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Receptor Characteristics of Specific Estrogen Binding in the Renal Adenocarcinoma of the Golden Hamster[1]

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SUMMARY

Linear sucrose gradient analyses reveal that all estrogen-induced and -dependent primary renal tumor cytosols examined contain an 8 S and variable amounts of 4 S receptor in low ionic buffer concentrations. Similar results were obtained with extracts of primary metastases of these tumors. Sucrose gradients containing high salt (0.4 M KCl) convert the 8 S receptor in both the hamster renal tumor and uterus to a 4 to 5 S complex. Scatchard plot analysis reveals that the renal tumor cytosol estradiol-receptor complex has a K_a of 1.7×10^9 M^{-1} and 9.2×10^{-10} M binding sites. Competition for the tritiated 17β-estradiol binding sites in the renal tumor was similar to that in the uterus with respect to estrogenic compounds. Nonestrogenic steroids exhibited minimal competition at the same concentrations or higher. Substitution in the ring structure, particularly in position 3 of the phenolic A-ring, resulted in a considerable loss in the ability of such compounds to compete for these receptors. Antiestrogens were effective competitors for these estrogen receptors only at higher concentrations relative to the tritiated estradiol.

INTRODUCTION

The renal adenocarcinoma of the Syrian golden hamster (*Cricetus auratus*) represents an interesting system to study estrogen carcinogenesis, since only estrogenic hormones are necessary for its induction and maintenance (8). The carcinogenic action of estrogen in the initiation of these kidney tumors appears to be direct and peculiar to this species.

The selective uptake and retention of steroid hormones by steroid-dependent target tissues has been well documented, and their binding to cytoplasmic receptor proteins has been implicated as an initial step in the action of these hormones on their respective target cells (4, 5, 15). Specific estrogen-binding protein receptors have been identified in such estrogen-sensitive tissues as the uterus, mammary gland, and hypothalamus (6, 17, 19), as well as in the dimethylbenz(a)anthracene-induced mammary carcinoma in the rat and in human endometrial carcinoma (2, 20). King *et al.* (7) and Steggles and King (18) have reported the presence of cytoplasmic 4 S or 8 S + 4 S and nuclear 5 S estrogen-binding complexes in the estrogen-induced renal adenocarcinoma of the golden hamster with some properties similar to the uterine receptors. However, these studies indicated that the 8 S cytosol receptor was not a consistent finding in the transplanted primary renal tumor, whereas the serially transplanted, less estrogen-dependent tumor consistently contained both of these estrogen-binding moieties.

This study was undertaken to describe more fully these receptors in the primary renal tumor and its metastases and to compare the specificity of these receptors with those found in the hamster uterus. Moreover, since antiestrogen treatment of tumor-bearing animals causes tumor regression (1), we have investigated the ability of such agents to compete for these estrogen-binding sites.

MATERIALS AND METHODS

Chemicals and Reagents. 17β-[2,4,6,7-^{3}H]Estradiol (105 Ci/mmole) was obtained from New England Nuclear, Boston, Mass., and was found to be more than 98% pure by thin-layer chromatography on Eastman Kodak silica gel plates in a solvent system described previously (12). Unlabeled 17β-estradiol, estrone, and estriol were provided by Calbiochem, San Diego, Calif., and all other unlabeled steroids were purchased from Sigma Chemical Co., St. Louis, Mo. Antiestrogens: CC[4] was a gift from Dr. Alfred Richardson, Jr., Merrell-National Laboratories, Cincinnati, Ohio, and U-11,100A was a gift of Dr. Harold M. Woodward and Dr. Gary L. Neil, The Upjohn Company, Kalamazoo, Mich. Trizma base, Norit A, dextran 80, dithiothreitol, protamine sulfate, catalase, bovine serum albumin, ovalbumin, potassium chloride, sodium chloride, and TACE were obtained from Sigma. Ultrapure sucrose (RNase-free) was purchased from Schwarz/Mann, Orangeburg, N. Y.

Animals and Tumor Induction. Young, mature castrated

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[4] The abbreviations used are: CC[clomiphene citrate (*cis*)], 1-[p-(β-diethylaminoethoxy)phenyl]-1, 2-diphenyl-2-chloroethylene monocitrate; U-11,100A (nafoxidine hydrochloride), 1-{2-[p-(3,4 dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl}pyrrolidine hydrochloride; TACE (chlorotrianisene), tri-p-anisyl chloroethylene; DES, diethylstilbestrol.

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male and ovariectomized female Syrian hamsters (LVG:LAK, outbred strain, Charles River; Lakeview Hamster Colony, Newfield, N. J.), weighing 80 to 100 g and 100 to 110 g, respectively, were used. Tap water and Ralston Purina laboratory chow were available *ad libitum*. Castrated males were acclimated at least 2 weeks prior to use, whereas the females were used 48 to 60 hr after ovariectomy. DES or estradiol-17β (Sigma) pellets (20-mg estrogen pellets were prepared by Dr. George M. Krause, Copley Pharmaceutical Inc., Boston, Mass.) were implanted subpannicularly as described previously (10). Additional hormone pellets were implanted every 3 months in each animal to maintain estrogen levels. Renal tumors weighing 1 to 5 g/animal appeared after 8 to 10 months of hormone treatment, and abdominal metastases were common. A few male hamsters treated similarly had tumor weights greater than 10 g/animal. The earliest tumor nodules were detected after 5 months of estrogen treatment. The pellets were removed 60 to 68 hr prior to sacrifice to clear endogenous hormone. All animals were exsanguinated under ether anesthesia and the tissues were immediately washed twice in cold 0.15 M NaCl in 10 mM tris-HCl-1.5 mM EDTA-1mM dithiothreitol buffer, pH 7.4.

Preparation of Cytosol. The tumor tissue was freed of necrotic or hemorrhagic areas and tumor fragments were weighed and then homogenized in 2.5 or 3.0 volumes of cold 10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol buffer, pH 7.4, using a chilled Teflon Potter-Elvehjem-type homogenizer. Cytosols from uteri were prepared similarly, except that homogenization was carried out in a conical ground-glass homogenizer. Tissue homogenates were centrifuged in a Spinco L2 ultracentrifuge for 60 min at 5° and 100,000 $\times$ *g*. After centrifugation, the supernatant fluids (cytosols) were then filtered through a Millex filter, 0.45 μm), (Millipore Corp., Bedford, Mass.) to remove residual lipid and cell debris.

Protein concentration of the cytosols was determined by the method of Lowry *et al.* (13), using bovine serum albumin as a standard.

***In Vitro* Incubation of Specific Estrogen Binding.** Aliquots (0.5 or 1.0 ml) of the cytosol fraction were incubated *in vitro* with 1×10^{-9} M 17β-[^{3}H]estradiol prepared in 30% ethanol in 0.15 M NaCl solution. In competition studies, similarly dissolved unlabeled compounds were added immediately prior to the addition of the labeled estradiol. After incubating the samples for 2 hr on ice with gentle agitation, appropriate amounts were taken for measurement of radioactivity. Free (unbound) and some of the low-affinity [^{3}H]estradiol binding were next removed, using dextran-coated charcoal with gentle shaking on ice by a method previously reported (12). No cytosolic preparations were stored frozen prior to study, because preliminary experiments indicated that extensive receptor aggregation was observed after sucrose gradient analyses.

To determine the affinity constant and the number of binding sites in the renal tumor cytosol receptor-[^{3}H]estradiol complex, the cytosol was diluted to 1 mg/ml in 10 mM Tris-HCl-1.5 mM EDTA-1 mM dithiothreitol buffer, pH 7.4 and incubated with varying concentrations of labeled estradiol for 27 hr on ice to attain equilibrium, according to Ellis and Ringhold (3).

Sucrose Density Gradient Analyses. Tissue extracts (0.3 to 0.4 ml) were layered on 4.6-ml linear 5 to 20% sucrose gradients prepared in 10 mM Tris-HCl-1.5 mM EDTA-1 mM dithiothreitol buffer, pH 7.4 using a gradient former (Buchler Instruments, Inc., Fort Lee, N. J.). Sucrose gradients containing 0.4 M KCl were prepared similarly. The samples were centrifuged for 17 hr at 39,000 rpm in a Spinco L2 ultracentrifuge using either a SW 39 or SW 50.1 rotor at 4°. Gradient tubes were pierced with a 20-gauge needle and 9-drop fractions were collected in scintillation vials containing 0.5 ml protosol (New England Nuclear) and assessed for radioactivity.

Sedimentation coefficients were determined by the method of Martin and Ames (14), using catalase (11.3 S), bovine serum albumin (4.6 S), and ovalbumin (3.7 S) as sedimentation markers.

Uterine and tumor cytosols, when incubated with protamine sulfate (7.5 mg/ml) for 10 min, precipitated the receptor according to the procedure described by Steggles and King (18).

Measurement of Radioactivity. The radioactivity in the sucrose gradient fractions was measured after adding 10 ml of a scintillation fluid mixture containing toluene-Liquifluor-protosol (New England Nuclear) with a final protosol concentration of 10%. All non-gradient samples were counted in a similar mixture containing 5% protosol. Samples were counted at 5° in a Packard Tri-Carb liquid spectrometer with a counting efficiency of approximately 38% for tritium.

RESULTS

Relative Binding of Tritiated Estradiol in the Untreated Kidney, Estrogen-treated Kidney, and Renal Tumor Cytosols. A comparison of estrogen binding in renal cytosol fractions from untreated castrate, estrogen-treated castrate, and tumor-bearing hamsters was made. We have reported previously that a specific 4 S estrogen-binding protein is present in the renal cytosols of castrated and estrogen-treated hamsters (12). It is estimated, based on total radioactive estradiol binding after dextran-charcoal minus competition with 100-fold excess of unlabeled estradiol, that the level of specific estradiol binding in the renal tumor cytosol is 8- to 9-fold and 7-fold higher than that observed in the kidney cytosols obtained from castrate and estrogen-treated animals, respectively (Chart 1).

Sedimentation Profiles of Bound Estradiol in Cytosols of Hamster Renal Tumor and Uterus. When the estradiol binding in the hamster renal tumor cytosols was examined with 5 to 20% linear sucrose gradients, binding peaks were observed either in the 8 S or 8 S + 4 S region of the gradient, whereas uterine cytosols exhibited only an 8 S binding component with minimal estrogen binding in the 4 S region. Competition studies with unlabeled estradiol at 100-fold excess indicate that part of the 4 S binding moiety in those tumor samples containing a large amount of binding in this region is nonspecific. Sucrose gradient profiles of extracts obtained from primary renal tumor metastases also contained similar estrogen-receptor complexes as the primary lesion. The behavior of both the renal tumor and uterine

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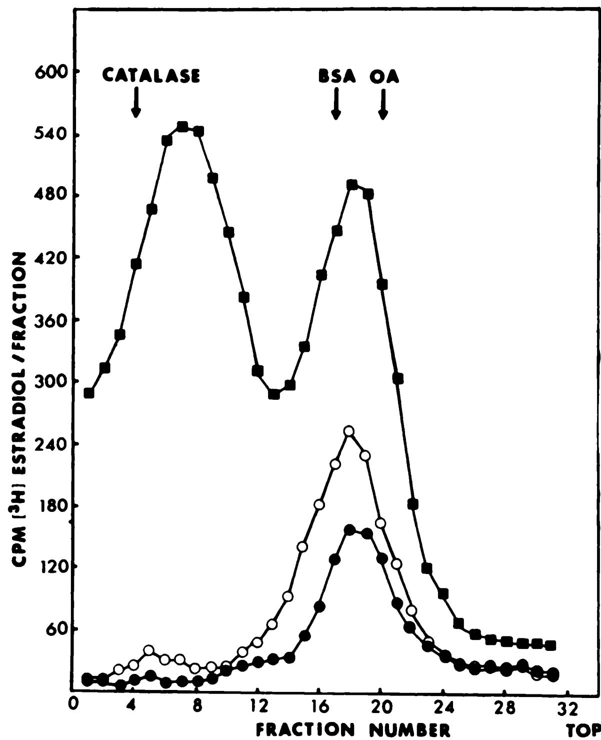

Chart 1. Representative sedimentation patterns of renal cytosol fractions from untreated castrate, DES-treated, and renal tumor-bearing male hamsters after *in vitro* incubation with 1 nM 17β[³H]estradiol at 0°. Dextran-charcoal treatment (4 hr) was used to remove free steroid from cytosols of untreated control (●), 6.0-month DES-treated (○), and primary renal tumor (■). Protein concentration was 13 to 15 mg/ml. Aliquots of 0.4 ml were layered on each gradient. Catalase, bovine serum albumin (*BSA*), and ovalbumin (*OA*) served as sedimentation coefficient standards.

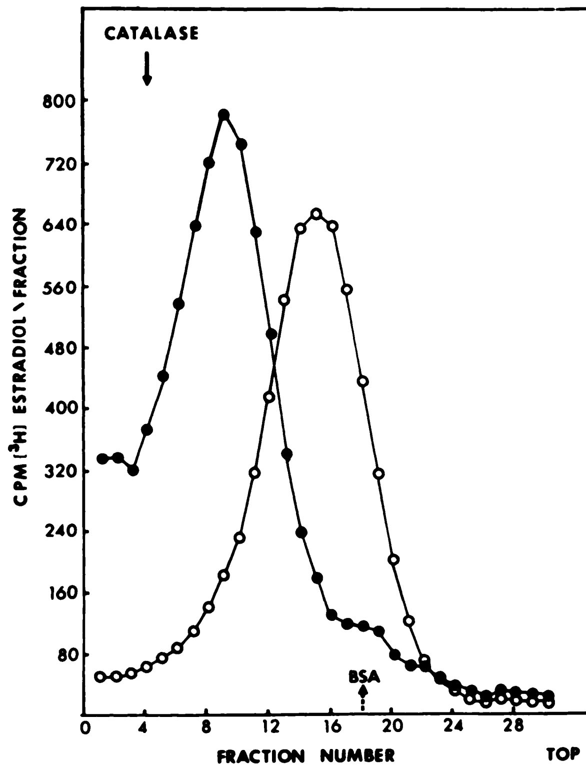

Chart 2. Sucrose gradient centrifugation of uterine cytosol from 48-hr ovariectomized female hamsters incubated *in vitro* with 1 nM tritiated estradiol at 0° for 2 hr. Dextran-charcoal treatment (3 hr) was used to remove free steroid. Protein concentration was 14 mg/ml. Aliquots of 0.4 ml were layered on 5 to 20% sucrose gradients (●) and 5 to 20% sucrose + 0.4 M KCl (○) gradients. Bovine serum albumin (*BSA*) standard sedimented in sucrose + 0.4 M KCl (- - ▶).

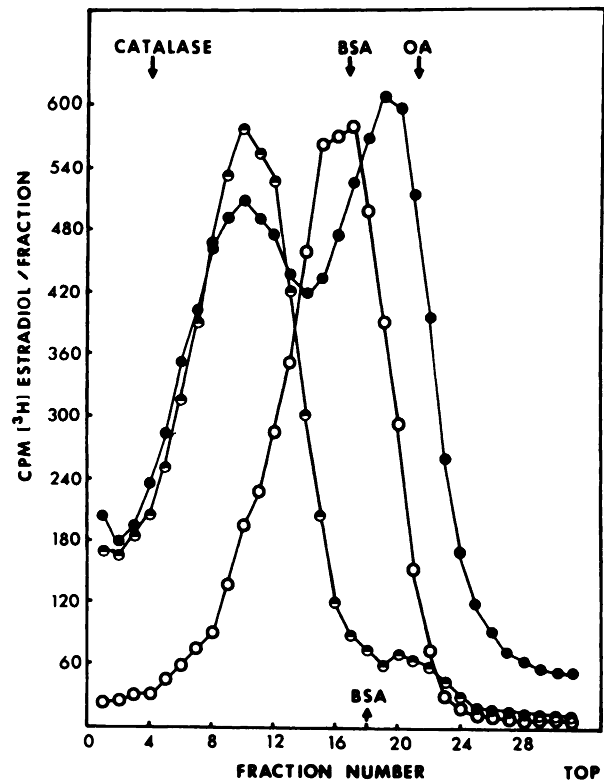

Chart 3. Sedimentation profiles of primary renal tumor cytosols from 8.0- to 10.0-month DES-treated castrate male hamsters incubated *in vitro* with 1 nM 17β[³H]estradiol at 0° and subsequent dextran-charcoal treatment. Protein concentration was 14 to 16 mg/ml. Aliquots were layered on 5 to 20% sucrose gradients (●, 0.4 ml; ◐, 0.3 ml) and 5 to 20% sucrose + 0.4 M KCl (○, 0.3 ml) gradients. Bovine serum albumin (*BSA*) standard sedimented in sucrose + 0.4 M KCl (- -▼); *OA*, ovalbumin.

receptors in the presence of 0.4 M KCl is similar, sedimenting as 4 to 5 S binding components (Charts 2 and 3).

Determination of Affinity Constant and Concentration of Binding Sites for Estradiol Renal Tumor Receptor Protein. Renal tumor cytosol fractions were incubated with varying concentrations (1.0 to 100 × 10^{-10} M) of radioactive estradiol at 0° to estimate the affinity constant (K_a) and the number of binding sites for the estradiol-receptor complex. A Scatchard (16) plot of the data revealed a high-affinity, low-capacity component with a K_a of 1.7 × 10^9 M^{-1} and 9.2 × 10^{-10} M binding sites at a protein concentration of 1.0 mg/ml (Chart 4). These values are within the range of uterine and hypothalamic values reported previously (19). The presence of a small amount of low-affinity, nonsaturable binding sites at much higher estradiol concentrations (70 to 100 × 10^{-10} M) represents nonspecific protein binding. Although the dextran-charcoal treatment used removes much of the steroid bound to low-affinity proteins, the tritiated estradiol is not removed completely from these sites even after many hours of charcoal incubation (12).

Comparison of Estradiol Binding Specificity in Renal Tumor and Uterine Cytosols. The competition for estradiol

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binding *in vitro* by estrogenic compounds, ascertained by dextran-coated charcoal analyses and sucrose gradient profiles, is very similar for both the renal tumor and uterine receptors. The specificity of estrogen binding in these tissues was demonstrated by the inability of dihydrotestosterone, cortisol, progesterone, and 5-, 17-, and 20-substituted metabolites of progesterone to compete for binding even at 500-fold excess concentrations. In contrast, nearly complete competition for the renal tumor receptor is exhibited at concentrations of 100- and 50-fold excess of unlabeled estradiol. Table 1 summarizes the results of these competition studies. Competition for these receptors by estrogen metabolites, synthetic estrogens, and substituted estrogens at lower competing concentrations (5- and 10-fold) was consistently less than when estradiol itself was used as an inhibitor. At 5- and 10-fold excess of unlabeled 17α-estradiol, the tritiated estradiol was displaced from the receptor to a slightly greater extent in the renal tumor than in the uterus. However, at 50- and 500-fold excess concentrations, all estrogenic compounds competed to similar extents with the tritiated estradiol for the receptors, except for those substituted estrogens containing either acetate or propionate ester moieties in positions 3 and 17 on the steroid molecule. Unexpectedly, estradiol 3-benzoate, unlike the other ester-substituted estrogens, was nearly as effective an inhibitory agent as DES and the estrogen metabolites examined in its ability to compete for both the renal tumor and uterine receptors. Since this compound was found to be stable and essentially free of contamination (97% pure by thin-layer chromatography), it is possible that this steroid may have undergone some hydrolysis during the prolonged incubation period despite the low temperature used. Preliminary results using estra-1,3,5(10)-16-tetraen-3-ol (courtesy of Dr. James C. Orr), in which no functional group is present in position 17 of the steroid, indicate only negligible competition with this compound at 5- and 10-fold excess concentrations (6 to 15%), whereas at 50- and 500-fold excess there is considerable competition (30 to 80%) in both the renal tumor and uterus.

Incubating labeled receptor protein from renal tumor or uterine cytosols with protamine sulfate precipitated virtually all of the bound tritiated estradiol, indicating the acidic nature of these receptor proteins in the hamster, which confirms previous studies (7, 18).

Competition with Antiestrogens in Renal Tumor and Uterine Cytosols. Competition for the estradiol-binding re-

Table 1

Competitive binding of estrogens and antiestrogens with [³H]estradiol in cytosols of renal adenocarcinoma and uterus

Renal tumor and uterine cytosols were incubated *in vitro* for 2 hr with 1 nM tritiated estradiol and nonradioactive compounds at 5×10^{-9} M (5-fold), 1×10^{-8} M (10-fold), 5×10^{-8} M (50-fold), and 5×10^{-7} M (500-fold) at 0°. Free estradiol was removed by dextran-charcoal adsorption (4 hr). [³H]Estradiol concentration in these cytosols, without inhibitor, corresponded to 0% inhibition. Values are based on the mean of triplicate determinations in separate experiments.

Competitors	% inhibition							
	Renal tumor (5-fold)	Uterus (5-fold)	Renal tumor (10-fold)	Uterus (10-fold)	Renal tumor (50-fold)	Uterus (50-fold)	Renal tumor (500-fold)	Uterus (500-fold)
Estrogens[a]								
17β-Estradiol	51	48	73	65	86	81	93	92
Estrone	29	25	51	44	80	82	92	92
Estriol	35	25	57	43	83	83	93	92
Ethinyl estradiol	21	24	48	53	87	89	95	94
17α-Estradiol	35	16	50	28	79	76	92	91
DES	30	31	59	50	90	84	95	95
Estradiol 3-benzoate	26	31	42	54	81	81	94	89
Estradiol diacetate					31	40	86	87
Estradiol dipropionate					21	44	81	84
DES dipropionate						60	93	93
Estradiol 3-methyl ether					8	14	46	65
Estrone 3-methyl ether					8	4	18	40
Estriol 3-methyl ether					0	0	26	55
Antiestrogens								
CC	0	0	14	8	46	50	87	89
U-11,100A	0	0			29	35	79	85
TACE					16	17	55	72

[a] Trivial names for steroids were used. 17β-Estradiol, 1,3,5(10)-estratrien-3,17β-diol; estrone, 1,3,5(10)-estratrien-3-ol-17-one; estriol, 1,3,5(10)-estratrien-3,16α, 17β-triol; ethinyl estradiol, 1,3,5(10)-estratrien-17α-ethinyl-3,17β-diol; 17α-estradiol, 1,3,5(10)-estratrien-3,17α-diol; DES, 3,4-bis(4′-hydroxyphenyl-)3-hexene; estradiol-3-benzoate, 1,3,5(10)-estratrien-3,17β-diol-3-benzoate; estradiol diacetate, 1,3,5(10)-estratrien-3,17β-diol diacetate; estradiol dipropionate, 1,3,5(10)-estratrien-3,17β-diol dipropionate; estradiol 3-methyl ether, 1,3,5(10)-estratrien-3,17β-diol-3-methyl ether; estrone 3-methyl ether, 1,3,5(10)-estratrien-3-ol-17-one-3-methyl ether; estriol 3-methyl ether, 1,3,5(10)-estratrien-3,16α,17β-triol-3-methyl ether.

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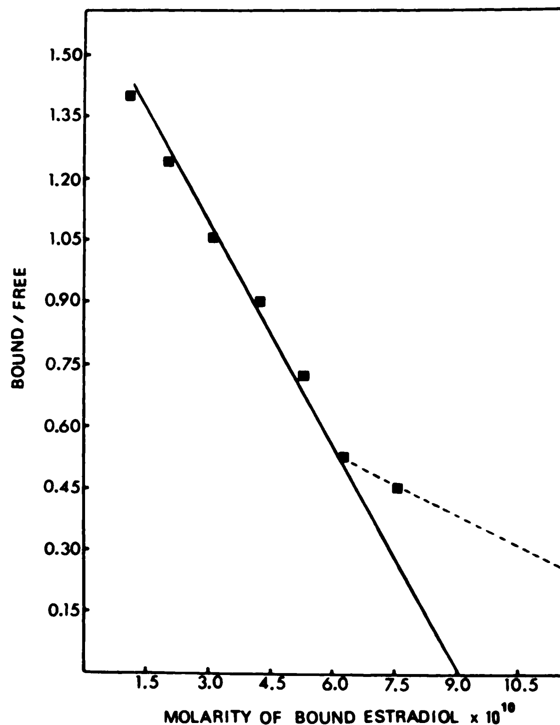

Chart 4. Scatchard plot analysis of the binding of tritiated estradiol in primary renal tumor cytosol (1.0 mg protein per ml) incubated for 27 hr at 0° to attain equilibrium and then treated with dextran-charcoal (4 hr). (■——■), high-affinity binding and (■----■), low-affinity binding.

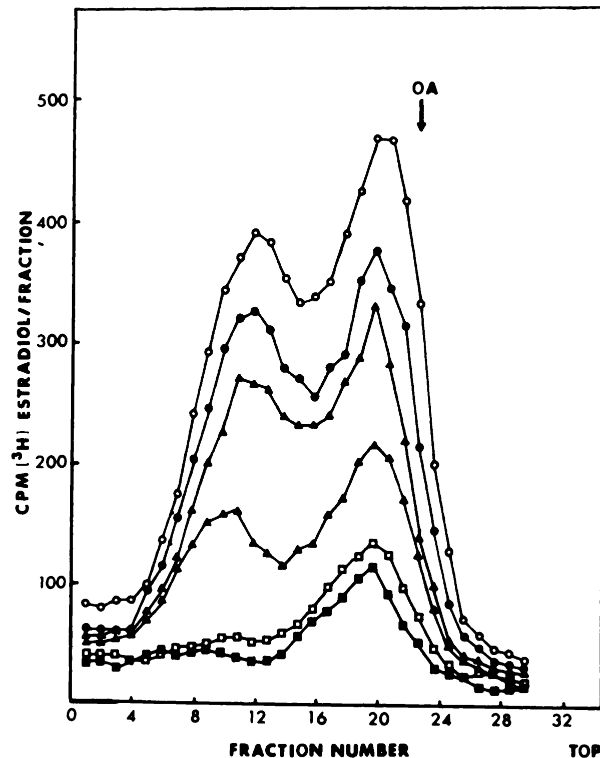

Chart 5. Competition by various estrogens and antiestrogens for 17β-[³H]estradiol binding sites in primary renal tumor cytosol from 7.0- to 9.0-month DES-treated castrate male hamsters. Aliquots of the cytosol were incubated with 1 nM tritiated estradiol alone (○), or in combination with unlabeled estrone-3-methyl ether (●), estradiol-3-methyl ether (△), TACE (▲), U-11,100A (□), and CC (■). Concentration of each competing agent was 5 × 10^{-7} M; 0.4 ml was applied to each gradient; *OA*, ovalbumin.

ceptor in the renal tumor and uterus in the hamster with CC or U-11,100A was marked only when higher concentrations (50- and 500-fold) of the competing agent were used (Chart 5). Competition at lower concentrations (5- and 10-fold) of unlabeled compound was either nil or negligible (Table 1). TACE was considerably less effective in inhibiting the binding of radioactive estradiol to the receptor than were the above-mentioned compounds and supports the contention that this agent must be metabolized to a more active compound to elicit its antiuterotrophic effects.

DISCUSSION

The estrogen-induced and -dependent renal adenocarcinoma is essentially an estrogen target tissue. This conclusion is based on the present observations and is supported by morphological studies (9) and isozyme profiles (11). Steggles and King (18) have reported the presence of either 4 S or 8 S + 4 S estrogen receptors in extracts of transplanted primary estrogen-dependent renal tumors. In contrast, in over 50 renal tumors examined, we have always detected the presence of an 8 S and variable amounts of 4 S receptor in cytosols obtained from the primary renal tumor or its metastases, using sucrose gradient analyses. The cause of the variability in the size of the 4 S peak is unclear. It is possible that differences either in the state of aggregation of the receptor protein or in the amount of nonspecific (low-affinity) protein capable of binding estradiol in the 4 S region of the gradient could be a result of a mixed tumor cell population. We have found that the amount of tritiated estradiol bound to the high-affinity receptors in the tumor cytosol remains relatively constant following 4 to 24 hr of dextran-charcoal treatment. Sucrose gradient analyses reveal that no dissociation of the 8 S receptor has occurred after 3 to 6 hr of dextran-charcoal treatment.

Competition studies, sedimentation characteristics, affinity constants, and the number of binding sites are similar for both the renal tumor and uterine estradiol-binding macromolecules, indicating that the receptors from these tissues are nearly identical. It is evident from competition studies that the 3- and 17-hydroxyl groups on the estradiol molecule are important for receptor binding since ester substitutions (except for estradiol 3-benzoate) in these positions result in a marked reduction in ability to compete with estradiol for the receptor in both the renal tumor and uterus. The data from competition studies with 3-methyl ether substitutions and estra-1,3,5(10)-tetraen-3-ol also indicate that the integrity of the phenolic A-ring is relatively more important than the 17β-hydroxyl group in the binding of estradiol to the receptor in both the hamster renal tumor and uterine systems.

Antiestrogens such as CC and U-11, 100A compete effectively for the estrogen receptor complex in the renal tumor and uterus only when higher concentrations of the antiestrogen are used. These findings may account for the observation that U-11, 100A treatment inhibited the growth of renal tumor transplants in the hamster (1).

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