Identification of High Affinity Estrogen Binding Sites in Calf Uterine Microsomal Membranes*

(Received for publication, October 3, 1979, and in revised form, July 21, 1980)

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Membrane-associated binding sites with high affinity and specificity for estrogens have been identified in calf uterine microsomes. The binding of 17β-[3H]estradiol is specific and saturable at low hormone concentrations (2 nM) of high affinity (Kd = 0.5 nM) and sensitive to trypsin and other proteolytic enzymes. Binding of [3H]estradiol to membranes is inhibited by low concentrations of unlabeled 17β-estradiol and diethylstilbestrol (50 to 100 pm) while high concentrations of nonsterogenic steroids have little effect. The nondisplaceable binding is low and never exceeds 15% at the half-maximal point of specific binding. The maximum amount of ligand bound per mg of membrane protein is in the range of 0.4 to 1.8 pmol. Specific estradiol binding associated with microsomal fractions varies between 7 to 15% of the total binding sites. Estradiol appears not to be metabolized to any significant extent after binding to uterine membranes. Whereas the affinities of the estrogens tested are similar, the affinity of the antiestrogen, Tamoxifen, for the cytosolic receptor is at least 10 times higher than for the microsomal binding sites. In contrast to rat uterus and ovaries, the microsomal membranes from various non-target tissues do not show any specific binding.

A large amount of information is available concerning the cytosolic (1-4) and nuclear (5-7) estrogen receptors in a variety of tissues, and their relationship to the ultimate effect of estrogens on nuclear events leading to increased protein synthesis (8-10). However, little is known about the primary events leading to incorporation of estrogen into the target cells. It is usually hypothesized that steroids, being lipophilic compounds, are taken up passively into the cells by a diffusion-mediated process. Since target cells have an intrinsic ability to differentiate among various steroids, it is reasonable to speculate that a highly selective, membrane receptor-mediated process may exist for the selective uptake of steroids. In the early studies of [3H]estradiol incorporation by uterus, it was noted that a significant proportion of the steroid was bound to uterine microsomes (11). Several investigators have postulated that high affinity membrane receptors could play a role in the recognition and transport of corticosteroids into target cell (12-14). Furthermore, such a receptor-mediated uptake for the steroidal glycoside, ouabain, has been well documented in HeLa cells (15). A recently reported study with Xenopus laevis oocytes suggests that progesterone acts at the level of the cell surface and induces changes in intracellular Ca2+ distribution (16). A membrane-bound receptor, possibly involved in mediating the uptake of estradiol into the cell, has recently been postulated to exist in rat uterine slices (17) and isolated rat endometrial cells (18).

Identification and characterization of estrogen-specific membrane-bound receptors might help in investigations leading to an understanding of the primary action of estrogens in target tissues. The present study was initiated to establish the presence, in target tissues of estrogen-specific membrane-bound receptors and was performed with calf uterine microsomes, which allow investigation of estradiol binding separately from transport and metabolism of the steroid. The microsomal preparation also enables study of the binding process in the absence of cytosolic and nuclear receptors and acceptors which may complicate interpretation of results. The properties of estrogen-specific membrane-associated binding sites in calf uterus are described in this communication.

MATERIALS AND METHODS

All reagents were of analytical grade. [2,4,6,7-3H]Estradiol (American/Searle; 104 Ci/mmol) was more than 98% pure as determined by thin layer chromatography. Unlabeled 17β-estradiol, estrone, progesterone, prednisone, testosterone, and dihydrotestosterone were obtained from Steraloids, Inc., Wilton, N.H. Dienestrol, 17β-diethylstilbestrol, 17α-estradiol, estriol, iodoacetamide, p-chloromercuribenzenesulfonic acid, N-ethylmaleimide, dithiothreitol, and Nonidet P-40 were purchased from Sigma. Tamoxifen1 was a kind gift of Imperial Chemical Industries, Wilmington, Delaware. Buffer A consisted of 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA, 5 mM MgCl2, 250 mM sucrose and 1 mM dithiothreitol, while Buffer B consisted of 50 mM Tris-HCl, pH 7.5, containing 1 mM EDTA.

Preparation of Membranes—All steps were performed at 0-4°C unless otherwise stated. Calf uterus weighing less than 15 g were collected fresh, and connected and adipose tissues were dissected away. Cleaned tissues may be frozen in liquid nitrogen and stored at -90°C until required. Various cellular fractions were prepared by a modification of a previously described method (19). Calf uterus were minced and homogenized with a Polytron (Brinkmann) at setting No. 3 for six times at 15 s each in 1 to 2 volumes of Buffer A. The homogenate was filtered through one layer of nylon mesh (120 mesh) and subsequently through four layers of nylon mesh. A crude nuclear fraction was obtained by centrifugation at 900 × g for 10 min. Further debris were removed at 600 × g for 10 min and discarded. The supernatant was centrifuged at 10,000 × g for 15 min to obtain a heavy microsomal pellet and at 200,000 × g for 20 min to obtain a light microsomal pellet and the cytosol. All three particulate fractions were individually suspended in Buffer A with a Potter-Evans homogenizer and washed three times by centrifugation with approximately 100 volumes of Buffer A.

Alternatively, membranes were occasionally prepared after initially removing cell debris at 1000 × g for 10 min by applying the crude homogenate to either a continuous sucrose gradient (10 to 90%) or a discontinuous gradient with steps of 10, 25, 35, and 50% sucrose. The

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2 3,4-Bis(p-hydroxyphenyl)-2,4-hexadiene.

3 1-p-3-Dimethylamino-ethoxyphenyl-trans-1,2-diphenylbut-1-ene.

10266
properties and binding characteristics of these membranes were identical with those prepared as above, and these methods were not used routinely.

The nuclear pellet was finally resuspended in Buffer A containing 1 M sucrose and centrifuged for 60 min at 100,000 x g on a discontinuous gradient of 1.65 M sucrose in Buffer A over 2.4 M sucrose in the same buffer. The nuclei were collected from the lower interface and were incubated by microscopic examination to be essentially free of contamination. Nuclear membranes were prepared from the purified nuclei by sonication as described earlier (20). The various cellular fractions were analyzed for protein by the Coomassie blue method (21), nucleic acid (22), and marker enzymes (23-25) as shown in Table I. The light microsomal fraction was used for routine binding analyses. The yield of microsomal receptors was not affected by using previously frozen uteri or by exclusion of 0.25 M sucrose from Buffer A.

Female 40- to 45-day-old Sprague-Dawley rats weighing 120 to 150 g were decapitated, and various organs (uteri, ovaries, mammary gland, heart, lung, spleen, adrenals, pancreas, brain, liver, and kidneys) were removed for homogenization. The microsomal membrane fractions from the various tissues were prepared as described above. The membranes from calf or rat uteri may be stored frozen in Buffer B for more than two weeks without appreciately losing binding capacity for [3H]estradiol.

**Binding Assay**—The binding of 17β-[3H]estradiol to membrane preparations was determined by filtration on Whatman glass fiber (GF/B) filter discs. The choice of filter discs is critical since estradiol adsorbs strongly to various cellulose-derived filter papers. The membranes (50 to 100 µg of protein) were incubated in a final volume of 220 µl of Buffer B with 0.5 µM [3H]estradiol (20 pmol [3H]estradiol) at 22°C for 1 to 4 h. The reaction was stopped by adding 3 ml of ice-cold Buffer B, filtered through the glass fiber filters, and washed with 10 ml of ml of ice-cold Buffer B under suction. All determinations were performed in duplicate or triplicate. The radioactivity on the filters was determined by liquid scintillation with 10 ml of Aquasol.

Cytosol from three uteri, removed from 30-day-old rats, was prepared and assayed essentially as described above. The preliminary method was used for protein determination as well as for [3H]estradiol binding to the membranes from the various tissues were prepared as described above. The membranes from calf or rat uteri may be stored frozen in Buffer B for more than two weeks without appreciably losing binding capacity for [3H]estradiol.

**Preparation of Prelabeled Cytosolic Receptor**—The cytosol from rat uteri was prepared and assayed essentially as described earlier (4). Cytosol from three uteri, removed from 30-day-old rats, was prepared in a total volume of 5 ml of Buffer A [17β-estradiol was added to the cytosol at a final concentration of 10 nm]. After 1 h of incubation at 4°C the labeled cytosol was chromatographed through a 15-ml Bio-Gel P10 column at 4°C. The void volume fractions containing the [3H]estradiol receptor complex were pooled and stored at 4°C until use.

### RESULTS

**Characterization of Cellular Fractions**—Various subcellular markers were used to define cellular compartments as shown in Table I. Plasma membranes, as defined by 5'-nucleotidase and K⁺-dependent, ouabain-sensitive p-nitrophenolphosphatase, a component of Na⁺,K⁺-ATPase (26), were largely in the light microsomal fraction, though considerable activity of the former enzyme was also found in the cytosol. Succinic dehydrogenase (a mitochondrial enzyme) was predominantly in the heavy microsomes, and lactate dehydrogenase was almost exclusively found in the cytosol, in contrast to the estradiol binding sites which had significant levels in both microsomal fractions.

Three uteri were homogenized separately, and enzymes and various parameters were determined in duplicate on each of the subcellular fractions from the individual uteri (Table I). The numbers in parentheses indicate the variations among the three uteri. The experimental error within duplicate determinations was less than 6%.

**Effect of Various Estrogens and Nonestrogens on Binding of [3H]Estradiol**—The specific binding of [3H]estradiol to the calf uterine membranes is a time-dependent process (Fig. 1). A steady state equilibrium of specific binding is attained within 3 to 4 h at 22°C (15 to 20 min at 37°C). Dissociation of the bound estradiol from uterine membranes at 37°C, as studied by 50-fold dilution of membranes with Buffer B, is very rapid with a half-life of 15 min (data not presented). At 22°C the half-life of dissociation is approximately 300 min.

### Table I

<table>
<thead>
<tr>
<th>Cellular fraction</th>
<th>Binding</th>
<th>Protein</th>
<th>DNA</th>
<th>p-Nitrophenolphosphatase</th>
<th>Glucose-6-phosphatase</th>
<th>5'-Nucleotidase</th>
<th>Lactate dehydrogenase</th>
<th>Succinate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>nm</td>
<td>Kd</td>
<td>Rmax</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole homogenate</td>
<td>ND*</td>
<td>ND</td>
<td>650 (97)</td>
<td>99 (7)</td>
<td>0.27 (0.1)</td>
<td>8.9 (0.8)</td>
<td>35.7 (2.6)</td>
<td>496.3 (87)</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.13 (0.01)</td>
<td>146 (159)</td>
<td>567 (41)</td>
<td>3.2 (0.7)</td>
<td>0.0</td>
<td>9.4 (1)</td>
<td>27.2 (2.4)</td>
<td>599.9 (136)</td>
</tr>
<tr>
<td>Nuclei</td>
<td>0.13 (0.05)</td>
<td>198 (20)</td>
<td>40.5 (6)</td>
<td>510 (60)</td>
<td>0.0</td>
<td>3.4 (0.4)</td>
<td>4.5 (0.6)</td>
<td>13.2 (19)</td>
</tr>
<tr>
<td>Heavy microsomes</td>
<td>0.29 (0.16)</td>
<td>837 (166)</td>
<td>9.6 (2)</td>
<td>0.0</td>
<td>2.5 (0.6)</td>
<td>5.6 (0.6)</td>
<td>13.2 (5.4)</td>
<td>330.3 (85)</td>
</tr>
<tr>
<td>Light microsomes</td>
<td>0.19 (0.10)</td>
<td>523 (268)</td>
<td>13.5 (6)</td>
<td>0.0</td>
<td>9.3 (5)</td>
<td>19.4 (5.5)</td>
<td>50.4 (14)</td>
<td>87.7 (12)</td>
</tr>
</tbody>
</table>

* Femtomoles per mg of protein.
* ND: not determined.
* K⁺-dependent, ouabain-sensitive phosphatase; micromoles of nitrophenol released per mg of protein per min.
* Micrograms of inorganic phosphate released from substrate per mg of protein per h.
* Nanomoles of NADH oxidized per mg of protein per min.
* Nanomoles of dichlorophenolindophenol reduced per mg of protein per min.
* ND, not determined.

The numbers in parenthesis represent the variations among three calf uteri homogenized and fractionated separately.
Membrane-associated Estrogen Binding Sites

binding to uterine membranes (Fig. 3). The nonsteroidal estrogens, diethylstilbestrol and dienestrol, are almost as effective as estradiol. In contrast, the nonestrogenic steroids, progesterone, prednisone, testosterone, dihydrotestosterone, cholesterol, and 17α-estradiol are considerably less potent in competing with [3H]estradiol for binding to calf uterine microsomal membranes. For comparative purposes the binding characteristics for the cytosol and microsomal membranes from a single calf uterus were investigated in parallel. Table II presents the \( K_d \) values for the inhibition of [3H]estradiol binding by various steroids and nonsteroids. The affinities of the nonestrogenic steroids for both receptors were at least 10-fold lower than that for 17β-estradiol, while the nonsteroidal estrogens had affinities comparable to that of estradiol. For the estrogens compounds investigated the ratio of \( I_50 \) values for the microsomal to cytosolic receptors were from 0.5 for estrol to 1.5 for estrone. However, the \( I_50 \) of the antiestrogen, Tamoxifen, for the cytosolic receptor was about 11-fold lower than that for the microsomal receptor. It is conceivable that this difference in affinity is due to metabolism of Tamoxifen.

Nature of [3H]Estradiol Bound to Membranes—Estradiol appears not to be chemically transformed to any significant extent after binding to uterine membranes under the experimental conditions used. The membrane-bound estradiol can be quantitatively extracted with ether. The radioactivity extracted from the centrifuged membranes as well as from the membrane-free supernatant was analyzed by thin layer chromatography on silica gel plates (Brinkmann) using cyclohexane:chloroform:acetic acid (70:20:10) as the solvent system (28). More than 95% of the radioactivity extracted from the membranes or from the membrane-free supernatants co-migrated with estradiol.

Effect of Enzymatic Digestions of Membranes—Various proteolytic enzymes and micrococcal nuclease were tested at two different concentrations for their effect on binding of [3H]estradiol to uterine membranes (data not presented). Incubation of uterine membranes at 22°C for 50 min with trypsin or chymotrypsin (1 μg/ml) reduces the specific binding of [3H]estradiol by more than 50%. Micrococcal nuclease (10 μg/ml) is ineffective in altering the binding characteristics. The data clearly indicate the protein nature of the membrane-associated estradiol binding sites.

Effect of Various Treatments on Binding—Binding of [3H]estradiol to uterine membranes is about 40% higher after pretreatment of the membranes with 1.5 M KCl, but lower KCl concentrations (0.15 to 0.5 M) have no effect. The increase in specific binding with high concentrations of KCl is reminiscent of similar findings observed with the insulin receptors in rat liver membranes (29). Extraction with 1 mM EDTA in distilled water also did not affect the binding. Sonication of the membranes for a short time (up to 20 s) has no effect on binding while prolonged sonication (60 s) produces a small but detectable decrease (up to 20%). Among the four thiol reagents tested, N-ethylmaleimide, iodoacetamide, dithiobis-2-nitrobenzoic acid, and β-chloromercuribenzenesulfonic acid, only the latter (10 mM) substantially lowers (55%) [3H]estradiol binding after pretreatment of the membranes.

Binding of [3H]Estradiol to Various Rat Tissues—Comparative characteristics of [3H]estradiol binding to microsomal preparations from various rat tissues were investigated (Table III). As in the case of calf, rat uterine membranes show specific, saturable, high affinity binding for [3H]estradiol. The \( K_d \) value calculated from Scatchard plots is 0.2 nM with a maximum ligand binding of 267 pmol per mg of membrane protein. The microsomal membranes prepared from rat ovaries show a \( K_d \) value similar to that of rat uterus with comparable binding sites. The light microsomal fraction from mammary, from either mature or lactating rats, showed very little specific binding of estradiol which could only be detected by using large quantities of membranes. However, microsomes from the mammarys of immature and ovarietomized rats displayed no specific binding (horizontal Scatchard plots). In

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**Table II**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Microsomes</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-Estradiol</td>
<td>0.8</td>
<td>1.3</td>
</tr>
<tr>
<td>17α-Estradiol</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Estriol</td>
<td>16</td>
<td>30</td>
</tr>
<tr>
<td>Estrone</td>
<td>35</td>
<td>23</td>
</tr>
<tr>
<td>Diethylstilbestrol</td>
<td>0.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Dienestrol</td>
<td>4.7</td>
<td>5.9</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>1,400</td>
<td>130</td>
</tr>
<tr>
<td>Progestrone, testosterone, prednisone, cholesterol</td>
<td>~80,000</td>
<td>~80,000</td>
</tr>
</tbody>
</table>

FIG. 1 (left). Time course of [3H]estradiol binding to calf uterine microsomes at 22°C. The concentration of [3H]estradiol in each incubation was 0.5 nM. The nondisplaceable binding was determined in the presence of 1 μM unlabeled estradiol. Inset, the specific binding represents the difference between the total and nondisplaceable binding.

FIG. 2 (center). Binding to calf uterine microsomes as a function of [3H]estradiol concentration at 22°C. Inset, Scatchard plot.

FIG. 3 (right). Competition of [3H]estradiol binding to calf uterine microsomes with various estrogens and nonestrogenic steroids. [3H]Estradiol (0.5 nM) and various steroids were incubated simultaneously with the membranes (80 μg) for 4 h at 22°C. The 100% binding corresponds to 10,300 dpm. Δ, diethylstilbestrol; O, unlabeled estradiol; □, dienestrol; ●, estrone; ▲, estradiol; □, Tamoxifen; and X, testosterone, dihydrotestosterone, progesterone, prednisone, cholesterol, and androstenedione.

Effect of Proteolytic Enzymes and Micrococcal Nuclease—Several proteolytic enzymes and micrococcal nuclease were tested for their effect on the binding of [3H]estradiol to uterine membranes (data not presented). Incubation of uterine membranes at 22°C for 60 min with trypsin or chymotrypsin (1 μg/ml) reduces the specific binding of [3H]estradiol by more than 50%. Micrococcal nuclease (10 μg/ml) is ineffective in altering the binding characteristics. The data clearly indicate the protein nature of the membrane-associated estradiol binding sites.

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contrast to rat uterus, where approximately 15 to 20% of the total receptors are found in the microsomes, in mature or lactating mammary tissues only 1 to 3% of the estradiol binding sites are membrane-associated. Due to such low levels it is difficult to draw any conclusions regarding the presence or absence of microsomal binding sites for estradiol in rat mammary tissues. The microsomal membranes from the non-target rat tissues (heart, lung, spleen, pancreas, thymus, adrenals, liver, kidney, and whole brain) do not show any specific binding, although the degree of nonspecific binding did vary among the tissues.

Adsoption of Cytosolic Receptor to Rat Microsomes—The adsorption of rat uterine cytosolic receptor to rat target and nontarget tissue microsomes was tested by two different experimental designs. The nontarget tissues (kidney and diaphragm) from 30-day-old rats were homogenized separately in aliquots of freshly prepared rat uterine cytosol, and the microsomal fraction was prepared as described above. Scatchard analysis showed no specific \([^{3}H]\)estradiol binding sites in either of the microsomes prepared in the presence or absence of uterine cytosol (horizontal Scatchard plots, data not presented).

In a separate series of experiments the binding of prelabeled cytosolic receptor to rat diaphragm microsomes was studied. Three diaphragms from 30-day-old rats were homogenized in 3 ml of prelabeled rat uterine cytosol (740,000 dpm per 3 ml). Unlabeled estradiol (1 \(\mu\)m) was included to prevent binding of any dissociated label with microsomal binding sites. The exchange of \([^{3}H]\)estradiol on cytosolic receptor for unlabeled hormone cannot be very substantial as the rate of competitive dissociation of estradiol at 4°C is less than 5% in 4 h. The light microsomal fractions of diaphragm, thus prepared, retained 11,000 dpm or 1.5% of the total label added. This corresponds to 2,200 dpm or 7.6 fmol of \([^{3}H]\)estradiol per mg of protein.

Detergent Extraction of Uterine Microsomes—The effect of various detergents on binding of \([^{3}H]\)estradiol to calf uterine light microsomes was tested. Microsomes were preincubated with 0.01 to 0.05% (v/v) of various detergents such as sodium cholate, Lubrol-PX, Triton X-100, and Nonidet P-40 (NP-40) for 30 min at 4°C. The membranes were collected by centrifugation, washed twice with 100 volumes of Buffer A, and resuspended to their original volume. Protein determinations were made on trichloroacetic acid-precipitated, ether-ethanol-extracted aliquots of the membrane suspension (30). Control membranes were treated similarly without the detergent. Binding of \([^{3}H]\)estradiol to the membranes was determined under standard assay conditions as described under "Materials and Methods." Lubrol-PX and cholate caused a decrease in specific binding of estradiol. The effect of Triton X-100 (data not presented) and NP-40 (Fig. 4) on binding of estradiol was biphasic. While the absolute binding of the ligand remains virtually unchanged at detergent concentrations of 0.05 to 0.5%, the recovery of the microsomal proteins decreases, and as a result the specific binding of estradiol increases by up to 3-fold. Scatchard analysis of binding data for microsomes extracted with 0.15% (v/v) NP-40 (Fig. 4) indicates that the \(B_{\text{max}}\) is increased by about 50% but the \(K_{d}\) remains unaffected by such treatment. The increased binding of estradiol seems to be due to selective removal of nonreceptor protein and possibly in part due to uncovering of previously buried binding sites.

Similar results have been obtained using 0.015 to 0.5% (w/v) \(\beta\)-octyl glucoside. More than 80% of the microsomal proteins were solubilized resulting in an almost 5-fold increase in the specific binding of the remaining microsomal pellet. The lowest effective concentration of NP-40 was around 0.05% which is slightly above its critical micellar concentration (0.03% v/v), whereas \(\beta\)-octyl glucoside was effective well below its critical micellar concentration (0.74% w/v).

**DISCUSSION**

We have presented data indicating the presence of high affinity estradiol binding sites associated with the microsomal fraction of calf uteri (in addition to those routinely found in cytosol and nuclei) which are 10 to 15% of total cellular estradiol binding sites. In contrast the nuclear membranes appear to have insignificant amounts of estrogen-specific binding sites (data not presented). It has been speculated that an estradiol receptor occurs in endometrial plasma membranes (18). While our results do not negate this possibility, we do not find enrichment of the estradiol binding sites parallel to the \(5'\)-nucleotidase or the \(K\)-dependent, ouabain-sensitive \(\beta\)-nitrophenylphosphatase in the light microsomal fraction of whole uteri, and it appears to be more prevalent in the heavy microsomal fraction. The presence of specific binding of estrogens at very low concentrations and the absence of interaction of nonestrogenic steroids with the same binding site indicate estrogen-specific receptor interactions. None of the (nine) nontarget tissues investigated showed any specific binding of \([^{3}H]\)estradiol. The exclusive presence of estradiol-specific membrane-associated binding sites in target tissues suggests that these putative receptors may be important biologically. The \(K_{d}\) of estradiol for these binding sites (0.2 nM) is compat-

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**Table III**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Cytosolic</th>
<th>Microsomal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uterus</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(B_{\text{max}})*</td>
<td>(K_{d})*</td>
</tr>
<tr>
<td></td>
<td>(K_{d})</td>
<td>(K_{d})</td>
</tr>
<tr>
<td></td>
<td>438</td>
<td>236</td>
</tr>
<tr>
<td></td>
<td>0.30</td>
<td>0.21</td>
</tr>
<tr>
<td>Ovary</td>
<td>81</td>
<td>224</td>
</tr>
<tr>
<td>Mammary</td>
<td>48(a)</td>
<td>27</td>
</tr>
<tr>
<td>Mature</td>
<td>31(b)</td>
<td>17</td>
</tr>
<tr>
<td>Lactating</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

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\(a\) Maximal binding of \([^{3}H]\)estradiol, expressed as femtomoles per mg of protein.

\(b\) Pancreas, thymus, lung, adrenal, liver, kidney, whole brain, and heart.
Membrane-associated Estrogen Binding Sites

rable to that for the cytosol receptor (0.3 nM) as measured in calf uterine preparations (4).

The fact that pretreatment of uterine membranes with buffers of high or low ionic strengths does not lead to the release of binding sites to the supernatant suggests that these sites are not an "adsorbed" form of cytosolic receptor in contrast to a recent report (31). Table I indicates that a portion of the total estradiol binding partitions in opposition to lactate dehydrogenase, which is a marker for cytosolic proteins. Less than 0.5% of the total lactate dehydrogenase activity is recovered in the microsomal fraction, whereas approximately 7% of the total estradiol binding was associated with this fraction.

In some instances, as much as 15% of the total estradiol binding was recovered in the microsomal fraction, though this may reflect the relative instability of the cytosolic receptors as opposed to the microsomal receptors.

The presence of a membrane-associated estradiol receptor is further supported by the fact that microsomal membranes from a non-target tissue (such as rat kidney or diaphragm) do not show any specific binding (horizontal Scatchard plot) of [3H]estradiol even when these are prepared in the presence of excess rat uterine cytosol. In an alternative approach the rat diaphragm microsomes, prepared in the presence of rat uterine cytosol (prelabeled with [3H]estradiol) showed no appreciable binding of radioactivity, indicating that cytosolic receptors are not being adsorbed to the microsomal membranes. Under these conditions a maximum of 1.5% of the cytosolic receptor was adsorbed onto the membranes. Prolonged sonication of the microsomal membranes of high or low ionic strengths does not lead to the release of binding sites to the supernatant suggesting further that trapped or vesicularized cytosolic receptors may be tightly linked by protein-protein interactions.

Examples of such tightly linked proteins, that are not disrupted by detergents, have been described for coated pits or microvillus diaphragm microsomes, prepared in the presence of rat uterine preparations (4).

The presence of estrogen-specific membrane-bound receptors may be tightly linked by protein-protein interactions. Nonionic detergent, NP-40, indicates that these putative receptors are not dislodged by detergents, have been described for coated pits or microvillus diaphragm microsomes, prepared in the presence of rat uterine preparations (4). Puromycin, under the conditions of Table I, reduced the binding of estradiol by 20%.

As much as 15% of the total estradiol binding was associated with the supernatant suggests that these sites are not dislodged by detergents. These putative receptors are not dislodged by detergents, have been described for coated pits or microvillus diaphragm microsomes, prepared in the presence of rat uterine preparations (4). Puromycin, under the conditions of Table I, reduced the binding of estradiol by 20%.

The finding that the microsomal binding sites for estradiol are stable to the relatively harsh treatment with the nonionic detergent NP-40 indicates that these putative receptors may be tightly linked by protein-protein interactions. Examples of such tightly linked proteins, that are not disrupted by detergents, have been described for coated pits or microvillus diaphragm microsomes, prepared in the presence of rat uterine preparations (4). Puromycin, under the conditions of Table I, reduced the binding of estradiol by 20%.

The data presented in this communication strongly suggest the presence of estrogen-specific membrane-bound receptors in target tissues. Unlike many other hormones, where a biochemical function for the membrane receptor has been suggested, the functional role of the postulated membrane-bound estrogen receptors is still not clear.

Acknowledgments—We thank Dr. P. Cuatrecasas for his very helpful suggestions and continued encouragement during the course of this work.

REFERENCES