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A Comparative Study of Electrophoretic Mobilities of [^{3}H]-Estradiol and Monohydroxytamoxifen Binding Components in the Cytosols of Human Breast Carcinomas and Sera of Healthy Adult Females

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Abstract—*Cytosols from human breast carcinomas rich in estrogen receptors (ER) were examined for the presence of* [^{3}H]*-estradiol* (E_2) *and* [^{3}H]*-monohydroxytamoxifen* (OH-*TX*) *binding components. Polyacrylamide gel electrophoresis was used to examine the comparative anodal mobilities of ER-*[^{3}H]*-*E_2 *and ER-*[^{3}H]*-*OH-*TX complexes, and also to identify any cytosol or serum component that may exhibit preferential high affinity to* OH-*TX. We have demonstrated that* [^{3}H]*-*OH-*TX binds ER with high affinity and the anodal mobility of ER-*[^{3}H]*-*OH-*TX complexes is identical to that of ER-*[^{3}H]*-*E_2 *complexes. We were unable to identify an antiestrogen-specific component in ER-positive or ER-negative cytosols or in sera of healthy adult females. A serum component exhibiting a higher affinity to* [^{3}H]*-*OH-*TX and* [^{3}H]*-DES than to* [^{3}H]*-*E_2 *has been identified in all the female sera examined, but this binding is of high capacity and is unsaturable by a* 1000*-fold molar excess of unlabeled* E_2 *or antiestrogens. The electrophoretic mobility of this component is comparable to that of serum albumin.*

INTRODUCTION

CYTOPLASMIC estrogen receptors of human breast carcinomas complexed with ^{3}H-labeled estradiol or monohydroxytamoxifen have been shown to have identical sedimentation characteristics in sucrose density gradients [1, 2]. Biochemical evidence for the presence of a cytosol component with a high affinity exclusively for antiestrogens was first presented by Sutherland *et al.* in the chick oviduct and human breast carcinomas [3, 4], and similar observations have been reported for the immature rat uterus [5, 6]. In the lamb and calf uterine cytosols, however, such an antiestrogen binding component distinct from estrogen receptors could not be detected by Borgna and Rochefort [7].

In our laboratory we have developed a procedure for electrophoretic identification of estrogen receptors (ER) in human breast cancer cytosols labeled with [^{3}H]-estradiol ([^{3}H]-E_2) using highly porous polyacrylamide gels [8]. With the help of this electrophoretic method we have been able to identify differences in the anodal mobility of cytoplasmic ER-[^{3}H]-E_2 complexes of human breast cancer and ER-E_2 complexes of calf and rat uterine cytosols. The present study was undertaken with the purpose of: (a) comparing the electrophoretic characteristics of ER complexed with [^{3}H]-E_2 or [^{3}H]-monohydroxytamoxifen ([^{3}H]-OH-TX); and (b) identifying any component(s) in the human breast cancer cytosols or normal female sera that exhibit preferential high affinity to non-steroidal antiestrogen OH-TX.

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MATERIALS AND METHODS

Radiolabeled compounds and other reagents

[^{3}H]-Labeled estradiol-17β was purchased from New England Nuclear, Boston, MA, U.S.A.

The specific activity was 100 Ci/mmol with 98% purity. [^{3}H]-Labeled diethylstilbestrol was obtained from Amersham, Arlington Heights, IL, U.S.A. at 97% purity with a specific activity of 113 Ci/mmol. It was used within 2 months after purchase, during which time it retained its capacity to bind type I cytoplasmic receptors with high affinity. [^{3}H]-Monohydroxytamoxifen, a 1:1 mixture of *cis* and *trans* isomers, was a gift from ICI, plc. (Pharmaceuticals Division), Mereside, Macclesfield, Cheshire, U.K. The specific activity of this compound was 42 Ci/mmol and its purity 97% in absolute ethanol. It was used without further purification.

Cytosols of human breast carcinomas

Repeated electrophoretic analysis of freshly prepared ER-rich breast tumor cytosols and cytosols frozen at -80°C for varying lengths of time and thawed prior to analysis failed to show any differences in the [^{3}H]-E_2 binding components. There were no alterations induced by freezing and thawing either in the quantity or anodal mobility of ER-[^{3}H]-E_2 complexes. Therefore, for the present study excess cytosols from breast cancer sent to our laboratory for quantification of steroid receptors that were stored frozen were utilized. The cytosols rich in ER ($\geqslant$300 fmol/mg cytosol protein; type I saturable sites; K_d of ER-E_2, complexes $\leqslant 1 \times 10^{-10}$M) were frozen individually or, if the quantity was insufficient, cytosols from different ER-rich tumors were pooled together and frozen.

ER-Negative cytosols included in this study were from post-menopausal women with breast cancer.

Sera

Sera collected from healthy females with no history of breast cancer or any other carcinomas were frozen at -20°C and also examined for [^{3}H]-E_2, [^{3}H]-OH-TX or [^{3}H]-DES binding components.

Handling of monohydroxytamoxifen

We experienced great difficulty in preparing aqueous solutions of monohydroxytamoxifen. [^{3}H]-OH-TX adhered to the glass containers and its solubility in aqueous buffer solutions was very poor. In these characteristics it resembled R5020, the synthetic progestogen. Since inclusion of glycerol in the buffer (30% v/v) alleviated the solubility problems of R5020, we tested the efficacy of glycerol for increasing the solubility of OH-TX. Buffers containing 5, 30 or 60% glycerols were used to prepare solutions containing different [^{3}H]-OH-TX concentrations. Since OH-TX is highly soluble in ethanol, parallel dilutions of [^{3}H]-OH-TX were made in absolute ETOH for comparison. The ratio of observed concentration of [^{3}H]-OH-TX over expected concentration of each of the solutions was determined.

Preparation of cytosols from tumors

Cytosols were prepared from tumor powders processed by percussion of tumor pieces which were frozen hard using liquid N_2. The homogenizing buffer was composed of 0.01 M Tris, 0.0015 M EDTA and 0.005 M dithiothreitol, pH 7.2. The tumor powders were homogenized using a polytron homogenizer as described previously [8]. The cytosols were prepared by centrifuging the homogenized pulp at 100,000 *g* for 1 hr in a Beckman ultracentrifuge equipped with a Type 50 rotor.

Incubation of cytosols with radiolabeled ligands

The cytosols were incubated in 100 or 200 μl quantities with 0.4 pmol of ^{3}H-labeled E_2, OH-TX or DES alone or in the presence of unlabeled competitors in 200- or 1000-fold molar excess. All the ^{3}H-labeled ligands were competed with an excess of unlabeled E_2, OH-TX or DES. Cytosols were incubated with the ligands for 18 hr. The incubation mixtures were either subjected to electrophoresis without removal of unbound ligands or they were mixed and shaken with charcoal pellets made from 0.5 ml of DCC solution (composition of DCC solution as described by McGuire and Dela Garza [8]) and centrifuged to sediment the charcoal. The radioactivity in the supernates was quantified in measured aliquots of the supernates mixed with scintillation liquid using a Delta 300 scintillation counter with a ^{3}H counting efficiency of 48–50%.

Since the solutions of [^{3}H]-OH-TX were made in a buffer containing 60% glycerol, when the cytosols were incubated either with [^{3}H]-OH-TX alone or in the presence of unlabeled competitors the final concentration of glycerol in the incubation mixtures was 12%. No glycerol was present in incubation mixtures containing cytosols and ^{3}H-labeled E_2 alone or with unlabeled competing ligands. The presence of even 10% glycerol was found to increase the dissociation constant of ER-[^{3}H]-E_2 complexes by 1 log (from 1×10^{-11}M to 1×10^{-10}M) and the use of glycerol was found to be detrimental to electrophoretic analysis of ER-[^{3}H]-E_2 complexes because [^{3}H]-E_2 dissociated from the receptor and hence did not migrate as a sharp band, as they did in the absence of glycerol (unpublished observation).

Polyacrylamide gel electrophoresis

Polyacrylamide gels containing 4% acrylamide and 0.5% cross-linking were used in a continuous buffer system. For the preparation of acrylamide gels the following solutions A, B and C were prepared in electrode buffer (0.09 M Tris base, 0.0025 M EDTA, 0.09 M borate, pH 8.3, prepared in metal-distilled water, non-deionized):

Solution A: Acrylamide, 16 mg; bis-acrylamide, 0.8 g in 100 ml electrode buffer.

Solution B: TEMED, 240 μl per 100 ml buffer.

Solution C: Ammonium persulfate 140 mg/100 ml buffer.

Solutions A, B and C were mixed at a proportion of 1:1:2 v/v and poured into glass tubes of (outside diameter) 7 × 75 mm dimensions. Polymerization was allowed to proceed under fluorescent light for 1 hr at room temperature. The electrode buffer, as well as the gels, were stored in the refrigerator until use.

Electrophoresis was performed at +5°C. This temperature was maintained by the use of a refrigerated circulating water bath for cooling the buffer chamber. Only pre-chilled gels and electrode buffers were used for electrophoresis. The protein load per gel was never allowed to exceed 50 μg and the volume of the solution was maintained equal to or below 30 μl per gel. Three parts of the protein solutions were mixed with one part of the sucrose–dye solution (40% sucrose, 0.1% bromophenol blue in electrode buffer) and 25–30 μl of this mixture was subjected to electrophoresis.

Electrophoresis was performed at a constant current of 2 mA/gel for 1 hr 20 min, during which time the free dye (bromophenol blue) migrated to the bottom of the gel. None of the cytosols (ER-rich or ER-negative) contained a sufficient quantity of albumin so as to give a visible albumin–dye band. All the normal sera, however, did yield an albumin–dye band and therefore the distance of migration of this band from the point of origin could be measured. In addition, at the end of the electrophoresis one of the gels was stained with Coomassie blue to visualize the protein bands and the rest of the gels were sectioned into 3 mm thick slices using a Biorad gel slicer. Each slice was transferred to a scintillation vial, soaked in scintillation cocktail and the ^{3}H-activity counted.

Protein determination

Protein content of the cytosols was determined using BioRad reagent and the protein standard was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. The protein standard solution was a mixture of human serum gamma globulins and albumin.

RESULTS

Effect of glycerol in the buffer on the solubility of [^{3}H]-OH-*TX*

[^{3}H]-OH-TX was diluted ×130 from our ETOH stock solution containing 52 pmol of OH-TX/50 μl. When the glycerol concentration in the diluting buffer was increased from 0 to 60% there was an increase in the ratio of observed/expected radioactivity in the resultant solution. The solubility of [^{3}H]-OH-TX was comparable to its solubility in ethanol when 60% glycerol was present in the diluent buffers. Inclusion of 60% glycerol prevented OH-TX from sticking to the glass pipette tips. Furthermore, the reproducibility of the recovery of radioactivity in the diluted solutions was superior when 60% glycerol was included in the buffer, as shown in Table 1.

Table 1. Reproducibility in the recovery of radioactivity of [^{3}H]-OH-*TX in the diluent buffer containing 60% glycerol (using stock* [^{3}H]OH-*TX in ethanol)*

	Diluent with 60% glycerol (pmol/50 μl)		Diluent with no glycerol (pmol/50 μl)	
	cpm/50 μl	Observed / expected	cpm/50 μl	Observed / expected
ETOH → diluent	28,774		6170	
	30,732	0.62 / 0.62	6295	0.126 / 0.62
	30,074		5916	
ETOH → diluent → diluent (serial double dilution)	14,217		1891	
	13,911	0.31 / 0.309	1714	0.04 / 0.32
	14,110		1816	

Comparison of ER-[^{3}H]-E_2 sites and ER-[^{3}H]-OH-TX sites in ER-rich cytosols

Table 2 contains the comparative data on total saturable ER-[^{3}H]-E_2 sites and ER-[^{3}H]-OH-TX sites quantified in 11 individual ER-rich cytosols and 5 different pools of ER-rich cytosols. The cytosols were prepared without any glycerol in the homogenizing buffer. The aliquots of cytosols that were incubated with [^{3}H]-E_2 also lacked glycerol (see Materials and Methods for effect of glycerol on the stability of ER-E_2 complexes), whereas the portions of cytosols incubated with [^{3}H]-OH-TX or [^{3}H]-OH-TX plus molar excess of unlabelled E_2, OH-TX or DES contained a final concentration of 12% glycerol. When the ER sites were quantified it was observed that the presence of 12% glycerol did not affect the stability of ER-[^{3}H]-OH-TX complexes. Of 17 determinations, the percentage of ER-[^{3}H]-OH-TX/ER-[^{3}H]-E_2 binding was $\geqslant$99% in 4 instances, $\geqslant$70% in 13 cases, and in 4 cases the [^{3}H]-OH-TX binding was below 70% of [^{3}H]-E_2 binding (Table 2; AUT, PER, pooled cytosols 3, 4). In no case was the concentration of [^{3}H]-OH-TX binding higher than those obtained with [^{3}H]-E_2.

Anodal migration of protein binding [^{3}H]-E_2 *or* [^{3}H]-OH-*TX*

The data pertaining to electrophoretic analysis of ER-rich cytosols are given in Table 3 and Figs 1–3. A comparison of electrophoretic mobility of ER-[^{3}H]-OH-TX complexes in the glycerol-free system (Fig. 1) with glycerol-containing buffer system (Figs 2, 3) revealed that the glycerol does not alter the electrophoretic mobility of [^{3}H]-OH-TX binding components. The results of ER-negative cytosols are discussed below (Fig. 4), and information regarding normal human sera (female) is given in Fig. 5.

ER-rich cytosols. Of 11 samples of ER-rich cytosols analyzed by electrophoresis (from 7 individual tumors and 4 cases in which several cytosols were pooled together), in all but 1 sample (MILL) only a single radioactive peak which migrated 1.8 cm (6th slice) was evident (Table 3) (Figs 2, 3). There was no difference in the position of this peak whether [^{3}H]-OH-TX or [^{3}H]-E_2 was the ligand. This radioactive peak was observed only when the cytosol was exposed to ^{3}H-ligands in the absence of unlabeled competitors. No radioactive peak was evident in this area (gel slices 4–8) in the cytosols when incubated with ^{3}H-ligand in the presence of molar excess of E_2, OH-TX or DES (Figs 1, 2). When the cytosols labeled with [^{3}H]-E_2 or [^{3}H]-OH-TX in the absence of unlabeled competitors were examined by electrophoresis after removal of free ligands by DCC treatment, the radioactive peak was seen in the same position as found in the cytosols which were subjected to electrophoresis without prior removal of free ligands (Fig. 3, curves A, B, C). The affinity of the bond between the ligand and the protein is high enough to withstand DCC treatment and subsequent electrophoresis.

Table 2. Comparison of saturable binding sites in ER-rich cytosols incubated with [^{3}H]-E_2 *or* [^{3}H]-OH-*TX**

Cytosols (n = 17)	[^{3}H]-E_2 bound (fmol/mg cytosol)	[^{3}H]-OH-TX bound (fmol/mg cytosol)	[^{3}H]-OH-TX bound / [^{3}H]-E_2 bound (%)
Single cytosols			
BER	237	186	76
BR	151	107	71
MAR	263	229	87
AUT	1614	691	43
DAL	434	340	78
ID	911	645	71
MIL	1013	706	70
PER	1021	373	37
FER	628	619	99
NAD	786	778	99
NY	999	769	77
Pooled cytosols			
(1)	589	511	87
(2)	445	347	78
(3)	493	308	62
(4)	553	346	63
(5)	365	365	100
(6)	353	358	101

*Saturable binding sites = total binding sites minus sites unsaturable in the presence of molar excess of unlabeled DES in the case of [^{3}H]-E_2 and OH-TX in the case of [^{3}H]-OH-TX binding.

Table 3. Electrophoresis of ER-rich cytosols

Sample code	Total cpm/25 μl subjected to electrophoresis* [^{3}H]-OH-TX†	[^{3}H]-E_2†	ER-bound cpm/25 μl DCC supernate‡ [^{3}H]-OH-TX†	[^{3}H]-E_2†	Position of radioactive peak in gel§ [^{3}H]-OH-TX†	[^{3}H]-E_2†
ID	961	5287	734	2503	6th (slice)	6th
AUT	1454	4963	692	3905	6th	6th
DALT	1361	4839	418	1287	6th	6th
FER	1548	5107	564	1257	6th	6th
NAD	1357	4840	425	944	6th	6th
NY (see Figs. 2A, B)	2226	7460	1240	4443	6th	6th
MILL‖	1654	5151	869	3009	6th	5th, 9th
Pooled cytosols						
P - 1	1503	3811	423	1072	6th	6th
P - 2	3059	8625	344	970	6th	6th
P - 3	Not analyzed without DCC treatment		435	1682	6th	6th
P - 4	Not analyzed without DCC treatment		633	2457	6th	6th

*The incubation mixtures containing the cytosol and the radiolabeled ligand with or without competing unlabeled ligands were subjected to electrophoresis without prior DCC treatment. Seventy-five microliters of each sample were mixed with 25 μl of electrode buffer containing 40% sucrose and 0.1% bromophenol blue; 25 μl of this mixture was layered on the gel column.

†Specific activity of [^{3}H]-OH-TX = 42 Ci/mmol; dpm/pmol = 93,240; specific activity of [^{3}H]-E_2 = 100 Ci/mmol; dpm/pmol = 255,300.

‡An aliquot of the incubation mixtures was treated with DCC to remove unbound ligands and the cpm given in this column is the protein-bound radioactivity in the DCC supernate calculated for volume comparable to that of the incubation mixture loaded on the gel column without prior DCC treatment.

§Slices were 3 mm thick; 6th slice means 6th from the top of the gel.

‖This is the only case where a discordancy in the position of the radioactive peak was noted between [^{3}H]-OH-TX and [^{3}H]-E_2 incubated samples. Technical difficulties were experienced in this case.

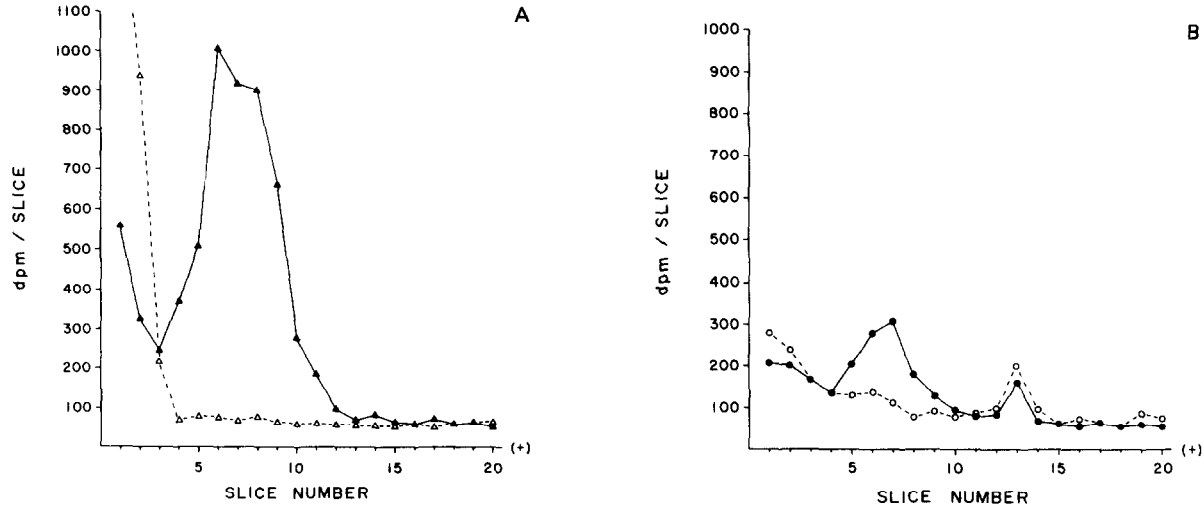

Fig. 1. Electrophoretic analysis of [^{3}H]-E_2 and [^{3}H]-OH-TX binding components in ER-rich breast cancer cytosol in glycerol-free buffer system. Cytosols from several ER-rich tumors were pooled and aliquots were incubated for 18 hr *with [^{3}H]-E_2 or [^{3}H]-OH-TX alone or in the presence of 1000 molar excess of unlabeled E_2, OH-TX or DES. All the ligands were solubilized in a glycerol-free buffer system. Three parts of the incubation mixtures (without prior removal of free ligands by DCC) were mixed with one part sucrose-dye buffer (40% sucrose, 0.1% bromophenol blue) and 25 μl of these mixtures were subjected to electrophoresis. At the end of electrophoresis the gels were cut into* 3 mm *slices and the radioactivity in each slice was counted. Counts per minute (cpm) were converted to dpm and plotted. The electrophoresis was anodal and the anode (+) was towards the bottom of the gel. (The specific activity of [^{3}H]-E_2* was 100 Ci/mmol, *while [^{3}H]-OH-TX had a specific activity of only* 42 Ci/mmol.) *(A) ▲——▲ The cytosol was incubated with [^{3}H]-E_2 only. △---△ The cytosol was incubated with [^{3}H]-E_2* and 1000-*fold molar excess of DES. Same results were obtained when the competing ligand was E_2 or* OH-TX. *Ligands were dissolved in a glycerol-free buffer. (B) ●——● The cytosol was incubated with [^{3}H]-OH-TX only and subjected to electrophoresis. ○---○ The cytosol was incubated with [^{3}H]-OH-TX plus* 1000-*fold molar excess of unlabeled* OH-TX. *The same results were obtained when the competing ligand was unlabeled E_2 or DES. The ligands were solubilized in a glycerol-free buffer. While the radioactive peak with an electrophoretic mobility identical to [^{3}H]-E_2 binding component (Fig. 1A) is completely saturable by a molar excess of E_2,* OH-TX *or DES, the anodally fast moving peak is not. Therefore, this fast-moving component is not 'antiestrogen specific'. The fast-moving component co-migrated with albumin.*

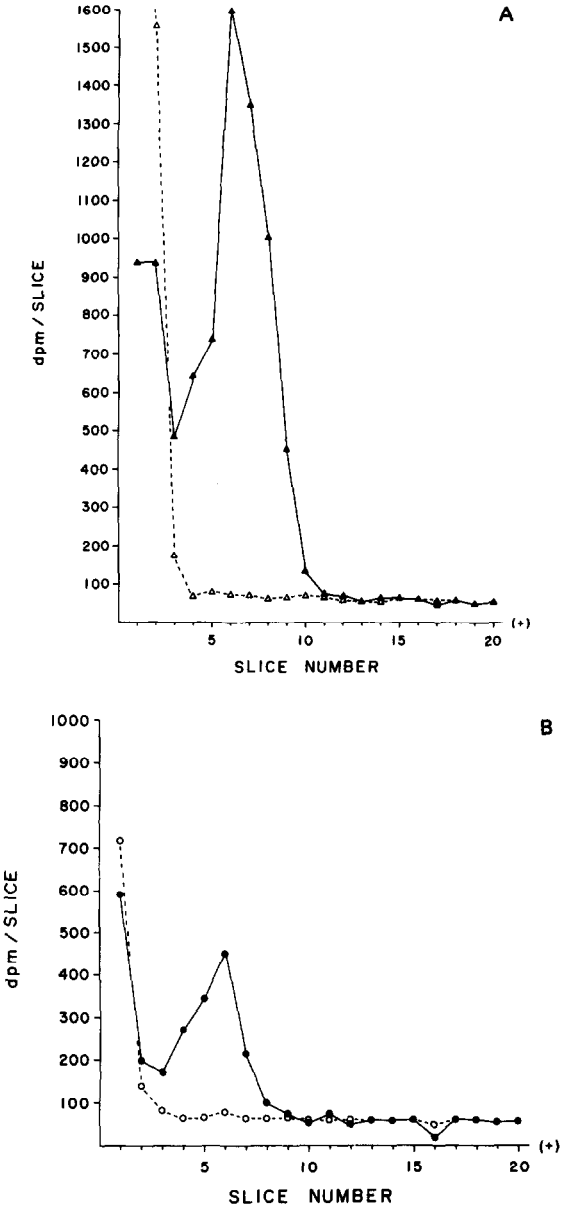

Fig. 2. ER-[³H]-E_2 complexes and ER-[³H]-OH-TX complexes show identical electrophoretic mobility even when OH-TX solutions are in a glycerol buffer system. ER-rich cytosol NY (see Table 3) was incubated for 18 hr with [³H]-E_2 or [³H]-OH-TX alone or in the presence of 1000-fold molar excess of unlabeled E_2, OH-TX or DES and subjected to electrophoresis as described for Fig. 1. (A) ▲——▲ NY cytosol incubated with [³H]-E_2 only (glycerol-free buffer). △---△ NY cytosol incubated with [³H]-E_2 in the presence of 1000-fold molar excess of unlabeled OH-TX. The same results were obtained with a molar excess of DES or E_2. (B) (Ligands solubilized in glycerol buffer) ●——● NY cytosol incubated with [³H]-OH-TX only. ○---○ NY cytosol incubated with [³H]-OH-TX in the presence of 1000-fold molar excess of unlabeled E_2. The same results were obtained with a molar excess of unlabeled OH-TX or DES.

ER-negative cytosols. Three ER-negative cytosols from post-menopausal women were incubated with [³H]-E_2 and [³H]-OH-TX and analyzed by electrophoresis. No radioactive peaks were evident in any of these samples (Fig. 4) or

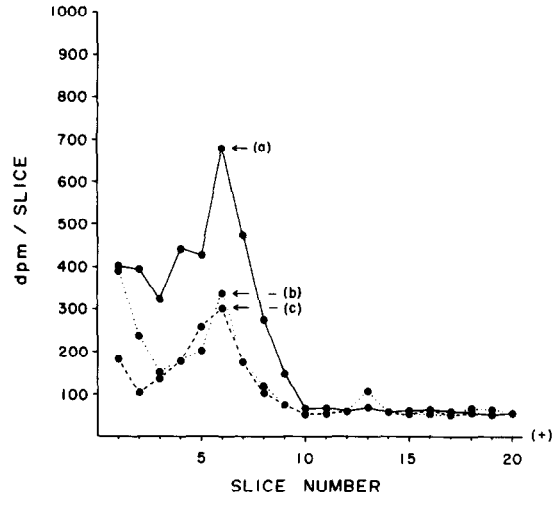

Fig. 3. [³H]-OH-TX binds ER with high affinity and [³H]-OH-TX-ER complexes withstand DCC treatment and subsequent electrophoresis. A pool was made from several ER-rich cytosols. It was incubated for 18 hr with [³H]-E_2 or [³H]-OH-TX with or without a molar excess of unlabeled competing ligands (E_2, OH-TX or DES). The incubation mixtures were subjected to electrophoresis (as described for Fig. 1) either after the removal of unbound ligands by prior DCC treatment (curves A, B) or without such pre-treatment (curve C). The anodal mobility of the radioactive peaks in the DCC treated and untreated samples were identical. Curve A: ●——● cytosol + [³H]-E_2. Unbound ligand was removed by DCC treatment prior to electrophoresis. Curve B: ●····● cytosol + [³H]-OH-TX. Unbound ligand was removed by DCC prior to electrophoresis. Curve C: ●---● cytosol + [³H]-OH-TX. The free ligands were not removed by DCC treatment. No radioactive peaks were seen when the cytosols incubated with ³H-ligands in the presence of a molar excess of DES, E_2 or OH-TX were analyzed without prior DCC treatment.

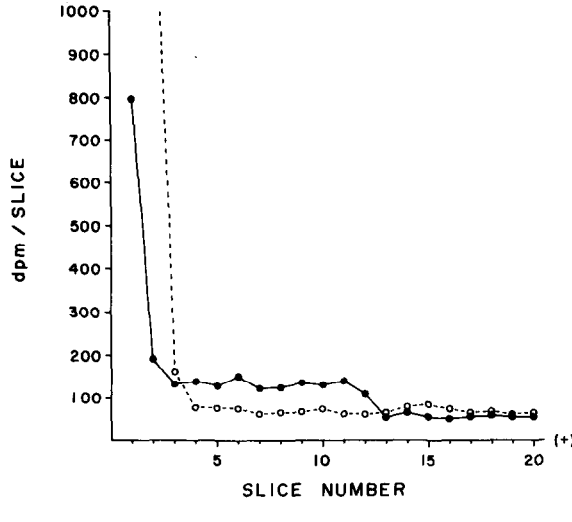

Fig. 4. No [³H]-E_2 or [³H]-OH-TX binding components could be detected by ER-negative cytosols. ER-negative cytosol (KUSH) was incubated for 18 hr with ³H-labeled ligands and subjected to electrophoresis without prior removal of free ligands by DCC treatment. ●——● Cytosol + [³H]-OH-TX. ○---○ Cytosol + [³H]-E_2.

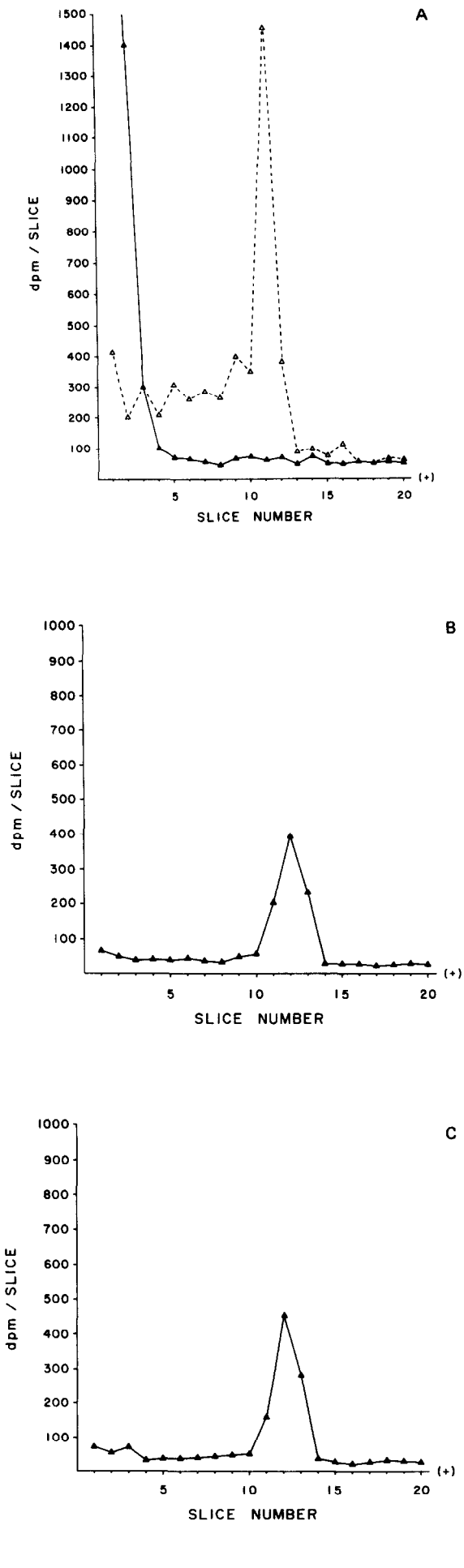

Fig. 5. [^{3}H]-OH-*TX and* [^{3}H]-*DES bind to an anodally fast moving component in the female sera. This binding is unsaturable in the presence of a* 1000*-fold molar excess of unlabeled* E_2, OH-*TX or DES. Serum from a healthy adult female (H. Mn) was incubated with radiolabeled ligands with or without a molar excess of competing ligands. The incubation mixtures were subjected to electrophoresis without prior DCC treatment. (A)* ▲——▲ *Serum +* [^{3}H]-E_2. △---△ *Serum +* [^{3}H]-*DES (specific activity of* [^{3}H]-*DES,* 113 Ci/mmol). *This radioactive peak was not obliterated in the presence of unlabeled ligands* E_2, *DES or* OH-*TX. (B)* ▲——▲ *Serum +* [^{3}H]-OH-*TX only. (C)* ▲——▲ *Serum +* [^{3}H]-OH-*TX plus a* 1000*-fold molar excess of unlabeled* OH-*TX. (D)* ▲——▲ *Serum +* [^{3}H]-OH-*TX plus a* 1000*-fold molar excess of unlabeled* E_2. *The radioactive peaks of* 5*B–D were retained in the serum exposed to* [^{3}H]-OH-*TX in the presence of a molar excess of unlabeled DES. DCC treatment of these samples (*5*A–D) resulted in dissociation of protein-bound radioactivity. In all the gels the radioactive peak co-migrated with serum albumin, which appeared as a broad band* (0.6 cm *in width) in the gels stained with Coomassie blue.*

when these cytosols were exposed to the radiolabeled ligands in the presence of excess of unlabeled competitors.

Normal sera. Five normal sera incubated with either ^{3}H-labeled E_2, OH-TX or DES alone or in combination with unlabeled competitors were subjected to electrophoresis without prior removal of free ligands by DCC treatment. In all the sera incubated with [^{3}H]-E_2 only, no radiactive peak was evident in the gels (Fig. 5A); on the other hand, a single radioactive peak was observed in sera exposed to [^{3}H]-OH-TX or [^{3}H]-DES (Figs 5A and B) and the position of this peak co-migrated with the albumin–dye band visible in these gels or was immediately adjacent to this albumin–dye band. This radioactive peak was present even when the sera were exposed to [^{3}H]-OH-TX or [^{3}H]-DES in the presence of 1000-fold excess of unlabeled E_2, OH-TX or DES (Figs 5C and D). When the free ligands were removed by

DCC treatment, very little residual protein-bound radioactivity was seen in the DCC supernates.

DISCUSSION

Sutherland and Murphy [4] investigated the comparative binding of [^{3}H]-E_2 and [^{3}H]-tamoxifen ([^{3}H]-TX) in ER-positive and ER-negative cytosols of human breast carcinomas and presented evidence for the presence of a component in the cytosols to which only the antiestrogens bind. Their conclusion was based on the fact that tamoxifen was able to saturate all the [^{3}H]-E_2 binding sites but estradiol was unable to saturate the [^{3}H]-TX binding sites completely. Nicholson *et al.* [9], using cytosols from human mammary carcinomas, were unable to demonstrate such tamoxifen-specific, E_2-unsaturable high-affinity binding. [^{3}H]-OH-TX binding components in the cytosol of human breast sediment in the 8S region of sucrose density gradients [1, 2], but an exclusively OH-TX specific binding component was not demonstrated by sucrose density gradient analysis.

Our observations on 17 samples of breast carcinoma cytosols in which DES saturable-[^{3}H]-E_2 binding and OH-TX saturable-[^{3}H]-OH-TX binding were compared by DCC analysis (Table 2) are in agreement with the conclusions of Nicholson *et al.* [9]. In the presence of antiestrogen binding protein, in addition to ER in the cytosols, one would expect a greater quantity of [^{3}H]-OH-TX binding sites than [^{3}H]-E_2 binding sites. Our data indicate to the contrary that in all but 4 of the 17 cytosols the [^{3}H]-OH-TX binding sites were considerably less than E_2 binding sites. None showed a greater quantity of [^{3}H]-OH-TX sites than [^{3}H]-E_2 sites. It is to be emphasized that [^{3}H]-OH-TX shows a very high degree of non-specific binding (>30% of the total binding) and DCC treatment of [^{3}H]-OH-TX alone in the absence of cytosol (buffer blank) revealed that DCC at the concentration used was not sufficient to absorb all the [^{3}H]-OH-TX whereas it was efficient in removing all the [^{3}H]-E_2. High non-specific binding in the presence of cytosol combined with an incomplete removal of free [^{3}H]-OH-TX might cause an underestimation of specific [^{3}H]-OH-TX binding in a saturation analysis experiment similar to ours. Therefore we adopted polyacrylamide gel electrophoresis to study all the protein components that bind [^{3}H]-OH-TX or [^{3}H]-E_2 and their specificity for these ligands.

Polyacrylamide gel electrophoresis also failed to reveal the presence of an exclusively OH-TX binding component unsaturable by E_2 in the ER-positive cytosols (Table 3), ER-negative cytosols or in female sera (Figs 1–5). [^{3}H]-E_2 binding sites were completely saturable by OH-TX and the [^{3}H]-OH-TX binding component bound E_2, OH-TX and DES equally well. In one of the ER-rich cytosol pools examined (Fig. 1B) and in all of the human sera examined, [^{3}H]-OH-TX and [^{3}H]-DES were able to bind to a serum component which co-migrated with bromophenol blue–albumin complex or very close to it (Figs 5A–D). This could not be classified as specific antiestrogen binding component because the binding was unsaturable even in the presence of a 1000-fold excess of E_2, OH-TX or DES. From our experimental data one cannot, however, conclude that the non-saturable, high-capacity [^{3}H]-OH-TX or [^{3}H]-DES binding represents serum albumin complexed with these labeled ligands. Normal sera in which albumin is completely removed by absorption with anti-albumin antibodies should be exposed to the ^{3}H-labeled antiestrogens and the reaction products analyzed by polyacrylamide gel electrophoresis in order to investigate whether the serum component that binds OH-TX or DES is indeed albumin. Whatever this component is [^{3}H]-E_2 does not bind to it (Fig. 5A). The affinity of binding of [^{3}H]-OH-TX or [^{3}H]-DES to the serum component is far lower than the affinity of these ligands to ER because DCC treatment of serum proteins labeled with these two ligands dissociates the complexes formed between this serum component and these two ligands.

The low-capacity, high-affinity binding component found in all the ER-rich cytosols possesses all the characteristics attributed to ER. They include the following: (1) this component binds [^{3}H]-E_2, [^{3}H]-OH-TX and [^{3}H]-DES with high affinity and these sites are saturable with a 200- or 1000-fold excess of all the three unlabeled ligands equally well (Figs 1–3); (2) this component is not detected in ER-negative cytosols or in sera labeled with [^{3}H]-E_2, [^{3}H]-OH-TX or [^{3}H]-DES (Figs 4, 5); (3) removal of free ^{3}H-ligands from ER-rich cytosols labeled with [^{3}H]-E_2, [^{3}H]-OH-TX or [^{3}H]-DES did not dissociate the complexes formed between the cytosol component and these three ^{3}H-labeled ligands (Fig. 3); therefore, the radiolabeled peak found in the ER-rich cytosols analyzed by electrophoresis does not represent free ^{3}H-ligands. Furthermore, none of the three ^{3}H-labeled compounds penetrates the gel upon electrophoresis. They are all of neutral charge and do not exhibit anodal migration. That this radiolabeled peak present in ER-rich cytosols is indeed ER is also substantiated by the fact that rabbit antibodies raised against this component [8] react with ER of human breast cancer cytosols and with ER of calf [8], human [10] rat and mouse

uterine cytosols (V. C. Jordan; unpublished observations).

An interesting observation that emerged out of the present study is the difference in the effect of glycerol on the stability of ER-[^{3}H]-E_2 vs ER-[^{3}H]-OH-TX complexes. Glycerol even at 5% either dissociated the ER-E_2 complexes or aggregated them to form a large molecule and, therefore, we failed to observe a distinct radiolabeled peak in the polyacrylamide gels (unpublished observation). The ER-[^{3}H]-OH-TX complexes, on the other hand, seem to be unaffected by the presence of glycerol (Figs 2, 3). The differential effect of glycerol implies that E_2 and OH-TX may bind to the ER differently. This may have important implications for understanding the mechanisms of antiestrogen action.

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