 (v/v), R_F. 0.45. Radioactive tamoxifen was stored in methanol at −20°C, protected from light.

Protamine sulphate precipitation of oestrogen and tamoxifen binding proteins

Cytosol preparations (100 μl) from rat uteri and also DMBA-induced mammary tumours containing known quantities of oestrogen and tamoxifen binding sites were mixed with an equal volume of a protamine sulphate solution (1 mg/ml) and allowed to stand for 15 min at 4°C. The precipitates were pelleted at 800 *g* for 10 min and incubated for 16 hr at 4°C with either: (1) [^{3}H]oestradiol-17β (5 nmole/l) or (2) [^{3}H]tamoxifen (20 nmole/l) in the presence or absence of a 1000-fold excess of unlabelled non-radioactive ligand (200 μl). After incubation, the precipitate was washed five times in medium A (2 ml) and radioactivity extracted from the final washed pellet with absolute ethanol (500 μl). The method of determining specific counts was as described above.

Sedimentation analysis of [^{3}H]oestradiol-17β and [^{3}H]tamoxifen binding components

Samples of cytosol (400 μl) prepared from a rat mammary tumour were layered on linear 5–20% (w/v) sucrose density gradients under low salt conditions (sucrose solutions contained 10 mmole/l Tris–HCl buffer pH 7.4, which also included 5 mmole/l EDTA and 1 mmole/l dithiothreitol) and centrifuged for 18 hr at 100,000 *g* average at 3–4°C. At the end of the spin, gradients were fractionated and the individual fractions incubated for 2 hr at 4°C with either (a) [^{3}H]oestradiol-17β (5 nmole/l) in the presence or absence of competitors or (b) [^{3}H]tamoxifen (5 nmole/l) with or without cold oestradiol-17β. Free and non-specifically bound steroid were removed by treatment with dextran-coated charcoal.

Competitive binding studies

(a) Constant amounts of cytosol (200 μl) were incubated with either a saturating concentration of [^{3}H]oestradiol-17β (5 nmole/l) or [^{3}H]tamoxifen (20 nmole/l) in the presence of varying concentrations of non-radioactive competitors [200 μl] (see "Results"). After incubation (2 hr at 4°C) 400 μl of charcoal suspension was added and the samples processed as described earlier.

(b) A similar procedure was adopted to that described in (a) except that the incubation time varied from 30 min to 48 hr.

(c) Cytosol fractions were incubated with [^{3}H]oestradiol-17β (5 nmole/l) in the presence or absence of either diethylstilboestrol (500 nmole/l), tamoxifen (5 μmole/l) or metabolite B (2.5 μmole/l) for 5 min to 48 hr. Samples were then processed as described earlier.

Kinetic studies

(a) *Association rates of labelled oestradiol-17β and tamoxifen with oestrogen receptors.* Cytosol

fractions (200 μl) were incubated at 4°C with either [^{3}H]oestradiol-17β (5 nmole/l) or [^{3}H]tamoxifen (20 nmole/l) in the presence or absence of a 1000-fold excess of unlabelled ligand (200 μl). At various time intervals the reactions were stopped by the addition of a solution (100 μl) containing an excess of unlabelled diethylstilboestrol (2 μmole/l). This was immediately followed by the addition of charcoal suspension (500 μl). The total receptor concentration was determined independently by Scatchard analysis [11].

(*b*) *Dissociation rates of labelled oestradiol-17β and tamoxifen from oestrogen receptors.* Cytosol fractions were incubated with either [^{3}H]oestradiol-17β (5 nmole/l) or [^{3}H]tamoxifen (20 nmole/l) in the presence or absence of a 1000-fold excess of unlabelled ligand for 4 hr at 4°C. After the preincubation period a further excess of diethylstilboestrol (2 μmole/l) was added to the tubes containing radioactivity alone. Aliquots (400 μl) were then removed at various time intervals, and added to a charcoal suspension (400 μl). Specifically bound radioactivity was monitored in the normal manner.

(*c*) *Dissociation rates of unlabelled compounds from oestrogen receptors.* Cytosol fractions were preincubated for 4 hr at 4°C with various ligands (see "Results"). After incubation the tubes were maintained at 4°C and excess ligand removed by charcoal absorption. Aliquots (200 μl) of the charcoal free supernatant were incubated at 4° or 15°C for periods up to 24 hr with an equal volume of medium A containing 10 mmole/l phenylmethylsulphonyl fluoride together with [^{3}H]oestradiol-17β (5 nmole/l) in the presence or absence of a 1000-fold excess of unlabelled diethylstilboestrol. The remaining experimental procedure was as described earlier.

Chemical analyses

The protein concentrations of cytosol fractions was estimated using the method of Lowry *et al.* [12].

RESULTS

The data presented in Table 1 show the receptor content and dissociation constants [*KD*] determining for tamoxifen and oestradiol-17β binding to cytosol fractions from various oestrogen receptor containing tissues. Direct binding studies of the [^{3}H]ligands were carried out on the same cytosol preparations to establish any relationships between the binding characteristics of the two compounds. In all tissues examined tamoxifen binds to approximately the same number of sites as does oestradiol-17β but with 2.5–5% the affinity of oestradiol-17β.

Saturation curves generated for [^{3}H]tamoxifen and [^{3}H]-oestradiol-17β binding to a cytosol preparation from a DMBA-induced mammary tumour clearly show a higher non-specific binding component for [^{3}H]tamofixen binding [Fig. 1(a)]. Nevertheless, subtraction of this value from the value determined in the presence of [^{3}H]tamoxifen alone produced saturability of binding sites. Scatchard analysis [11] of the corrected data indicated a single class of binding sites for both oestradiol-17β and tamoxifen [Fig. 1 (c and d)]. The above data is representative of similar curves observed for the binding of the radioligands to cytosol preparations from human mammary tumours and also rat uteri (not illustrated).

Sucrose density gradient analysis of tamoxifen and oestradiol-17β binding is consistent with the concept of a common 7–8S binding

Table 1.

	Oestradiol-17β		Tamoxifen	
	KD	Receptor level	*KD*	Receptor level
	(nmole/l)	(fmole/mg Protein)	(nmole/l)	(fmole/mg Protein)
Human mammary tumour	0.12 ± 0.034	34.1 ± 8.63(4)	3.70 ± 1.02	31.2 ± 8.43(4)
Rat mammary tumour	0.102 ± 0.052	73.0 ± 24.9(5)	4.34 ± 1.76	67.0 ± 20.6(5)
Rat uteri	0.134 ± 0.052	60.9 ± 13.0(3)	4.58 ± 1.56	55.9 ± 11.7(3)

Results are expressed as mean ± standard deviations. Figures in parenthesis show the numbers per group.

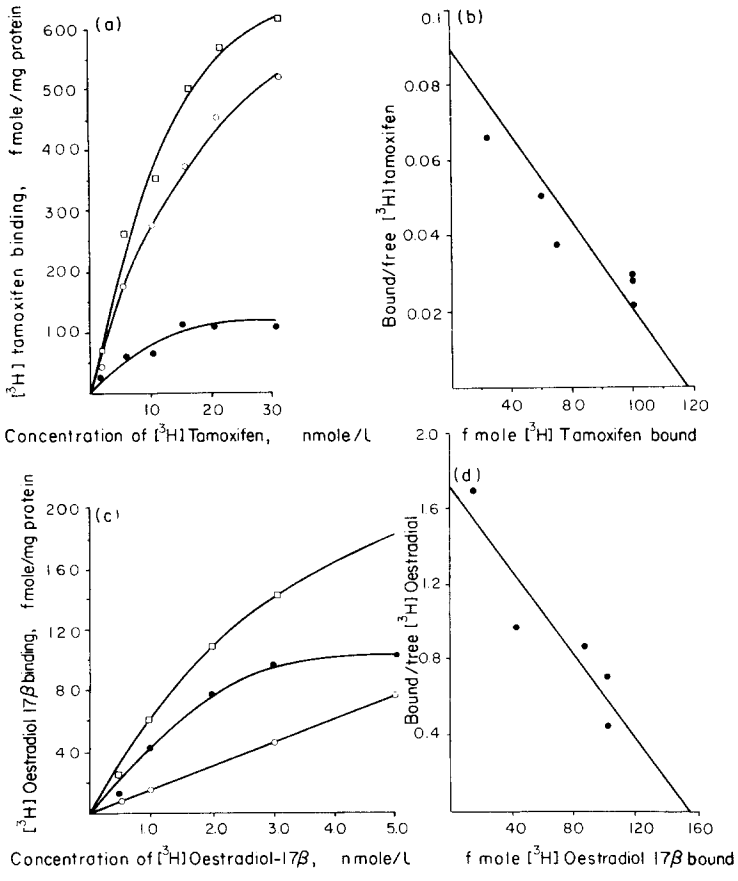

Fig. 1. Determination of the number of specific oestradiol-17β and tamoxifen binding sites. Cytosol fractions prepared from DMBA-induced mammary tumour tissue were incubated for 16 hr at 4°C with various concentrations of either [^{3}H] tamoxifen or [^{3}H]oestradiol-17β (□) in the presence or absence of a 1000 fold excess of unlabelled ligand (○). Specific binding was obtained by subtraction of the counts obtained in the presence of cold ligand from those obtained in its absence (●) [Figs. (a and c)] and plotted according to Scatchard [12] [Figs. (b and d)].

protein for the two compounds [Fig. 2 (a and b)]. This view is reinforced by three sets of data: (1) cytosol preparations from a mammary tumour with no specific binding peak for oestradiol-17β also showed no displaceable tamoxifen binding [Fig. 2 (c and d)]; (2) protamine sulphate precipitation of the oestrogen receptor also precipitates the binding protein for tamoxifen (Table 2); (3) competitive binding analysis indicates a similar specificity of association for the two radioligands, competitive efficiency increasing in the sequence, tamoxifen, metabolite B, oestriol, diethylstilboestrol and oestradiol-17β [Fig. 3 (a and b)]. No competition for either tamoxifen or oestrogen binding sites was evident with either progesterone or cortisol.

Comparison of the relative affinities of tamoxifen and oestradiol-17β binding obtained by either direct (Table 1) or indirect [Fig. 3(a)] methods demonstrates a considerable discrepancy between the results. Relative affinity values calculated from competitive binding experiments (2 hr incubation at 4°C) were consistently lower than those determined by the ratio of *KD* values (cf. 0.33% and 3–5% respectively). In addition, the capacity of tamoxifen to compete with oestradiol-17β for oestrogen receptor sites apparently decreases with increasing incubation time [Fig. 4(a)], such that, following a 48 hr incubation period at 4°C, only a 16% displacement of [^{3}H]oestradiol-17β binding by a 2000 fold excess of tamoxifen was observed. The effect was not so marked for metabolite B [Fig. 4(b)], a compound whose relative affinity for the oestrogen receptor was approximately 25% that of oestriol [Fig. 3(a)].

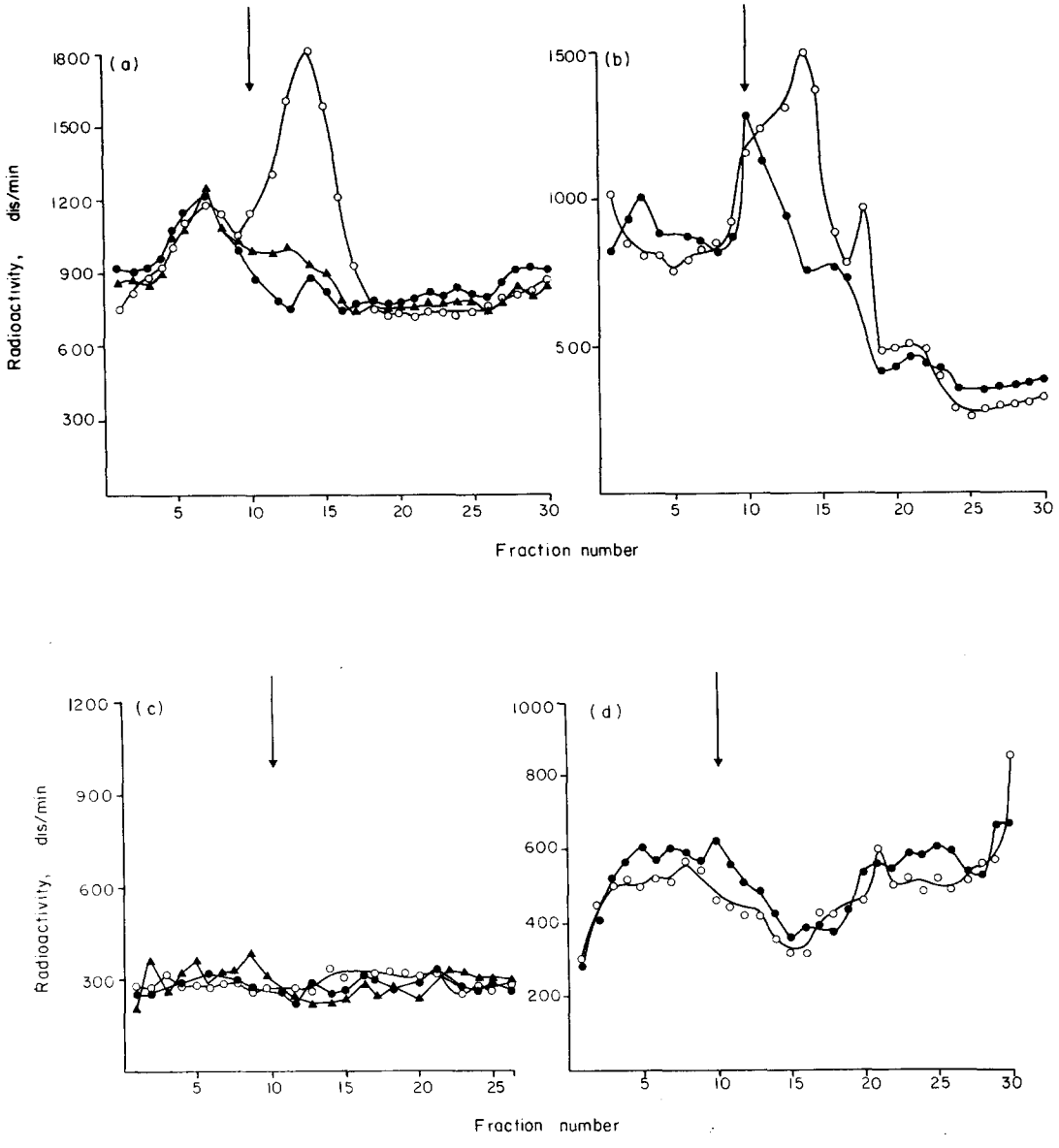

Fig. 2. Sucrose density gradient profiles of cytosol fractions. Cytosol fractions (400 μl) *prepared from DMBA-induced mammary tumours were centrifuged through linear* 5–20% (w/v) *sucrose density gradients under low salt conditions prior to their incubation with either* [^{3}H]*oestradiol-17β* (○) *in the presence or absence of an excess of cold oestradiol-17β* (●) *or tamoxifen* (▲) [*gradients* (*a*) *and* (*c*)] *or* [^{3}H]*tamoxifen* (○) *with or without cold oestradiol-17β* (●) [*gradients* (*b*) *and* (*d*)]. *Free steroid was removed from each fraction by charcoal adsorption. Sedimentation marker* (*arrow*) *was bovine serum albumin* (4.6 *S*) *in each case.*

Table 2. Protamine sulphate precipitation of oestrogen and tamoxifen binding sites

	Percentage precipitation of binding sites	
	Oestradiol-17β	Tamoxifen
Rat uteri	98.3(4)	96.4(4)
DMBA-induced mammary tumour	94.0(4)	95.7(4)
Human mammary tumour	93.1(2)	96.4(2)

Figures in parenthesis show numbers per group.

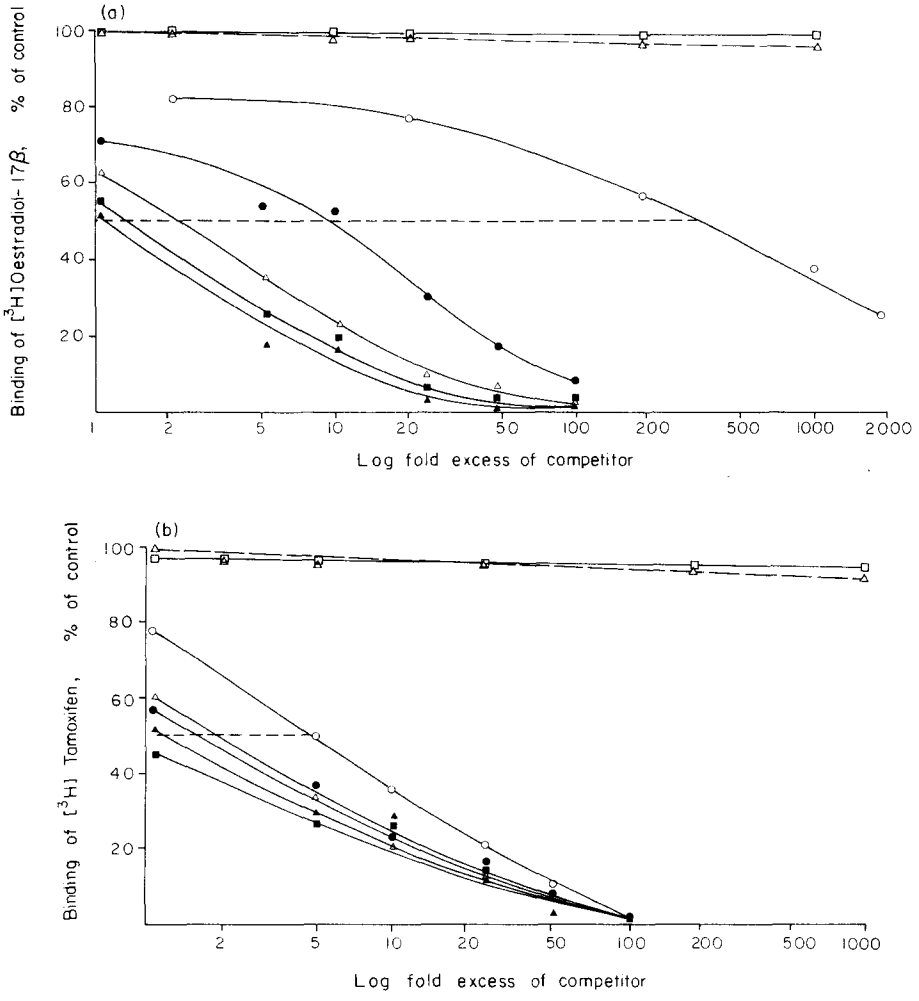

Fig. 3. Competition for oestrogen and tamoxifen binding sites. Samples of cytosol were incubated with either (a) [^{3}H]*oestradiol-17β* (5 nmole/l) *or (b)* [^{3}H]*tamoxifen* (20 nmole/l) *alone or in the presence of increasing concentrations of the other ligands for* 2 hr *at* 4°C *(range:* 1–100 *or* 1000 *fold excess non-radioactive ligand). Free steroid was removed by charcoal adsorption and a correction for the non-specific binding component made by subtraction of the counts bound in the presence of a* 100 *fold excess of non-radioactive ligand (either oestradiol-17β or tamoxifen in (a) and (b) respectively). Competing ligands were tamoxifen* (○), *metabolite B* (●), *oestradiol-17β* (▲), *oestriol* (△), *cortisol* (□), *progesterone* (- - △ - -) *and diethylstilboestrol* (■). *Binding is expressed as a percentage of charcoal-resistant binding in the presence of either (a)* [^{3}H]*oestradiol-17β* (73 fmole/mg *protein) or (b)* [^{3}H]*tamoxifen* (63 fmole/mg *protein*).

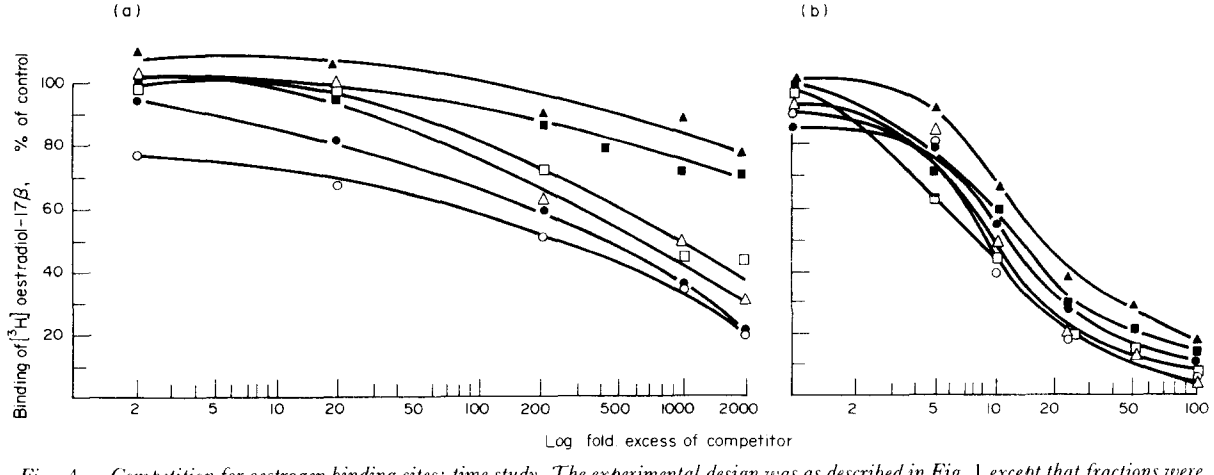

Fig. 4. Competition for oestrogen binding sites: time study. The experimental design was as described in Fig. 1 except that fractions were incubated with ligands for 30 min (○), 60 min (●), 120 min (△), 240 min (□), 24 hr (■) *and* 48 hr (▲) *at* 4°C. *Competing ligands were (a) tamoxifen* (5 μmole/l) *and (b) metabolite B* (2.5 μmole/l).

Incubation of tamoxifen (2 μM) and [^{3}H]oestradiol-17β (5 nM) for varying periods of time with a rat uterine cytosol preparation at 4°C clearly demonstrates that tamoxifen decreases the rate of uptake of oestradiol-17β onto the oestrogen receptor [Fig. 5(a)]. Binding equilibrium for oestradiol-17β in the presence of tamoxifen was not achieved in the period up to 48 hr. Recalculation of the results as the percentage binding of [^{3}H]oestradiol-17β indicated that tamoxifen exhibits a biphasic inhibitory action [Fig. 5(b)]. At early (<15 min) and late (>24 hr) time points tamoxifen had little effect on [^{3}H]oestradiol-17β binding. The maximum competition was observed at 60 min. Timed competition curves generated for metabolite B and diethylstilboestrol showed little if any secondary decrease in competition at 4°C [Fig. 5(b)]. Similar results were observed with oestrogen receptors from DMBA-induced mammary tumours (not illustrated).

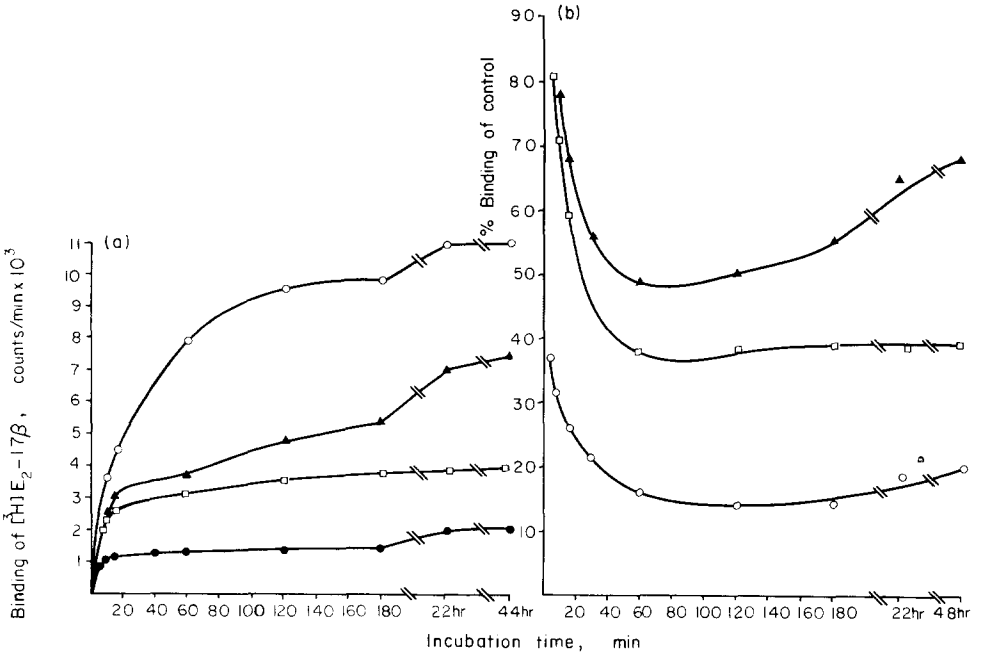

Fig. 5. Competition for oestrogen binding sites: time study at a single concentration of the competitors. The experimental design was as described in Fig. 1 except that fractions were incubated with [^{3}H]*oestradiol-17β for periods up to* 48 hr *with a fixed concentration of either diethylstilboestrol* (●) 5000 nmole/l, *tamoxifen* (▲), 5 μmole/l, *metabolite B* (□), 2.5 μmole/l *or buffer A* (○). *(a) Rate of association of* [^{3}H]*oestradiol-17β with oestrogen receptors; (b) competition for oestrogen receptors.*

The relatively low binding affinity of tamoxifen for cytoplasmic binding components is reflected by its rapid dissociation from the oestrogen receptor. Following an incubation period of 24 hr at 4°C endogenously bound tamoxifen was specifically replaced by [^{3}H]oestradiol-17β [Fig. 6(a)]. Under conditions identical to those described above, only small quantities of metabolite B and oestriol were released. No substantial release of either oestradiol-17β or diethylstilboestrol was observed. Elevation of the temperature to 15°C resulted in the total dissociation of tamoxifen and the partial dissociation of the remaining compounds [Fig. 6(b)].

These data are consistent with the rate of dissociation of tamoxifen and oestradiol-17β from their specific binding sites (Fig. 7). At 4°C, 85% of the specifically bound [^{3}H]-tamoxifen dissociates during the 48 hr experimental period (k_2, 20×10^{-4}/min). Under similar conditions no release of [^{3}H]oestradiol-17β was observed. Elevation of the incubation temperature to 15°C increased the dissociation rate of both tamoxifen and oestradiol-17β (k_2 values, 144×10^{-4}/min and 15.1×10^{-4}/min respectively). The association rates of [^{3}H]oestradiol-17β and [^{3}H]tamoxifen binding were measured at 4°C only, since, at higher temperatures, the association rate was too great to determine accurately. In contrast to the reverse reaction constants, the forward rate constant for tamoxifen association with cytosolic proteins was 4 times smaller than

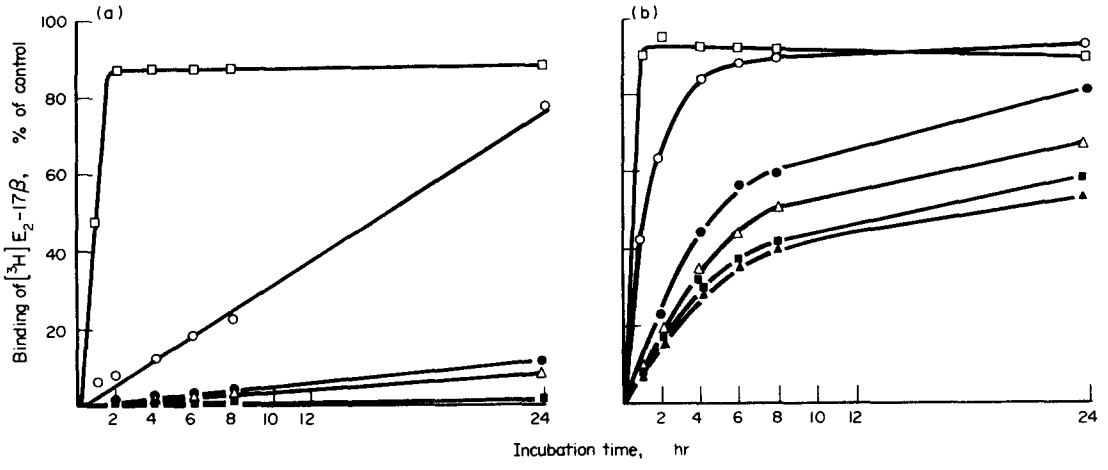

Fig. 6. *Effect of temperature on the exchange of specifically bound ligand. Cytosol preparations were preincubated for* 2 hr *at* 4°C *with a saturating concentration of either oestradiol-17β* (▲), 500 nmole/l, *diethylstilboestrol* (■), 500 nmole/l, *oestriol* (△), 500 nmole/l, *metabolite B* (●), 2.5 μmole/l, *tamoxifen* (○), 5 μmole/l *or medium A* (□). *Excess ligand was removed by charcoal adsorption and the ligand–receptor complex incubated for periods up to* 48 hr *at* (*a*) 4°C *or* (*b*) 15°C *with either* (i) *a saturating concentration of* [^{3}H]*oestradiol-17β* (5 nmole/l) *or* (ii) [^{3}H]*oestradiol-17β plus a* 1000*fold excess of unlabelled diethylstilboestrol. Specific binding was obtained by subtraction of* (ii) *from* (i). *Each value is expressed as a percentage of the value obtained by incubation of cytosol* (*untreated*) *with labelled steroid at* 4°C.

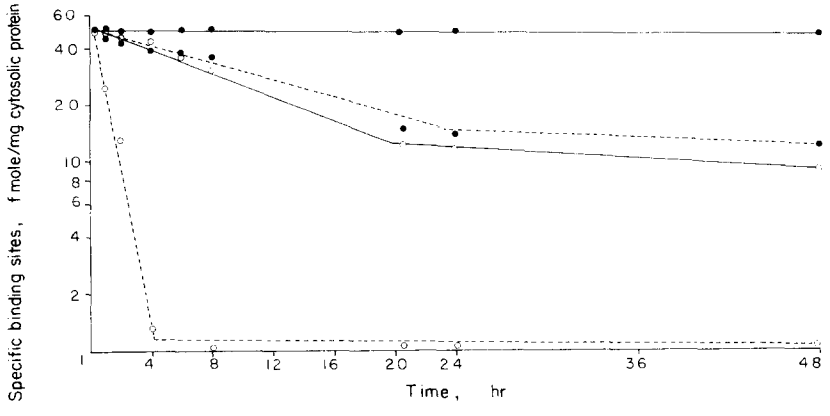

Fig. 7. *Dissociation kinetics of oestradiol-17β and tamoxifen from oestrogen receptor proteins. DMBA-induced mammary tumour cytosol fractions were incubated with either* [^{3}H]*oestradiol-17β* (●), 5 nmole/l *or* [^{3}H]*tamoxifen* (○), 20 nmole/l *for* 4 hr *at* 4°C *with* (i) *and without* (ii) *a* 1000*fold excess of non-radioactive ligand. Free ligand was removed by charcoal adsorption and specific binding estimated by subtraction of* (i) *from* (ii). *Rates of dissociation of the radioligand were measured by the isotopic dilution technique, where specific reassociation of dissociated radioligand is prevented by the addition of a large excess on non-radioactive ligand* (10 μmole/l) *to the cytosol. After various incubation times at* 4°C (—) *or* 15°C (- - - -), 400 μl *samples were treated with dextran coated charcoal and receptor bound radioactivity determined. Reverse rate constants were calculated from the initial slope using the equation,* $k_2t = \ln R_0/R$, *where* R_0 *and* R *are the number of receptor complexes at time* 0 *and time* t *respectively*

that observed for oestradiol-17β (k_1 values, 4.5×10^5l/mole/min and 17.2×10^5l/mole/min respectively) (Fig. 8). Comparison of dissociation constants (*KD*) for tamoxifen binding under equilibrium (*KD*) or non-equilibrium (k_2/k_1) conditions gave similar results (Table 3).

DISCUSSION

The present report clearly demonstrates that tamoxifen and oestradiol-17β share a number of binding characteristics: (1) in all tissues examined tamoxifen was found to bind to approximately the same number of cytosol

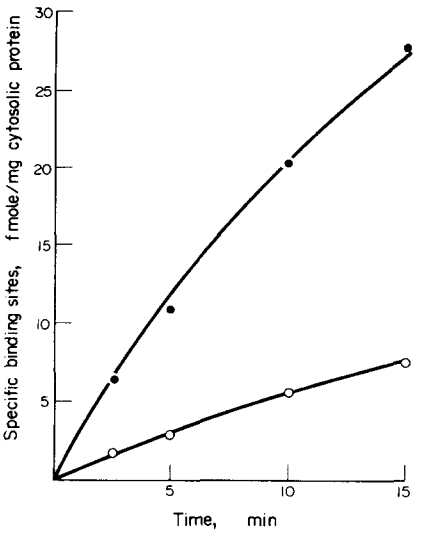

Fig. 8. Association kinetics of oestradiol-17β and tamoxifen with oestrogen receptor proteins. DMBA-induced mammary tumour cytosol fractions were incubated with [^{3}H]*oestradiol-17β* (●), 5 nmole/l *or* [^{3}H]*-tamoxifen* (○), 20 nmole/l *in the presence or absence of a* 1000*-fold excess of the non-radioactive ligand. Forward rate constants for the specific binding of the radioligands were calculated using the equation,* $k_1 = 1/t$ [Lo-Ro] ln [L]/[R] *assuming the reaction to be second order and where* Lo ≫ R. [Lo], [Ro], [L] *and* [R] *are the concentrations of ligand and receptor at time* 0 *and time* t *respectively. Total receptor content and dissociation constant* (KD) *were determined independently by Scatchard analysis.*

binding sites as did oestradiol-17β, although with a lower affinity; (2) common precipitation of the binding proteins was observed with protamine sulphate; (3) identical sedimentation coefficients were determined by sucrose density gradient analysis (a similar phenomenon has been described by Jordan and Prestwich [13] in rat uterine cytosol preparations using different methodology); (4) in the absence of oestrogen binding proteins no displaceable tamoxifen binding was evident [6] and (5) translocation of the oestrogen receptor to the nuclei of DMBA-induced mammary tumours [14–16] and also rat uteri [17] has been demonstrated to occur following the *in vivo* administration of tamoxifen. Such data, although not conclusive, is consistent with the concept of a common binding protein for oestradiol-17β and tamoxifen. Furthermore, similarities in the specificity of binding of the two ligands are indicative of a common binding site.

Hahnel, Twaddle and Ratajczak [18] postulated that the attachment of oestradiol-17β to the receptor probably occurs via two groups, the phenolic C-3 hydroxyl group binding to a highly specific site, thereby faci-

Table 3. Kinetic and equilibrium constants for oestradiol-17β and tamoxifen binding at 4°C to oestrogen receptors from rat mammary tumours

	*k*1 (10^5l/mole/min)	*k*2 (10^{-4}/min)	*k*2/*k*1 (nmole/l)	*KD* (nmole/l)
Oestradiol-17β	17.5±4.34(3)	—	—	0.102±0.052(5)
Tamoxifen	4.3±2.73(3)	21±6.7(4)	4.88	4.34±1.76(3)

Results are expressed as mean ± standard deviations. Figures in parenthesis indicate the number of tumours per group.

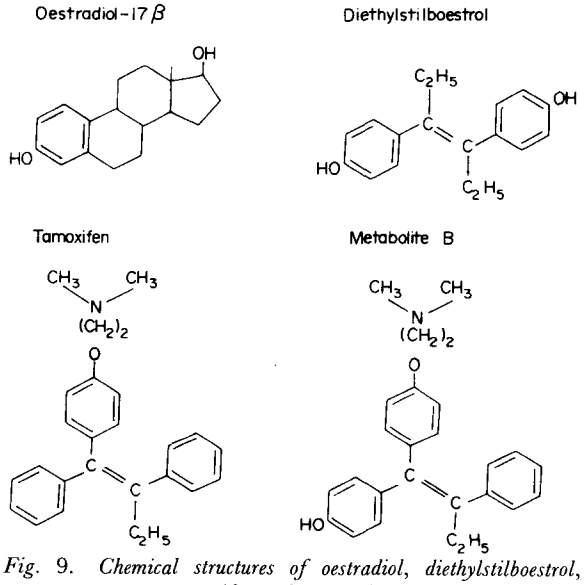

Fig. 9. Chemical structures of oestradiol, diethylstilboestrol, tamoxifen and metabolite B.

litating the attraction of the C-17β-hydroxy-function to a less specific binding site. The mechanism by which tamoxifen might bind to the oestrogen receptor is less easy to define. Its general structure, although devoid of hydroxyl groups, may be depicted in a manner similar to oestradiol-17β and diethylstilboestrol (Fig. 9). If this represents the active configuration of the molecule, one would predict that the incorporation of a single hydroxyl group in the *para* position of the C_1 phenyl of tamoxifen, forming metabolite B, would increase the affinity of the molecule for the oestrogen receptor. Competitive binding studies indicate this to be the case. Alternatively, the interaction between tamoxifen and the oestrogen receptor may be allosteric in nature [18].

Kinetic studies indicate that the rate of association of tamoxifen with its binding protein is lower than the association rate observed for oestradiol-17β. In addition, once formed the tamoxifen receptor complex is relatively unstable, dissociating with a primary half-life of approximately 8 hr at 4°C (Fig. 7). Application of the above data to the competitive binding studies may serve to explain the apparent time-dependent variations in affinity of tamoxifen for oestrogen receptor proteins and hence the affinity differences obtained between direct and indirect methods [19, 20]. It is likely that, during the early phase of competition, tamoxifen at high concentrations competes readily with [^{3}H]oestradiol-17β for oestrogen binding sites. At 4°C, however, tamoxifen, but not oestradiol-17β, dissociates from the receptor and may thus be replaced by [^{3}H]oestradiol-17β at the expense of the antioestrogen. Tamoxifen can therefore be seen as decreasing the association rate of oestradiol-17β to its receptor protein [Fig. 5(a)]. Metabolite B, having a higher affinity for the oestrogen receptor, would predictively show lower time-dependent affinity alterations. A similar effect for tamoxifen, nafoxidine and dimethylstilboestrol has been observed with oestrogen receptor from immature rat uteri [21]. Rochefort and Capony [21], however, proposed that, in the presence of oestradiol-17β, the receptor may be transformed into a form less sensitive to antioestrogen. The relative merits of these two possibilities awaits clarification.

As with tamoxifen [19, 20, 22], variations in relative affinities have also been recorded for androgens binding to oestrogen receptors [23–26]. Nevertheless, at high dose levels, androst-5-ene, 3β, 17β diol, 5α-dihydrotestosterone and testosterone are all capable of translocating the oestrogen receptor to the nuclei of rat mammary tumours [27]. Once present in the nucleus the androgen-translocated oestrogen receptor is then accessible to [^{3}H]oestradiol-17β *in vitro* at 4°C. Similar observations have been made with 5α-dihydrotestosterone in rat uteri by Ruh, Wassilak and Ruh [28], Rochefort and Garcia [29] and Rochefort, Lignon and Capony [30]. It is possible that the relatively high rate of dissociation of androgens from the oestrogen receptor [29] may account for these observations.

Logically, our studies on the antioestrogen and androgen mediated effects on oestrogen receptors suggests that the ability of the compounds to elicit the nuclear translocation process is a function of their capacity to associate with the oestradiol-17β binding site [13, 27], whereas the biological activity of the resulting complex is a function of the dissociation of the ligand from the complex. Rapidly dissociating compounds would therefore be seen as possessing low intrinsic oestrogenic activity and thus high antioestrogenic potential [31]. The hypothesis, although attractive, may represent an over simplification of the situation since metabolite B has a higher affinity for the oestrogen receptor than tamoxifen, yet it is a more potent antioestrogen in the rat [9]. Also, tamoxifen [16] and other antioestrogen receptor complexes [32] have prolonged nuclear retention times in comparison with the androgen-translocated oestrogen receptor [27]. Alternatively, the arguements against the hypothesis could represent artifacts associated with such characteristics as the clearance rates of the compounds from tissue and plasma. Following a single oral dose of [^{14}C]tamoxifen to female patients two half-lives of tamoxifen clearance from serum were described at approximately 7–14 hr and 7 days [7]. Similar values were found from studies in the female rat [8]. The disappearance of [^{3}H]testosterone from plasma also shows two half-lives [33]. Its clearance (half-lives approximately 7 and 30 min) is, however, much more rapid than tamoxifen. It seems likely, therefore, that over a prolonged period of time, tamoxifen, but not the androgens, is available for binding to the oestrogen receptor protein, thus ensuring a steady transfer of antioestrogen–receptor com-

plex to the nucleus. Maintenance of relatively high concentrations of androst-5-ene, 3β,17β-diol, by implantation or continuously injecting the steroid, results in an oestrogenic effect on both mammary tumours [34] and uterus [34, 35] of the rat. Interestingly, the short term oestrogenic properties of tamoxifen *in vivo* are more evident when the dose of the compound is increased [9, 36, 37].

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