Interaction of the Estradiol Receptor from Calf Uterus with Its Nuclear Acceptor Sites*

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The specific interaction between 17β-estradiol-receptor complex and nuclear acceptors was analyzed by immobilizing various nuclear proteins to CNBr-activated agarose. The specific, high affinity sites identified in a fraction of basic proteins that can be solubilized from purified nuclei of calf uterus (Puca, G. A., Sica, V., and Nola, E. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 979-983) were chromatographed on Sephadex G-100 columns. Elution of the acceptor activity depends on the pH and ionic strength of the buffer used. With 5 mM HCl, however, a peak of acceptor activity with a molecular weight of about 70,000 was partially dissociated from the other basic nuclear proteins. The high affinity binding of the receptor to the acceptor proteins was estradiol-, but not progesterone-, cortisone-, or testosterone-dependent; it was very sensitive to ionic strength and showed a physiological pH optimum. Low affinity binding, such as that seen between receptor and histone, showed no estradiol dependence and little ionic strength and pH sensitivity.

Native or heat-denatured DNA strongly modified the receptor-acceptor interaction, reducing the number of binding sites of acceptor for the receptor without changing the high affinity of the interaction. Heating of the acceptor protein before its covalent linkage to agarose considerably increased the affinity of the resulting agarose derivative. Free sulfhydryl groups of the receptor but not of the acceptor molecule play an important role in the acceptor-receptor interaction.

When receptor and acceptor preparations were incubated in solution, the resulting complex was included on a Sephadex G-100 column and eluted from DEAE-cellulose columns at lower ionic strength than the receptor alone. Even though not absolutely specific, these two properties allowed determination of the molecular weight (85,000) of the acceptor protein at neutral pH and more nearly physiological ionic strength. The apparent $K_d$ of the acceptor-receptor interaction was determined to be $2 \times 10^{-10}$ M at 0°.

Apparently similar, high affinity binding sites for estradiol receptors are also present in nuclei of other tissues.

Many experimental data suggest that the nucleus of target cells is the primary site of action of steroid hormones. It is believed, however, that the steroid molecules do not interact directly with the nucleus, but that they first bind to specific extranuclear receptor proteins which then move into the nucleus as a steroid-receptor complex (1-12). The function of the steroid is presumably to allow the receptor to recognize its nuclear partner, the "acceptor." How this occurs is still controversial. Small conformational changes (13, 14), association with the other cytosol entities (15), and enzymatic or nonenzymatic transformation (16-18) have been postulated as being responsible for directing the receptor to migrate from cytoplasm to nucleus once it has formed a complex with the specific steroid.

The interaction of cytoplasmic steroid receptors with nuclei has been studied in whole organs, isolated cells, and cell free preparations (1-13, 13-18). The estrogen-uterus system has been one of the most thoroughly investigated, but with inconsistent or conflicting results. These studies have been hampered by the estradiol receptor’s tendency to aggregate and to nonspecifically bind to numerous kinds of insoluble material including target or nontarget cell nuclei or chromatin (27, 37). We decided to investigate the estradiol receptor-nuclear interaction in the calf uterus following a new approach. This consists of solubilization from purified nuclei of those molecules which, when again immobilized on an inert support like agarose, interact with the extranuclear receptor in a way that mirrors closely the physiological receptor-nuclear interaction.

In a previous study (38) we have reported the identification of a high affinity nuclear acceptor for the estrogen receptor of calf uterus. These nuclear sites interact with high affinity only...
with the intact estradiol-receptor complex and not with either component alone. KCl concentrations between 0.3 and 0.5 M destroy this high affinity interaction. The protein nature of the acceptor sites was suggested by their sensitivity to proteolytic enzymes and lack of sensitivity to nucleases. Furthermore, direct interaction of the receptor with nucleotides immobilized on cellulose or agarose was insignificant. Purification of the nuclear acceptor on hydroxyapatite and on CM-cellulose showed that the high affinity binding sites were associated with basic nuclear proteins.

In this report the acceptor molecules solubilized from purified calf uterine nuclei are further purified and characterized. We also show that similar acceptor sites are present in nuclei from other tissues, and that interaction with the steroid-receptor complex requires free sulfhydryl groups in the receptor molecule. Nuclear acceptor sites can be readily distinguished from histones.

MATERIALS AND METHODS

The source of chemicals and reagents not mentioned below has been described previously (38). The buffer used was Buffer A (10 mM Tris-HCL, pH 7.5, 1 mM EDTA, 1 mM dithiobiotriol containing KCl as indicated).

Unless specified, all operations were carried out at 0 to 4°. Crude cytosol or partially purified 4.5 S receptor protein were prepared as described previously (16, 39). In studies of the elution of acceptor-receptor complexes from DEAE-cellulose columns, the receptor protein was further purified through DEAE-cellulose; only those fractions which were eluted below 0.08 M KCl in a linear gradient were collected. The nuclear fraction containing acceptor was prepared from purified nuclei washed with Triton X-100 as described earlier (38). Acceptor proteins were solubilized from this fraction, or directly from purified nuclei, by mild acid extraction.

The best condition for solubilization of nuclear material with high affinity for the receptor complex was acid extraction at a pH not lower than 4. HCl, pH 1 (containing 5 mM dithiothreitol and 1 mM NaH SO) was slightly less effective than HCl, pH 1, but was preferred because the extracted proteins could be precipitated more easily. Higher temperatures, lower pH values, and longer times decreased acceptor activity. After centrifugation for 30 min at 40,000 × g, the pH, H2SO4 extract was precipitated by 4 volumes of cold ethanol (-20°) and, after 40 to 60 min at -20°, was collected by centrifugation. The volume of the dissolving buffer was such that the total protein concentration was below 4 mg/ml. Longer precipitation times in ethanol or acetone impaired recovery of acceptor proteins.

Sepharose 4B was activated with CNBr (250 mg/g of packed agarose) according to Cuatrecasas (40). After activation, the chilled, activated agarose was rapidly filtered by means of a Buchner funnel, extensively washed with ice-cold distilled water followed by dioxane. The activated agarose was stored in dioxane at room temperature for as long as 3 months with no loss of coupling capacity. When needed, the agarose was washed free of dioxane with ice-cold distilled water and with the buffer to be used in the coupling reaction.

Coupling was carried out at 0° for 15 to 30 hours. Casein, histone, and H2SO4-solubilized DNA were coupled as described previously (38). Proteins were coupled either directly after neutralizing the pH, or after concentrating the protein by means of a small CM-cellulose column which was eluted with HCl, pH 2, or 0.6 M NaCl in phosphate buffer, pH 6.8. The concentration of nuclear protein to be coupled was maintained below 1 mg/ml. Substitution was estimated by determining the protein or nucleic acid content of the filtrates and washes. Usually 50 to 90% of the material initially present in solution was coupled to agarose. The efficiency of coupling did not vary in the range of pH from 6.2 to 7.5, but was clearly improved when the coupling was carried out in 0.8 to 1.2 M KCl. After coupling, the derivative was washed thoroughly with 1 M NaCl, then with water, and finally were incubated at room temperature for at least 2 hours in 2 mM glycine buffer, pH 0.4, to block unconjugated CNBr-activated groups. Glycine was removed by washing and the derivatives were suspended in 2 volumes of cold Buffer A/0.1 M KCl.

Binding of the estradiol-receptor complex to agarose derivatives was tested as previously described (38). Data are usually expressed as B/F, which represents the ratio of estradiol-receptor complex bound to agarose, i.e., which sediments at 500 × g together with the agarose, over complex which does not bind to the agarose. At 0° no tritiated estradiol is released by the receptor protein, and interaction of the estradiol-receptor complex with the substituted agarose does not favor dissociation of the hormone from the receptor site. The rate of dissociation of estradiol from receptor was actually reduced after interaction with the acceptor. The eluates of columns were tested for binding at 0.1 and 0.6 M KCl. The value of B/F at 0.1 M KCl relative to the value in 0.5 M KCl represents the acceptor activity of the various fractions. Fractions with greater acceptor activity also displayed high affinity and saturability when their interaction with receptor complex was analyzed on Scatchard plots.

Radioactivity was measured in a Mark 1 (Nuclear Chicago) liquid scintillation spectrometer with InstantGel (Packard) as scintillation fluid; counting efficiency was over 35%.

RESULTS

Presence of High Affinity Sites in Nuclei of Tissues Other Than Uterus

When fractions solubilized from non-uterine nuclei with 2 M NaCl were covalently attached to CNBr-activated agarose, specific binding sites for estradiol receptors could be detected (Table I). The specificity of this fraction for binding of estradiol receptors (high affinity and saturability) was similar or identical to that observed with the calf uterus nuclear fractions solubilized with 2 M NaCl. The approximate number of these sites was estimated from intercepts on the abscissa of Scatchard plots obtained using the same preparation of partially purified and labeled estrogen receptor.

Fractionation of Proteins Extracted from Uterine Nuclei

It was previously shown (38) that acceptor sites are associated with basic proteins which elute from CM-cellulose with the bulk of histones F1 and F2. However, when the mild acid extract of purified nuclei, the 2 M NaCl-soluble nuclear fraction, or the peak eluted from CM-cellulose is analyzed by Sephadex G-100 chromatography, it is possible to dissociate the steroid-receptor complex which does not bind to the agarose. At 0° no tritiated estradiol is released by the receptor protein, and interaction of the estradiol-receptor complex with the substituted agarose does not favor dissociation of the hormone from the receptor site. The rate of dissociation of estradiol from receptor was actually reduced after interaction with the acceptor. The eluates of columns were tested for binding at 0.1 and 0.6 M KCl. The value of B/F at 0.1 M KCl relative to the value in 0.5 M KCl represents the acceptor activity of the various fractions. Fractions with greater acceptor activity also displayed high affinity and saturability when their interaction with receptor complex was analyzed on Scatchard plots.

Radioactivity was measured in a Mark 1 (Nuclear Chicago) liquid scintillation spectrometer with InstantGel (Packard) as scintillation fluid; counting efficiency was over 35%.

TABLE I

Presence of saturable binding sites for estradiol receptors in nuclear extracts of various calf organs

The 2 M NaCl extract of purified nuclei was dialyzed against 25 mM phosphate buffer, and the insoluble precipitate was coupled to CNBr-activated agarose after solubilizing again with 2 M NaCl in 25 mM phosphate, pH 6.8. Various concentrations of partially purified 4.5 S receptor (labeled with [3H]estradiol) were incubated in a final volume of 1 ml for 5 hours at 0° with a constant amount of the nuclear fraction-agarose derivatives (from 10 to 50 mg according to the amount of substitution). After centrifugation, radioactivity was measured in the supernatant. Saturation points of the various nuclear-agarose derivatives were measured graphically on Scatchard plots and normalized to the amount of agarose-bound protein used. Activities were compared to those obtained with the uterine nuclear material.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Percent of binding</th>
</tr>
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<tbody>
<tr>
<td>Uterus</td>
<td>100</td>
</tr>
<tr>
<td>Lung</td>
<td>75</td>
</tr>
<tr>
<td>Spine</td>
<td>71</td>
</tr>
<tr>
<td>Kidney</td>
<td>40</td>
</tr>
<tr>
<td>Liver</td>
<td>35</td>
</tr>
<tr>
<td>Heart</td>
<td>15</td>
</tr>
<tr>
<td>Intestine</td>
<td>15</td>
</tr>
</tbody>
</table>

*Carried out with organs obtained from a different calf. The recovery of nuclei from intestine was very poor.
the acceptor activity from the major peaks of protein. On gel chromatography the elution pattern of protein (followed by absorbance at 230 nm) and of acceptor activity appears to be very dependent on the ionic strength and pH of the buffer used. In 5 mM HCl two principal protein peaks are included and the acceptor activity is localized in the ascending portion of the second peak (Fig. 1). By comparison with the elution volumes of standard proteins of known molecular weight, an apparent molecular weight of 70,000 can be estimated for the acceptor activity under these conditions. In a 50 or 100 mM acetate, pH 4.5 to 5, the protein profile is not changed but the acceptor activity appears spread and associated with the principal protein peaks. In 50 mM phosphate, pH 6.8, with or without KCl, and in Tris-HCl buffer, pH 7.4, 0.1 mM to 1 mM KCl, the protein pattern indicates the presence of large aggregates (data not shown). This behavior may be due to the well known phenomenon of aggregation of nuclear proteins at neutral pH and high ionic strength. Under the conditions described in Fig. 1, the ascending portion of the second protein peak contains the acceptor activity, whereas the descending portion lacks such activity. Many properties of the acceptor sites can be analyzed by comparing the behavior of the material present in the ascending and descending portions of these fractions.

Because of its tendency to aggregate, further purification of the acceptor molecules by conventional procedures has proven difficult. On CM-cellulose, acceptor activity was eluted by KCl or NaCl gradients together with the principal protein peaks. On electrofocusing on a pH 9 to 11 gradient, the activity coincided with the basic proteins, which appeared at the highest pH values of the gradient (data not shown).

**Receptor-Acceptor Interaction**

**Estradiol Dependence**—Fractions containing acceptor proteins, once coupled to agarose, bind with high affinity and saturability only those receptors which form complexes with estrogen (Fig. 2). Progesterone, $5 \times 10^{-8}$ M, and testosterone do not substitute for estradiol (Table II). In the absence of estradiol, Scatchard plots indicate only nonspecific, nonsaturable binding similar to that obtained in the presence of high salt (Fig. 2). Similarly, only nonspecific binding is seen when the descending part of the second protein peak of Sephadex G-100 (Fig. 1) is bound to agarose; at each value of bound estrogen-receptor complex, the ratio of B/F is constant and low.

**Effect of Ionic Strength**—The B/F values as used here reflect the specificity of the receptor-acceptor interaction. It is a convenient parameter for detection and study of acceptor activity when the amount of nonspecific interaction is constant. A high B/F value can be obtained when a large amount of nonspecifically interacting protein like protamine, histone or casein is coupled to agarose. In such cases, however, the B/F value at low salt (e.g. 0.1 M KCl) is usually lower and never higher than the B/F value at high salt (e.g. 0.5 M KCl); this is illustrated in Fig. 3A. The same amount of 4.5 S-labeled receptor was incubated with a fixed amount of various substituted agarose derivatives at increasing concentrations of KCl.

With specific acceptor-agarose, there is a sharp decrease in the B/F value in the range of 0.1 to 0.5 M KCl. With histone-agarose the decrease is very much smaller, and with casein-agarose the opposite effect is observed. It is thus evident that simultaneous determinations of binding in 0.1 and 0.5 M KCl are convenient for detecting the presence of acceptor sites without requiring determinations of saturability and affinity. The difference in binding observed between 0.1 and 0.5 M KCl can also be considered as being a specific property of the acceptor system because it occurs only when the receptor-estradiol complex is used.

**Effect of pH**—Fig. 3B illustrates the effect on the B/F values of varying the pH from 6.5 to 9 at 0.1 and 0.5 M KCl. The

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**TABLE II**

*Hormone specificity of receptor-acceptor interaction*

<table>
<thead>
<tr>
<th>Steroid</th>
<th>dpm bound to acceptor-agarose</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3H]Estradiol$</td>
<td>44,500</td>
</tr>
<tr>
<td>Cortisone</td>
<td>28,180</td>
</tr>
<tr>
<td>Testosterone</td>
<td>22,800</td>
</tr>
<tr>
<td>Progesterone</td>
<td>25,600</td>
</tr>
<tr>
<td>No steroid</td>
<td>23,300</td>
</tr>
</tbody>
</table>

*[^3H]Estradiol* $(10^{-8} \text{ M})$ was incubated with a fixed amount of crude cytosol (0.2 ml, 1.2 mg of protein) in a final volume of 1 ml containing the indicated steroids or no steroid. After 90 min of incubation, the agarose was sedimented by centrifugation, and $[^3H]Estradiol$ was added to the supernatant for an additional 2 hours of incubation. Receptor bound to agarose was assessed from the differences in $[^3H]Estradiol$ binding between the cytosol which had not been incubated with the acceptor agarose (64,800 dpm) and that which was left in the supernatants decanted after incubation with the acceptor agarose.
specific acceptor interaction (0.1 m KCl) is optimal at pH 7.5 to 8.5, while nonspecific interactions (0.5 m KCl) are relatively unaffected by these pH changes.

Effect of DNA—The presence of DNA modifies the receptor-acceptor interaction (Fig. 4). The number of binding sites for the receptor is decreased without changing the apparent affinity of the interaction. Nonspecific, KCl-insensitive binding is unaffected by DNA. In this, as in other experiments, heat denatured DNA is slightly more effective than native DNA.

The acceptor molecules bind strongly to DNA-agarose columns. These columns, however, do not bind receptor unless acceptor containing solutions are chromatographed before application of the receptor. As pointed out previously (38), several basic proteins like histone or lysozyme can nonspecifically mediate the binding of estradiol receptor to DNA-agarose, presumably through ionic interactions.

Effect of Nonionic Detergents and Chaotropic Salts—In order to improve the relative amount of specific binding, we attempted to inhibit nonspecific binding with nonionic detergents and chaotropic salts. Slightly higher B/F values were obtained using 0.01% Triton X-100. This concentration of detergent does not dissociate preformed estradiol-receptor complexes. With increasing detergent concentration, a parallel reduction of binding occurred at low and high salt concentrations. Chaotropic salts such as NaSCN or KI (0.1 m final concentration) decreased much more the high affinity, saturable binding than the nonspecific binding.

Effect of Heating—When the protein fraction containing the acceptor activity was heated for 10 min at increasing temperatures, and then cooled to 0° before coupling to agarose, the interaction with the receptor showed an increase in the B/F parameter which was more pronounced at 0.1 than at 0.4 M KCl (Fig. 5). Scatchard plots indicate that heating does not increase the number of the acceptor sites in the preparation but rather increases the apparent affinity between acceptor sites and the receptor (data not shown).

Effect of Sulfhydryl Reagents The optimum dithiothreitol concentration for the receptor-acceptor interaction is between 1 and 2 mM. The effect of this reagent (Fig. 6A) is much more pronounced for the high affinity interaction (0.1 m KCl) than

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**Fig. 3.** A, effect of KCl concentration on the binding of estradiol-receptor complex to different protein-agarose derivatives. Acceptor-agarose (50 mg packed, 0.6 mg of protein/g packed) (●), histone-agarose (80 mg packed, 5 mg of histone II/g packed) (△), and casein-agarose (150 mg packed, 15 mg of α-casein/g packed) (□) were incubated at 0° with partially purified 4.5 S estradiol-receptor complex (82,000 dpm) in a final volume of 1 ml of Buffer A with increasing concentrations of KCl. After 2 hours, the agarose was sedimented by centrifugation (5 min, 500 × g) and the radioactivity left in the supernatant was measured. B, effect of pH on the binding of the estradiol-receptor complex to acceptor-agarose. Assays were carried out at 0.1 (○) and 0.5 M (△) KCl. Incubation time was 40 min at 0° at the indicated pH, which was slowly changed by the addition to Buffer A of 1 N NaOH or HCl, at 0°, using a Mikro-Prasin-denspritzgerät (Braun).

**Fig. 4.** Effect of DNA on the binding of the estradiol-receptor complex to acceptor-agarose. Increasing concentrations of crude estradiol-receptor complex, in a final volume of 1 ml of Buffer A/0.1 M KCl, were incubated at 0° for 2 hours with 40 mg of packed acceptor-agarose. No DNA (○), 25 μg of native (△) or heat-denatured (□) calf thymus DNA was added to the incubation mixture. Parallel tests in 0.5 M KCl were carried out. Binding of the crude estradiol receptor complex to the agarose was measured as described in Fig. 3A.

**Fig. 5.** Effect of heating the acceptor protein on the subsequent binding of estradiol-receptor complex to acceptor-agarose. The acid extract of a nuclear fraction was dialyzed at 4° through a Sephadex G-25 column equilibrated in 0.1 M acetic acid containing 0.05 N NaOH (pH 4) and applied on a CM-cellulose column equilibrated in the same buffer. Acceptor protein were eluted with HCl, pH 2. Fractions of this solution were treated for 10 min at the indicated temperatures, cooled to 0°, neutralized, and finally coupled to CNBr-agarose. No significant change in the coupling efficiency was detected. The ability of the various agarose derivatives to retain estrogen-receptor complexes was tested as described in Fig. 5A in 0.1 m (●) and 0.5 m (△) KCl, using 60 mg of packed agarose and the partially purified 4.5 S estrogen-receptor complex (78,000 dpm). Incubation time was 3 hours.
The receptor, which is obtained as an estradiol-receptor complex.

These experiments require the use of partially purified reagent added).

The radioactivity left in the supernatant was measured. Data are expressed indicated times, the agarose was sedimented by centrifugation and the activity in the supernatant was measured. Data are expressed as percent of the KCl-sensitive binding activity of the control (no reagent added).

The specific interaction is very sensitive to sulfhydryl-group modifying reagents such as p-hydroxymercuribenzoate, n-ethylmaleimide and iodoacetate (Fig. 6B). The same reagents do not affect the preformed estradiol-receptor complex. If the acceptor-agarose derivative is incubated under identical conditions with these reagents and washed extensively before measuring its ability to bind the estradiol-receptor complex, no decrease in the receptor-agarose interaction is detected. However, prior treatment of the estradiol-receptor complex with these reagents, followed by elimination of the unreacted material by Sephadex G-25 gel filtration, results in impairment of binding to the acceptor-agarose derivative.

**Formation of Acceptor-Receptor Complexes in Solution**

Fractions containing acceptor proteins, obtained by the gel chromatography as shown in Fig. 1, were passed through Sephadex G-25, equilibrated in Buffer A/0.1 M KCl and incubated with the low salt-stable, partially purified 4.5 S estradiol receptor. Subsequent chromatography of this mixture on a Sephadex G-100 column shows a shift in the peak of radioactivity of the receptor protein (Fig. 7A). When compared to the elution volume of standard proteins, the formed acceptor-receptor complex exhibits a molecular weight which is 15,000 greater than that of the receptor alone. The pattern of elution of the major basic proteins is not modified by the addition of the receptor, which by itself does not contribute absorbance at the concentrations used.

When the receptor preparation is incubated with an identical amount of basic protein obtained from the descending part of the protein peak shown in Fig. 1, which is devoid of acceptor for the nonspecific one (0.5 M KCl). The specific interaction is sensitive to sulfhydryl-group modifying reagents such as p-hydroxymercuribenzoate, n-ethylmaleimide and iodoacetate.

The requirement for estradiol in these interactions could not be tested because these experiments require the use of partially purified receptor, which is obtained as an estradiol-receptor complex.

**Activity**, the complex as well as a new, major peak of protein elute in the void volume of the column (Fig. 7B). In both cases, then, there is a shift in the elution volume of the receptor, but in the presence of the specific, high affinity acceptor material the interaction is favored and the apparent increase in molecul-
lar weight appears to be unique since the new peak is nearly symmetrical and is not excluded from the column. This is consistent with the formation of stoichiometric receptor-acceptor complexes. When only nonspecific basic proteins (nuclear) are present, heterogeneous associations and aggregation appear to occur.

The receptor-acceptor interaction in solution was further investigated using DEAE-cellulose chromatography. Partially purified receptor is retained by DEAE-cellulose columns (3 ml) equilibrated with 0.1 m KCl, pH 8.3. However, when basic nuclear proteins are incubated with the tritiated estradiol-receptor complexes before chromatography, the radioactivity is not absorbed and is found in the breakthrough fractions of the column. The amount of radioactivity in this breakthrough material is strictly dependent on the receptor concentration at a fixed concentration of basic nuclear proteins (Fig. 8) or on the basic nuclear protein concentration if the receptor concentration is kept constant (Fig. 9). In both figures (8 and 9), Curves A represent data obtained with the proteins of the ascending portion of the second chromatography peak (similar to that shown in Fig. 1) and Curves D represent data obtained with the proteins of the descending portion of the same peak. Data similar to those depicted by Curves D were obtained when commercially available histone II was used instead of nuclear proteins. Histone III gave a curve with a much lower slope.

In both experiments more receptor appears in the breakthrough material of the DEAE-cellulose columns when the acceptor-containing fractions are used (Curves A, Figs. 8 and 9). The differences between Curves A and D are more pronounced at low concentrations of receptor or of basic protein. Subtracting Curve D from Curve A in Fig. 8 yields a rectangular hyperbola. Assuming a 1:1 receptor-acceptor complex (as suggested by the experiment of Fig. 7A), and that 1 molecule of 4.5 S receptor binds 1 molecule of estradiol, a $K_r$ of $2 \times 10^{-16}$ M can be calculated for the high affinity acceptor-receptor interaction.

**DISCUSSION**

The studies presented here were carried out with a protein preparation solubilized by a mild acid treatment of a crude nuclear fraction. The basic acceptor proteins, when immobilized on agarose, bind estradiol-receptor complexes highly specifically, i.e. demonstrating saturability and very high affinity. This binding is not observed in the absence of estradiol, or with nonestrogen steroid hormones. The existence of saturable nuclear binding sites for estradiol receptors has recently been questioned by Chamness et al. (27) on the basis of experiments carried out with intact, isolated nuclei. The apparent saturability observed in their system results from inhibition of receptor binding which occurs when the higher concentrations of cytosol protein are used. In our system, saturability is demonstrated on Scatchard plots in a range of protein concentration which varies from 5 to 50 $\mu$g/ml, levels which are from 100- to 1,000-times lower than are found in the crude cytosol. In this system saturability is observed when a fixed protein concentration is used and only the receptor concentration is varied, regardless of whether purified or crude receptor preparations are used.3

The receptor-nucleus interaction, as studied in vivo or in intact cells in vitro, is hormone-specific and sensitive to ionic strength. Receptor proteins are confined to the cytoplasm.

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3V. Sica and G. A. Puca, manuscript in preparation.

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**Fig. 8 (left).** Elution of the estradiol-receptor complex from DEAE-cellulose columns at varying concentrations of estradiol-receptor complex. Equal amounts (80 $\mu$g) of basic nuclear protein obtained from the ascending (A, ◦) and descending (D, △) portion of the second protein peak of chromatography (similar to that shown in Fig. 1) were incubated at 0° for 30 min in a final volume of 1 ml of Buffer A/0.1 m KCl with increasing concentrations of partially purified 4.5 S estrogen-receptor complex. The samples were applied on 3-ml columns of DEAE-cellulose equilibrated in Buffer A/0.1 m KCl, pH 8.3. After passing 5 ml of the same buffer, the breakthrough fraction was collected and 3 ml were counted for radioactivity. Difference between Curves A and D.

**Fig. 9 (right).** Elution of the estradiol-receptor complex from DEAE-cellulose columns at varying concentrations of basic nuclear protein concentrations. An equal amount of partially purified 4.5 S estrogen-receptor complex (123,000 dpm) was incubated with increasing concentrations of basic nuclear proteins obtained from the ascending (A, ◦) and descending (D, △) part of the second protein peak of chromatography (similar to that shown in Fig. 1). Experimental conditions were as described in Fig. 8.

Only after binding the specific steroid, the formed steroid-receptor complexes move rapidly to the nucleus. The complex can be subsequently released from the nucleus with 0.3 or 0.4 m KCl (17-20, 22, 29-30, 33, 35-36, 41). High affinity, saturable binding between the estradiol receptor and agarose-bound basic acceptor proteins can be detected only in the presence of the specific steroid. In the absence of estradiol, or in presence of nonspecific steroids, only nonsaturable binding identical to that obtained in 0.3 to 0.5 m KCl is noted. On the other hand, the interaction of cytosol receptor with casein-agarose or with histone-agarose shows very different sensitivity to KCl and it is not modified at all by the presence of estradiol.

DNA and RNA are completely absent from the acceptor preparation used in these studies, and the acceptor activity is unaffected by digestion with nucleases (38). Binding of estradiol receptors to DNA has been found to be mediated by the presence of other, nonspecific proteins (38). In agreement with our data, Higgins et al. (29) have found that estrogen acceptors are insensitive to DNase treatment that releases up to 83% of the nuclear DNA. Instead, new acceptor sites with identical affinity appear after such treatment. This is exactly the situation described in Fig. 4, where the presence of DNA decreases the number of acceptor sites for receptor but does not modify the apparent association constant of the interaction.

An interesting observation is the increase in affinity observed after heating acceptor preparations before coupling to agarose. This effect, which is also evident at physiological temperatures, could perhaps help to explain the much faster nuclear binding of receptor that occurs in cell-free systems when the temperature is raised from 0 to 37° (23-24, 42).

Free sulphydryl groups of the receptor molecules appear to
play an important role in the receptor-acceptor interaction. These results may help to resolve the controversial role of sulfhydryl groups in the estrogen-uterus system. Despite reports to the contrary (43), we were (44) unable to find any inhibition of estradiol binding to receptors using various sulfhydryl group modifying agents. We now show that although blocking of sulfhydryl groups of the receptor molecule does not affect the binding of estradiol, it thus impairs the ability of the receptor-estradiol complex to bind to the nuclear acceptor sites. Thus, receptor sulfhydryl groups may be important in the nuclear transfer of the estradiol-receptor complex. Identical sites. Thus, receptor sulfhydryl groups may be important in the receptor-acceptor interaction. These results may help to resolve the controversial role of acceptors, the effect of purified receptor on this interaction and with relatively simple systems. The interaction of DNA with chromatin structure. The approaches described in this report could facilitate investigation of these and other possibilities expected finding if it is considered that the acceptor proteins, which in turn permit facile and specific specificity of responses to particular steroid hormones. DNA immobilized on polymers can bind efficiently the acceptor proteins, which in turn permit facile and specific binding of the receptor-hormone complex. This may be an expected finding if it is considered that the acceptor proteins were originally bound to DNA in the nucleus. According to present theories, steroid hormones modulate the genome activity of their target cells. This modulation of genome activity by estrogen could, for example, be mediated through the release, modification or addition of regulatory elements to the complex chromatin structure. The approaches described in this report could facilitate investigation of these and other possibilities with relatively simple systems. The interaction of DNA with acceptors, the effect of purified receptor on this interaction and the possible modification of acceptor structure and function after interaction with receptor are under investigation.

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