The Cytoplasmic Estradiol Receptors of Bovine Uterus

THEIR OCCURRENCE, INTERCONVERSION, AND BINDING PROPERTIES*

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SUMMARY

The interconversions and properties of uterine cytoplasmic receptor proteins have been studied. In general the properties analyzed are those pertaining to the binding of the cytosol receptors to membranes or nucleohistone. Specific cytoplasmic estradiol receptors are found in both the endometrial and myometrial layers of both mature and immature heifers. The level of receptors is highest in immature tissues. The 4 S and 9 S receptors have similar binding properties and probably represent different levels of molecular complexity of the same receptor. The 4 S and 5 S receptors have very different binding properties in that the 4 S estradiol-receptor binds membranes in a temperature dependent process requiring the presence of divalent cations, whereas the 5 S receptor binds nucleohistone in a very rapid reaction even at 4°C, in the absence of divalent cations. A small amount of 4 S receptor also binds to nucleohistone but only at elevated temperatures (25°C) and in the absence of divalent cations. A small measure of specificity for target organ material is seen in the binding of 4 S estradiol-receptor to membranes. No specificity for target tissue was noted for 5 S estradiol-receptor binding to nuclear material. The 4 S form of the estradiol-receptor complex is converted into the 5 S estradiol-receptor in a reaction which does not require the presence of chromatin, but is dependent upon an elevated temperature and the presence of estradiol. Immature uterine tissue does not contain the 5 S receptor if assayed directly, but if the tissue is preincubated with estradiol, the formation of the 5 S receptor can be demonstrated, arguing that the 4 S-5 S conversion occurs within the cell in a reaction requiring the presence of estradiol. Since after incubation the 5 S estradiol-receptor is present in the cytoplasm in vivo and can bind nuclear material instantaneously at 4°C, we have raised the possibility that the 5 S estradiol-receptor found associated with nuclear material could have been translocated and bound during the homogenization of the tissue.

It has been proposed that an early event in the mechanism of estradiol action in the uterus involves transport of the hormone to the cell nucleus in order to modify nuclear activity. It has been argued that transport occurs by way of a hormone receptor which first binds the hormone in the cytoplasm and subsequently migrates and binds to nuclear material. Similar mechanisms have been suggested for the action of progesterone in the chick oviduct (1) and dihydrostestosterone in the ventral prostate (2). The transport hypothesis has been based upon three lines of evidence. These are: (a) If immature uterine tissue is incubated with estradiol and the cells subsequently disrupted and fractionated, 5 S estradiol-receptor complexes of cytoplasmic origin can be recovered from the nuclear material (3). (b) The amount of 5 S estradiol-receptor complex bound to nuclear material increases as a function of hormone concentration (4) and time of incubation (5). The increase in nuclear bound 5 S estradiol-receptor is paralleled by a concomitant decrease in the amount of cytoplasmic receptor. (c) A number of studies have argued that cytoplasmic receptors can bind specifically to target organ chromatin in vivo assays (6).

This paper is concerned with a re-examination of the interaction between cytoplasmic estradiol-receptors and chromatin in terms of the nature and location of the binding. We have also examined the nature of the interconversion between the two major species of cytoplasmic receptors and the relative capacities of these two receptor molecules to bind chromosomal material. The results argue that cytoplasmic estradiol-receptors (particularly the 5 S receptor) could bind to chromosomal material as an artifact of isolation and that there is doubt as to the physiological significance of cytoplasmic estradiol-receptor complexes found in association with nuclear material.

MATERIALS AND METHODS

Preparation of Estradiol-Receptor Complexes—Bovine uterine tissue (40 g) was blended in 50 ml of either Buffer B (10 mM Tris-Cl, 1.5 mM EDTA, pH 7.5) or Buffer A (100 mM NaCl, 5.0 mM KCl, 3.0 mM MgCl₂, 1.5 mM CaCl₂, 10 mM Tris-Cl, pH 7.0) as described in the text. After centrifugation at 27,000 × g for 20 min the supernatant was added to the required amounts of [3H]estradiol-17β and centrifuged at 130,000 × g for 60 min. The supernatant (containing the cytoplasmic receptor) was treated with freshly prepared charcoal-dextran G-60 (7) to a final concentration of 0.25%, charcoal-0.0025% dextran. After incubation for 20 min at 4°C, the charcoal was removed by centrifugation at 10,000 × g for 5 min. In cases where a number of
different concentrations of estradiol were used for Scatchard plots, the $[^{3}H]$estradiol-17β was added to the cytosol solution after the centrifugation at 130,000 × g.

Preparation of Nuclei and Microsomes—Nuclei and microsomes from thymus, lung and immature uteri (4 to 8 week old heifers) were prepared by the procedure previously outlined (8). For experiments involving incubations in Buffer B, the nuclear pellets from the centrifugation in 2.4 M sucrose-Buffer A were washed twice with Buffer B prior to use.

In order to avoid the presence of free estradiol during incubation with nuclear material, such incubations were conducted in the presence of fresh charcoal-dextran which was added immediately before mixing cytoplasmic receptors with nuclei or membrane material. The final concentration of nuclei or microsomes was such that incubation systems contained 3 to 5 mg of DNA per ml or 3 to 5 mg of protein per ml (microsomes), respectively. After incubation, 0.2-ml aliquots were collected both before and after centrifugation at 27,000 × g for 5 min, and the samples were assayed for radioactivity. A correction for charcoal adsorption was made by a control incubation in the absence of nuclear material. The nature of such a correction is described in the legend to Fig. 2.

Dissociation of Cytoplasmic Receptor-Nuclear Complexes—After incubation with estradiol-receptor complex, the nuclei (or microsomes) were collected by centrifugation at 10,000 × g for 1 min (nuclei) or at 27,000 × g for 5 min (microsomes). The pellets were washed once in the incubation buffer and then in Buffer B. The pellets were extracted in 0.4 M KCl-3% sucrose-10 mM Tris-Cl-1.5 mM EDTA, pH 8.5 (Buffer C) for 1 hour at 4°C and layered on 5 to 20% sucrose gradient in Buffer C. Centrifugation was in a Beckman SW 55 rotor at 300,000 × g for 19 hours at 4°C. Bovine serum albumin, (β₉m = 4.5) was used as a marker in a separate tube (9). After centrifugation, fractions (10 drops) were collected and assayed for $[^{3}H]$estradiol by counting in Bray’s solution (10).

Fractionation of Nuclear Material into Membrane and Nucleohistone—After incubating nuclei and chromatin with $[^{3}H]$estradiol-receptor the material was washed twice with Buffer B and twice with distilled water to form a viscous gel which was sheared in a VirTis 45 homogenizer at 70 volts in 3 bursts of 15 s each over a 10-min span as described previously (8). After shearing, the solution was centrifuged at 150,000 × g for 90 min, which sediments ~95% of the membrane and leaves >90% of the DNA (as nucleohistone) in the supernatant. The ionic environment was adjusted to a final 10 mM Tris-Cl, 1.5 mM EDTA, pH 7.2 and the solution was centrifuged at 150,000 × g for 90 min to pellet the nucleohistone. Aliquots (0.2 ml) were taken both before and after centrifugation to determine the amount of estradiol sedimented and for DNA analysis (11).

Receptor Binding to Chromatin, Microsomes, and Nucleohistone in Absence of Estradiol—A solution containing cytoplasmic receptor (estrogen not added) in Buffer B (5 ml) was incubated at 25°C for 30 min with a dispersed nucleohistone pellet (10 mg of DNA). The nucleohistone was removed by centrifugation at 27,000 × g for 5 min at 4°C. Subsequently a fresh pellet of nucleohistone (10 mg of DNA) was added for a second incubation. After removal of the nucleohistone, the supernatant (0.6 ml) was added to increasing concentrations of $[^{3}H]$estradiol-17β and incubated at 4°C for 4 hours to obtain a Scatchard plot for an analysis of the number of cytoplasmic receptor binding sites remaining in solution. An identical procedure was followed using chromatin (8 mg of DNA per pellet) or microsomes (20 mg of membrane protein per pellet).

$[^{3}H]$Estradiol-17β Binding in Tissue Incubations—The tissue (40 g) was incubated at 37°C for 45 min in Eagle’s medium (100 ml) (12) containing $[^{3}H]$estradiol-17β at a concentration of 3.3 × 10⁻⁸ M for immature uterine tissue and 5.5 × 10⁻⁹ M for mature endometrium in diestrous. After incubation the tissue was washed three times in water (4°C) and then incubated at 4°C for 5 hours in Eagle’s medium (50:1, v/v) containing a 5000-fold excess of unlabeled estradiol. After two such incubations the uteri were washed twice in distilled water and frozen or homogenized directly in the appropriate buffer.

RESULTS

Cytoplasmic Estradiol-Receptors of Bovine Uterus—Uterine tissue was collected from immature and mature heifers and dissected into the endometrial and myometrial layers. The sedimentation coefficients of the cytoplasmic receptors from these tissues were determined by sucrose density gradient analysis after binding $[^{3}H]$estradiol at 4°C in vitro with the results shown in Fig. 1. In agreement with many workers in other systems (13, 14) we find both 4 S and 9 S receptor proteins in these systems. These gradients contain no magnesium and it is possible that at least part of the 9 S material comes from aggregation of the 4 S protein, as the 4 S receptor is the primary component of adult uterine tissue when the cytoplasmic extract is prepared in the presence of magnesium (see below).

Dissociation of Estradiol-Receptors to Nuclei in Presence of Divalent Cations—The following experiments were performed in the presence of magnesium and calcium ions which inhibit the conversion of the 4 S and 9 S receptors to the 5 S form and its subsequent binding to nuclei (vide infra). The cytoplasmic binding proteins were incubated with $[^{3}H]$estradiol and unbound hormone removed at 4°C using charcoal as described under “Materials and

![Fig. 1. Sucrose gradient analysis of estradiol receptor proteins. A, mature endometrium (□—□), mature myometrium (■—■). B, immature uteri (○—○), immature endometrium (■—■). The tissue was homogenized in Buffer B, and centrifuged at 130,000 × g for 60 min and the supernatant labeled with $[^{3}H]$estradiol. After incubation at 4°C for 2 hours, charcoal-dextran was added to a final 0.25% charcoal, incubated for 20 min, and centrifuged at 10,000 × g for 10 min. The supernatant containing bound $[^{3}H]$estradiol was sedimented on a 5 to 20% sucrose gradient in Buffer B.](http://www.jbc.org/
Methods." The hormone-receptor complexes were incubated with either nuclei or microsomal membranes from immature uterine tissue and from thymus at either 25° or 4° for various time intervals, as shown in Fig. 2. The binding of the estradiol-receptor complexes is dependent upon temperature and at 4° the extent of binding amounts to less than 10% of the input radioactivity. At 25° we see considerable binding though there is little specificity of binding and the uterine estradiol-receptor complexes can bind efficiently to both nuclei and microsomal membrane. Both nuclei and microsomes from immature uterine tissue show a small additional binding capacity, relative to nuclei or microsomes from thymus.

The data of Fig. 2 concern, at least in part, a ternary complex of cytoplasmic receptor-estradiol-nucleus (or microsomes) rather than a shift of [3H]estradiol from the cytoplasmic receptor to a nuclear receptor. This was demonstrated by isolating uterine nuclei from such an incubation and extracting the bound hormone in 0.4 M KCl. As shown in Fig. 3A the estradiol is released as the 4 S estradiol-receptor. Not all of the nucleo-associated hormone is extractable in KCl and this may reflect a low affinity binary estradiol-nuclear complex of the type discussed previously (8).

Site of 4 S Estradiol-Receptor Binding to Nuclei and Microsomes—The observation that the estradiol-receptor complex binds microsomal membranes suggested that the complex might also be binding to the membrane component of the nucleus. Accordingly, chromatin was isolated from the nuclei after incubation and fractionated into the membrane and nucleohistone components. The data shown in Table I demonstrate that the bulk of the label was associated with the membrane fraction. The various subnuclear fractions were then extracted with 0.4 M KCl and analyzed on sucrose gradients as shown in Fig. 3.

**Table I**

| Fraction | cpm/0.2 ml | μg/0.2 ml | Specific activity | % bound |
|----------|------------|-----------|------------------|---------|
| Total sheared chromatin... | 1010 | 100 | 26,600/mg of membrane protein | 50 (membrane) |
| Membrane.... | 599 | 9 | 2,427/mg of DNA | 20 (nucleohistone) |
| Nucleohistone.... | 199 | 82 | 22 (free) |

**Fig. 2.** The binding of [3H]estradiol-receptor in Buffer A to nuclei and microsomes from thymus and immature uterus. A, thymus; B, immature uterus. Binding to microsomes at 25° (●—●), microsomes at 4° (■—■), nuclei at 25° (○—○), nuclei at 4° (□—□). The nuclear and microsomal binding of [3H]estradiol-receptor involves a correction due to the absorption of free [3H]-estradiol on to charcoal (particularly at 25°) which is necessary to prevent any dissociated estradiol from binding to membranes. The amount of label bound to nuclei and charcoal which co-sediments is compared to that bound and sedimented on charcoal alone in the absence of nuclei. Subsequently the hormone bound to nuclei is obtained by simple subtraction.

**Fig. 3.** Estradiol-receptor binding to nuclear and subnuclear fractions in the presence of divalent cations. The immature uterine nuclei were labeled by incubation with [3H]estradiol-receptor in Buffer A at 25° for 60 min. A, sedimentation profile of KCl extracts from nuclei (■—■), nucleohistone (●—●), nuclear membrane (□—□), nuclei not pretreated with Buffer B prior to KCl extraction (○—○). B, KCl extracts from microsomes labeled with [3H]estradiol-receptor at 25° for 60 min in Buffer A (●—●) and in Buffer B (○—○). Microsomes were washed once and nuclei twice with Buffer B prior to KCl extraction unless otherwise stated.
An estradiol binding component of sedimentation coefficient 4 S was extracted both from whole nuclear material and from the nuclear membrane fraction, but was not obtained from the nucleohistone fraction, again confirming (a) that the 4 S estradiol-receptor is unchanged after binding nuclei in the presence of divalent cations; and (b) that it binds primarily to the membrane fraction. The data of Fig. 3B also serve to document that the estradiol-receptor complex binds to microsomal membranes in a form which is also extracted as a 4 S protein.

Conversion of 4 S Estradiol-Receptor to 5 S Estradiol-Receptor—The cytosol extract containing the 4 S binding protein was incubated in Buffer B at 25° for 30 min in the presence of estradiol. Subsequently two binding complexes of sedimentation coefficients 4 S and 5 S were observed upon sucrose gradient analysis as shown in Fig. 4A. In a control experiment we incubated cytosol receptor under identical conditions except that the hormone was omitted. The incubation mixture was cooled to 4° and [3H]estradiol added to label the receptor proteins. Upon analysis of the sucrose gradient (Fig. 4) we can detect only a single 4 S peak under these circumstances. The ability of magnesium and calcium ions to inhibit the conversion of the 4 S to the 5 S receptor is documented in Fig. 4B. That the divalent cations inhibit the forward reaction rather than activating the reverse 5 S to 4 S conversion was shown by incubating a mixture of 4 S and 5 S receptor (prepared in Buffer B) with magnesium and calcium ions and showing that the 5 S receptor did not revert to the 4 S form (Fig. 4B). Finally, the cytosol solution from the incubation at 25° for 30 min which contains both 4 S and 5 S receptors was incubated with chromatin at 4° in Buffer B for 5 min; after centrifugation the supernatant was collected and analyzed on a sucrose gradient as shown in Fig. 4A. The 5 S estradiol-receptor has been effectively removed by binding to the chromatin in 5 min at 4°, an incubation condition under which binding of the 4 S receptor to chromatin is not observed, as described above.

Thus, the 4 S receptor protein can be converted in part to the 5 S receptor in a reaction which requires elevated temperature (25°) and the presence of estradiol, but is independent of the presence of chromatin. Similar observations have been reported by Jensen (15) for the rat uterus.

Binding of 5 S Estradiol-receptor to Nuclear Material—The following experiments were conducted in a divalent cation-free medium as this facilitates the conversion of the 4 S to the 5 S form. Cytoplasmic estradiol-receptor complex in Buffer B was prepared in the usual manner. It was then incubated for 30 min at either 4° or 25° so that we expect the 4° incubation to contain only the 4 S estradiol-receptor and the 25° incubation to contain a mixture of 4 S and 5 S estradiol-receptors. Immature uterine chromatin was added to each incubation system and the incubation continued according to the following protocols: (a) 4° preincubation—4° second incubation; (b) 4° preincubation—25° second incubation; (c) 25° preincubation—4° second incubation; and (d) 25° preincubation—25° second incubation. The rate of [3H]estradiol binding to chromosomal material as a function of time is shown in Fig. 5. Material which had not been incubated at 25° at either stage in the experiment (and therefore contains only 4 S estradiol-receptor) fails to bind, as expected, since the 4 S receptor does not bind chromosomal material at 4°. Preincubation at 25° followed by binding at 25° generates maximal interaction and sucrose gradient analysis of the 0.4 M KCl extract demonstrates that both 5 S and 4 S receptors are bound under these conditions (Fig. 6). Estradiol-receptor preincubated at 25° and subsequently treated with chromosomal material at 4° shows an immediate binding which amounts to 50% of the maximum, however, the extent of binding does not increase during the second incubation at 4°. We surmise that this might be due to an initial rapid binding of the 5 S estradiol-receptor produced during the preincubation, since the remaining 4 S receptor is converted neither to 5 S nor is it itself bound at the temperature (4°) of the second incubation. This interpretation was confirmed by extracting the chromosomal material with 0.4 M KCl after a 25°/4° incubation. The distribution of [3H]estradiol is primarily in the 5 S form as shown in Fig. 6.

Binding of Receptor to Nucleohistone, Microsomes, and Chromatin in Absence of Estradiol—A cytosol solution in Buffer B was incubated at 25° for 30 min with the appropriate substrate (chromatin, nucleohistone, or microsomes) as described under "Materials and Methods." After removal of these organelles (together with associated receptor proteins) by centrifugation, the receptors was incubated with immature uterine chromatin at 4° for 5 min (-----). B, effect of magnesium ion on the receptor conversion. Receptor in Buffer B was incubated at 25° for 30 min with [3H]estradiol, brought to 4°, treated with concentrated Buffer A to obtain a final Buffer A concentration, incubated at 4° for 30 min, treated with EDTA to remove divalent cations (-----). Receptor in Buffer A (i.e. in the presence of Mg+++) was incubated at 25° for 30 min with [3H]estradiol, brought to 4°, treated with EDTA (final concentration, 0.0015 M) (-----).
Fig. 5 (left). The rate of [3H]estradiol-receptor binding to uterine chromatin. Receptor isolated in Buffer B was incubated with [3H]estradiol at either 25° or 4°, as described below, treated with charcoal at 4° for 30 min, centrifuged to remove the charcoal, and then incubated at either 25° or 4° with chromatin. A, preincubation at 4° for 2 hours, incubated at 4° with chromatin (●—●). B, preincubation at 4° for 2 hours, incubated at 25° with chromatin (○—○). C, preincubation at 25° for 30 min, incubated at 4° with chromatin (■—■). D, preincubation at 25° for 30 min, incubated at 25° with chromatin (□—□). Incubations with chromatin at 25° were performed in the presence of charcoal to assay for free [3H]estradiol generated during the incubation and appropriate corrections were applied as discussed in the legend to Fig. 2.

Fig. 6 (right). The binding of uterine chromatin to [3H]estradiol-receptor in Buffer B. The receptor solution was preincubated at 25° for 30 min with [3H]estradiol and treated with charcoal at 4° for 20 min prior to addition to chromatin. Chromatin was extracted with 0.4 M KCl after incubation and the extract analyzed on a sucrose density gradient. Chromatin was incubated with [3H]estradiol-receptor at 4° for 30 min (○—○) and at 25° for 30 min (●—●).

The number of binding sites was compiled using the Scatchard procedure (16).

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Table II
Receptor binding to subcellular organelles in absence of estradiol

|                  | No. of binding sites | Receptor bound % |
|------------------|----------------------|----------------|
| Control (25°)    |                      |                |
| Receptor left in | 24.2                 |                |
| supernatant      |                      |                |
| Receptor left in | 19.2                 | 20.7           |
| supernatant after|                      |                |
| incubation with  |                      |                |
| chromatin        |                      |                |
| Receptor left in | 13.5                 | 44.2           |
| supernatant after|                      |                |
| incubation with  |                      |                |
| microsomes       |                      |                |
| Receptor left in | 10.8                 | 55.4           |
| supernatant after|                      |                |
| incubation with  |                      |                |
| nucleohistone    |                      |                |

Effect of Divalent Cations on Binding of Estradiol-Receptor to Nuclei from Immature Uterus—The estradiol-receptor complex was prepared at 25° in Buffer B (the solution will then contain both 4 S and 5 S receptors) and subsequently magnesium and supernatants were assayed for the number of receptor binding sites remaining in the supernatant using the Scatchard procedure. The results for the binding determinations are shown in Table II. To ensure that the receptor is actually binding to the various organelles and not being destroyed in some way (e.g. by proteolysis), the chromatin pellets from the incubation were incubated with [3H]estradiol in Buffer B at 4° for 2 hours. The nuclei were then extracted in 0.4 M KCl and sedimented on a sucrose gradient. Fig. 7 shows that the receptor is indeed extracted and exists as the 4 S receptor. A control experiment, utilizing chromatin which had not been treated with receptor, does not show the presence of cytoplasmic binding proteins upon KCl extraction.

Cytoplasmic receptors were isolated in Buffer B, incubated at 25° for 30 min with [3H]estradiol and free estradiol removed with charcoal in the usual manner. The receptor solution in the appropriate buffer was incubated with nuclei at 4° for 8 min in the absence (Buffer B) (■—■) and presence (Buffer A) (□—□) of 4.5 mM MgCl2.
TABLE III
Binding of estradiol-receptor to chromatin
subfractions in absence of divalent cations

The procedure for assay of hormone binding is as described in Table I.

| Fraction                                      | Hormone-associated | DNA in pellet | Specific activity | Hormone-bound |
|-----------------------------------------------|--------------------|---------------|------------------|---------------|
| Collagen (5 ml)                                | 4.1±0.2 mg/ml      | 19.7 mg/ml    | 11%              | 31%           |
| Collagen (10 ml)                               | 7.4±0.2 mg/ml      | 19.7 mg/ml    | 44%              | 31%           |
| Collagen (50 ml)                               | 33.7±0.2 mg/ml     | 15.5 mg/ml    | 16%              | 31%           |
| Collagen (100 ml)                              | 62.7±0.2 mg/ml     | 12.9 mg/ml    | 16%              | 31%           |

Fig. 9. Sucrose gradient analysis of estradiol-receptor binding to nuclear fractions in the absence of divalent cations. Immature uterine chromatin previously incubated at 25° for 60 min with [3H]estradiol-receptor in Buffer B was washed twice with Buffer B, once with water, and then fractionated by shearing and centrifugation. Prior to sucrose gradient analysis fractions were extracted in 0.4 M KCl. Total chromatin (O--O), nucleohistone (●-●), nuclear membrane (＊-＊).

calcium were added to a final concentration of 4.5 mM. This solution was then incubated with nucleo at 4°. A control incubation was performed in the absence of divalent cations. The data of Fig. 8 show that much more of the cytoplasmic hormone receptor binds to chromosomal material in the presence of Buffer B than when divalent cations are present. This is solely due to the binding of the 5 S estradiol-receptor complex (the 4 S form will not bind at 4°) to the chromosomal material as was shown in Fig. 5.

Site of 5 S Estradiol-Receptor Binding to Chromosomal Material—
The cytoplasmic estradiol-receptor complex was prepared and incubated with chromatin in Buffer B for 60 min at 25°. The chromatin was then collected and fractionated into membrane and nucleohistone components and each was assayed for associated [3H]estradiol. The data of Table III show that in contrast to an incubation in the presence of magnesium and calcium (Table I) a substantial part of the label is now associated with the nucleohistone moiety, although a somewhat smaller portion remains associated with the membrane fraction. Total chromatin as well as the membrane and nucleohistone subfractions were extracted with 0.4 M KCl and examined by sucrose gradient techniques. As seen in Fig. 9, chromatin contains both 4 S and 5 S receptor following such an incubation. However, the 5 S-receptor is bound primarily to nucleohistone, whereas the membrane fraction is much enriched in the 4 S receptor, confirming the notion that different estradiol-receptor complexes bind to different nuclear sites. There is also relatively little binding of the 5 S receptor to microsomal membranes (see Fig. 3) as had been predicted from the binding data above.

Specificity of 5 S Estradiol-Receptor Binding to Chromosomal Material—The cytoplasmic estradiol-receptor complex was prepared in Buffer B and incubated in the presence (Buffer A) or absence (Buffer B) of magnesium ions with subcellular fractions from uterus, thymus, or lung. The last two tissues do not respond specifically to estradiol. The binding data are presented in Fig. 10. There is a small degree of specificity seen in the binding to microsomal membranes particularly in the divalent cation-containing medium (Buffer A) as shown in Fig. 10A, however, nuclear material from all three tissue binds the estradiol-receptor complex in an identical fashion independent of the ionic environment (Fig. 10B). The association rate for the binding is also the same for all three in the presence or absence of divalent cations (Fig. 10C compared to Fig. 2). The binding also appears to be nonsaturable. Extraction (0.4 M KCl) of lung and thymus chromatin after binding gives rise to 4 S and 5 S estradiol-receptor complexes (data not shown) in essentially the same proportions and quantity to that found for uterine chromatin (Fig. 9).

Presence of 5 S Estradiol-Receptor within Immature Uterine Tissue—We have demonstrated that the conversion of 4 S to 5 S receptor occurs if the cytosol solution is incubated at 25° in the presence of estradiol. We now wanted to determine whether this same conversion could occur in the cytoplasm of the immature uterine cell. Immature uteri were incubated at 37° in the presence of [3H]estradiol for 45 min. The uteri were rinsed and suspended at 4° in a 5000-fold excess of unlabeled estradiol (two changes over 10 hours) so that the radioisotope could be depleted in the interstices of the tissue and thus avoid the artifact of binding of [3H]estradiol to cytoplasmic receptors upon tissue disruption. Any estradiol-receptor binding which had occurred within the cell is essentially irreversible at 4° for 10 hours (17). The tissue was disrupted in Buffer B and the cytosol solution analyzed on a 5 to 20% sucrose gradient with the results shown in Fig. 11. Both 4 S and 5 S estradiol-receptors were present in the cytosol. In a control experiment, immature uterine tissue was incubated at 4° with estradiol. The sucrose gradient analysis of the cytosol (Fig. 11) shows that only the 4 S receptor is present. These results indicate that 5 S receptor proteins are produced in the cytoplasm, only when estradiol is present at elevated temperatures.

If the cytosol solution from the incubation at 37° is treated with chromatin at 4° for 5 min, the 5 S estradiol-receptor is almost totally removed from solution (Fig. 11). Thus, we conclude that the conversion of 4 S receptor to the 5 S form takes place within the cytoplasm of the uterine cell at 37° in the presence of estradiol and that subsequently the 5 S estradiol-receptor is available for a rapid binding to nuclear material at 4° in the absence of divalent cations.

Availability of Receptor within Mature Endometrial Cell—Cytoplasmic receptors can bind estradiol rapidly at 4° in vitro
Fig. 10. [3H]Estradiol-receptor binding to nuclei and microsomes from immature uterus, lung, and thymus in Buffer A and Buffer B. A, [3H]estradiol-receptor binding in Buffer A: immature uteri microsomes (▪▪▪▪▪), thymus microsomes (■■■■■); and in Buffer B: immature uteri microsomes (〇〇〇〇〇), thymus microsomes (□□□□□). B, the binding of [3H]estradiol-receptor as a function of concentration in Buffer B at 25°C for 60 min: immature uterine chromatin (★★★★★), lung chromatin (●●●●●), thymus chromatin (■■■■■), immature uteri microsomes (〇〇〇〇〇), lung microsomes (〇〇〇〇〇), thymus microsomes (□□□□□). C, the binding of [3H]estradiol-receptor as a function of time in Buffer B at 25°C. Nomenclature is the same as B. All binding studies were done in the presence of charcoal and corrections made as outlined in the legend of Fig. 2.

Fig. 11 (left). Sucrose gradient analysis of [3H]estradiol-receptor isolated from immature uterine tissue incubated with [3H]estradiol. Tissue was homogenized in Buffer B and the homogenate centrifuged at 150,000 x g for 60 min. The supernatant was treated to final 0.4 M KCl with 2.0 M KCl and centrifuged on 5 to 20% sucrose gradients in Buffer C. [3H]Estradiol-receptor from tissue incubated at 37°C for 45 min with [3H]estradiol (●●●●●). [3H]Estradiol-receptor from tissue incubated at 4°C for 45 min with [3H]estradiol (○○○○○). [3H]Estradiol-receptor (from 37°C tissue though the rate of dissociation at this temperature is low indeed (17). Thus, the level of estradiol-receptor complex obtained after tissue disruption (at 4°C) will reflect the actual level occurring within the cell, together with any additional binding to estradiol located within the interstices of the tissue and made available to the receptor upon tissue homogenization. That the interstitial labeled estradiol can make a sizable contribution to the level of the estradiol complex is shown in Table IV. In this experiment endometrial tissue which had been incubated at 37°C with [3H]estradiol was divided into equal fractions, one-half was washed and disrupted immediately and the other half was washed and incubated at 4°C for 10 hours in the presence of fresh medium before tissue disruption. The level of free hormone falls from 14,000 dpm per ml in the tissue which was not postincubated to 3400 dpm per ml in the tissue which was. Similarly, the level of receptor-estradiol complex falls by a comparable amount indicating that as much as 60% of the hormone receptor complex can be generated during the homogenization procedures.

That such a postincubation is sufficient for removal of interstitial hormone is documented in Fig. 12 which shows the time-dependent release of [3H]estradiol from tissue previously incubated at 37°C (which permits hormone entry into the cell) and at 4°C in which case hormone does not enter the cell and the subsequent hormone release is simply that removed from the interstitium of the tissue (18). This last point is confirmed by the lack of receptor binding after homogenization of the latter tissue in the presence of charcoal.

The molar ratio of bound estradiol to free estradiol (B:F ratio) in 27 ml of homogenate at 4°C is obtained directly from Table IV (Column B) and has a value of 1.26. Since the mass and the density (1.03 g per ml) of this tissue are known, we can compute the volume of the tissue (10.3 ml) used for the incubations. Upon a 2.7-fold dilution (for homogenization) the B:F ratio decreases by a factor of 1.48, from which we estimate that in the cell the actual molar “bound to free” ratio is 1.48 × 1.26 = 1.87 at 4°C. In addition, since the cytosol receptor has ΔH° = −18 Cal per
mole (19), it is possible to compute that the dissociation constant for hormone receptor binding shifts from 1.35 × 10⁻⁹ at 4° (the temperature of homogenization) to 1.08 × 10⁻¹⁰ m at 37°. The attendant shift in B:F ratio is directly computed from the Scatchard equation and we conclude that the molar B:F ratio within the cell at 37° should be about 2.3.

The observed molar ratio of bound estradiol relative to that which is free within the cells after extensive postincubation treatment need not necessarily reflect the ratio existing within the cell prior to tissue disruption. The molar B:F ratio would be equal to that observed posthomogenization only if the hormone and the receptor are both as free to move within the cell as they are in the homogenization medium. Certainly, the observations reported previously concerning binding of free estradiol to the nuclear membrane (9) would argue that the internal domain open to the hormone is quite extensive. However, if the receptor were compartmentalized within the cell in such a way that it could not bind estradiol, then the B:F ratio within the cell would be appreciably less than that observed in the fully dispersed homogenization medium, particularly if the blending process exposes, or releases, receptor binding capacity. If the B:F ratio within the cell prior to homogenization could be estimated, then a simple calculation would give a direct indication of the extent of compartmentalization of the receptor molecules.

Fortunately, it is relatively simple to obtain an estimate of the amount of free and bound estradiol within the cell prior to homogenization by first removing interstitial estradiol as described above and then blending the tissue in the presence of a large excess of unlabeled estradiol. This will compete with the bulk of the free labeled intracellular hormone in binding any released receptors. The amount of free and bound hormone will then be a fairly accurate reflection of the amounts in the tissue before cooling and homogenizing. If such an experiment is performed, then as seen from the data of Table IV, the B:F ratio is 0.7. This value is some 30% of that obtained in the absence of unlabeled estradiol and as such argues that in the intact cell a substantial fraction of the receptors is unavailable for interaction with estradiol, though this is not the case after tissue rupture. It is possible to calculate the amount of receptor unavailable from the following relationship:

\[
K_D = \frac{[E_1 R_1]}{[E_2][R_1]_{\text{homogenization}}} = \frac{[E_2 R_2]}{[E_2][R_1]}_{\text{cell}}
\]

where \(R_2\) is the concentration of estradiol, \(R_1\) is the receptor concentration in the homogenate, \(R_2\) is the available receptor concentration in the cell. From the experiments above which measure the B:F ratio (which is \(E_2 R_2/E_2\)), we can write:

\[
\frac{2.33}{0.7} = \frac{R_1}{R_2}
\]

and we find that \(R_2/R_1 = 0.3\) indicating that receptor molecules are extensively restricted from interacting with cellular estradiol and that only 30% of the receptor molecules are involved in such interactions at any one time.

When we refer to the presence of free estradiol in the endometrial cell, we are measuring that estradiol which is not bound to the cytoplasmic receptor. This measurement with charcoal does not preclude the existence of lower affinity sites which can bind estradiol. Charcoal binds estradiol very strongly and can therefore destroy low affinity binding, providing there is a significant dissociation rate for this binding. Therefore, free estradiol may be bound to sites of lower affinity in the cell such as membranes or enzyme systems which might be controlled by this hormone and we would not detect these using this approach.

**DISCUSSION**

Cytoplasmic estradiol-receptors are found in the uterine tissue of both mature and immature heifers. These receptors are found in good yield in both the endometrial and the myometrial layers which compose the bulk of the uterine tissue. The endometrium and the myometrium respond differently to estradiol with only the endometrium of adult cows showing a proliferative response. The uterus of very young animals shows a small hyperemic response to estrogen. As the animal grows the uterine response increases until at a stage somewhat before puberty it demonstrates the full hyperemic and hyperplastic response to hormone administration (20). The high level of cytoplasmic receptors in very immature uteri was surprising as these receptors were thought to be involved in the proliferative response (21).

It has been argued that the cytoplasmic receptors are involved in transporting hormone through the cytoplasm to the nucleus where it was thought to be involved in differential gene activation. Since we had previously shown that estradiol binds nuclear membrane with high specificity during whole tissue incuba-
We have assayed the binding of the various receptor proteins to microsomal membranes and to chromosomal material (including the nuclear membrane). The 4 S estradiol-receptor protein binds primarily to membranes in a temperature-dependent process (very slow at 4°C) and shows a small specificity for uterine microsomal material over that of thymus microsomes. Furthermore, this binding occurs even if the 4 S receptor protein is not complexed with estradiol. The interaction between the 4 S receptor and membranes is disrupted at moderately elevated ionic strength (0.4 M KCl). The 4 S receptor in media containing divalent cations (Buffer A) has no capacity to bind to nonmembraneous chromosomal material (i.e. nucleohistone). In contrast, the 5 S estradiol-receptor complex in Buffer B binds predominately to nucleohistone in a rapid reaction even at 4°C. The conversion of the 4 S estradiol-receptor (and presumably the 9 S form) to the 5 S receptor requires elevated temperature (25°C), and the presence of estradiol. It is inhibited by hyperphysiological concentrations of divalent cations. Chromatin or other nuclear material is not required for this reaction. Thus, in uterine tissue (in the presence of estradiol) it is entirely feasible that the 5 S form of the receptor might be formed in the cytoplasm, and indeed this has been shown in the present work. Since the 5 S receptor can bind chromatin instantly even at 4°C, this raises the possibility that immediately upon tissue disruption at 4°C, the 5 S estradiol-receptor complex could rapidly bind to nuclei to give an appearance of having been bound to the chromosomal material before isolation and thus generate a disturbing artifact. If no compartmentalization of receptors occurs in the cell, then conditions of elevated temperature (37°C) should facilitate the binding of 4 S estradiol-receptor to nuclear material, a subsequent KCl extract of isolated nuclei would be similar to that seen in Fig. 6 for chromatin incubated at 25°C with estradiol-receptor. Yet as has been shown previously (5, 22), practically all of the estradiol bound to immature uterine chromatin isolated from tissue previously incubated with the hormone, is bound to the 5 S estradiol-receptor. These observations argue that the 4 S receptor is sequestered in a discrete part of the cell separate from the nucleus (for instance associated with the plasma membrane). We have demonstrated in this paper that 70% of the 4 S receptor is restricted even from binding estradiol within the cell. We have therefore developed a model to explain the observations made in this paper. The model is based on the following points. (a) The 4 S receptor can bind membrane (Figs. 3 and 10 and Table 1). (b) The 5 S estradiol-receptor has a much reduced affinity for membrane (Figs. 3 and 10). (c) The 4 S to 5 S estradiol-receptor conversion does occur within the cell (Fig. 11). The model envisages the following sequence of events for the transport of estradiol through the plasma membrane (see also Fig. 13). (a) 4 S receptor binds to the plasma membrane in such a way that it can pick up hormone from the circulatory system. (b) The 4 S estradiol-receptor complex is formed. (c) The 4 S to 5 S conversion occurs. (d) The 5 S estradiol-receptor dissociates from the membrane into the interior of the cell. (e) The estradiol dissociates from the 5 S receptor. (f) 5 S receptor is reconverted to 4 S receptor. (g) 4 S receptor reassociates with the plasma membrane. (h) Estradiol now in the free form is free to associate to the high affinity site on the nuclear membrane in mature endometrium or to bind allosterically to other proteins in the cytoplasm of both mature and immature tissues. The model has the merit that it fits all the available experimental data (except point f which is an assumption) and also that it provides an explanation for the ability of the hydrophobic estradiol to penetrate rapidly into the cell. It is clearly important to know more about the 4 S and 5 S conversion such as whether it is purely a conformational shift (albeit large) or if the 4 S estradiol-receptor complex associates with another molecule to give a complex with increased mass. In this model we have neglected to consider the 5 S estradiol-receptor protein as a protein involved in transport of estradiol to the nucleus. From the observations reported in this paper we have severe reservations about such a function.

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