REVERSIBLE DISAGGREGATION OF THE CYTOSOL-ESTROGEN BINDING PROTEIN OF UTERINE CYTOSOL

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The discovery by Jensen and Jacobson that the rat uterus is capable of selective uptake and retention of estradiol-17β (E₂)¹ has led to intensive investigation of the molecular species involved in binding. It has been shown that binding activity is present in both the high-speed supernatant (cytosol) and low-speed sediment (nuclear) fractions of uterine homogenates.²⁻⁴ Enzyme digestion experiments⁵⁻⁶ have indicated that a protein is probably involved. Sucrose density gradient centrifugation of the tritiated E₂³H-cytosol complex has suggested that the bound radioactivity migrates as a single peak with a sedimentation coefficient of 9.5S.

Recently, Jensen et al.⁷,⁸ reported that rehomogenization of the nuclear pellet in 0.3 M KCl resulted in solubilization of estrogen binding activity with a sedimentation coefficient of 5S, which they termed the "nuclear receptor." The suggestion was made that E₂ first binds to a cytosol protein and then is transferred to the cell nucleus, either associated with a fragment of the cytosol binder or with a new binder, and there it may produce certain biochemical effects.

This conceptual framework was based on studies purporting to show that after incubation with native E₂³H-cytosol-complex, uterine nuclei were found to contain 5S binding material after extraction with 0.3 M KCl.

We were unable to find any 5S binding protein in rabbit uterus after incubation of uterine minces with E₂³H. It was only after the report⁵ that KCl-containing sucrose density gradients were used in preparation of the 5S component that we were able to produce this material routinely. This suggested that the high concentration of KCl might have altered the buoyant density of the estrogen binder.

In this report we present evidence demonstrating that both in rabbit and rat uterus the high-molecular-weight cytosol-estrogen binding protein is an oligomer which may be reversibly disaggregated by 0.3 M KCl into 5S components.

Technical Details.—Chemicals were reagent-grade. Estradiol-17β-6, 7⁻³H, 42.5 c/mole was obtained from the New England Nuclear Corporation and was homogenous upon thin-layer chromatography. It was used without further purification. Norite A (activated charcoal) was obtained from Matheson, Coleman, & Bell, and dextran D grade from Mann Research Biochemicals. Agarose beads (Biogel A 5 M) were obtained from Calbiochem.

Protein preparation: Rabbit uteri were obtained on the sixth day of pregnancy before implantation. They were frozen immediately in solid CO₂ and either homogenized or kept at −15°C. Uteri were homogenized at 4°C in 3 vol of ice-cold buffer A (0.01 M Tris-HCl buffer, pH 8.0, containing 0.001 M EDTA and 0.25 M sucrose) either for 30 sec in a Waring Blendor or in a glass homogenizer using ten strokes of a motor-driven Teflon pestle. The homogenate was centrifuged at 2000 × g for 10 min and the supernatant centrifuged at 95,000 × g for 90 min in a Spinco model L-2 ultracentrifuge.
The "nuclear" binder was obtained by homogenizing the low-speed pellet gently with buffer A containing 0.3 M KCl. In *in vitro* binding experiments both cytosol and "nuclear" binding activity were allowed to reach equilibrium by incubation with E₂⁻H for at least 3 hr at 0°.

Rat uterine cytosol and "nuclear" binders were prepared exactly as for the rabbit, with the exception that the Tris-HCl buffer was brought to pH 7.4.

Scintillation counting was carried out in a Packard Tri-carb liquid scintillation spectrometer model 4322 with 10 ml of toluene phosphor at an efficiency of 51% for ³H. No sample-to-sample variation in quenching was found by monitoring with an external standard.

Density-gradient ultracentrifugation was carried out at 3° by the method of Martin and Ames with sucrose gradients of 5-20% prepared either in 0.01 M Tris-HCl buffer, pH 8.0, containing 0.001 M EDTA or 0.01 M Tris-HCl buffer containing 0.001 M EDTA and 0.3 M KCl. Samples were spun for 15.5 hr at 39,000 g in an SW 50 rotor in a Spinco model L-2 ultracentrifuge, and 15-drop aliquots were collected by gravity flow. Sedimentation constants were calculated as described. Radioactivity in fractions obtained from the gradients was extracted by shaking with 10 ml toluene phosphor and decanting into counting vials after brief centrifugation to separate the organic and aqueous phases.

**Results and Discussion.**—When rabbit uterine cytosol was incubated with E₂⁻H and centrifuged in KCl- and non-KCl-containing sucrose density gradients (Fig. 1), the KCl was seen to alter the apparent molecular weight of the complex from 220,000 (10S) to 78,000 (5S) (assuming globular molecules). This change in buoyant density occurred both with cytosol and with KCl extracts, whether or not the samples were incubated in KCl prior to centrifugation. Conversely, if the E₂⁻H-cytosol complex was preincubated in 0.3 M KCl for 16 hours at 0° and run in an ordinary sucrose density gradient with or without prior dialysis, a single peak with a sedimentation constant of 10.0S was found, suggesting that the KCl effect was reversible. This was verified when the E₂⁻H-cytosol complex,
after incubation overnight in 0.3 M KCl, was subjected to gel filtration through an agarose gel column. The bound radioactivity eluted in the high-molecular-weight protein peak just as the untreated complex did (Fig. 2). When the 5S binder peak obtained from sedimentation in KCl underwent vacuum dialysis against buffer A and recentrifugation in an ordinary gradient (Fig. 3), 10S binder resulted, showing that it was reversible alteration of binder structure rather than displacement of E23H which occurred in KCl.

Furthermore, incubation of fresh rabbit uterine minces at 37° with E23H resulted only in 10S binding material when centrifuged in ordinary gradients and 5S binding material in KCl-containing gradients, suggesting that binding activity in the cell-free state was the same as that found in whole cell preparations.

Although KCl-containing gradients had a slightly higher average density than ordinary gradients, we were able to show, as illustrated in Figure 4, that γ-globul

![Figure 3](image1.png)

**Fig. 3.**—Rabbit uterine E23H-cytosol complex was sedimented in a sucrose density gradient containing 0.3 M KCl. The peak tubes (15–21) were combined, subjected to vacuum dialysis against buffer A at 0–2° for 16 hr., and rerun in an ordinary gradient.

KCl gradient, ——— ; ordinary gradient, ———.

![Figure 4](image2.png)

**Fig. 4.**—Sedimentation pattern of human γ-globulin in ordinary and KCl-containing sucrose density gradients compared to the E23H-cytosol complex.

Cytosol binder ordinary gradient, ——— ; γ-globulin ordinary gradient, ——— ; γ-globulin KCl gradient, ———.

ulin sedimented similarly in both preparations. We calculated the molecular weight of the cytosol binder to be 220,000, using a molecular weight of 177,000 for γ-globulin, verifying the previously estimated molecular weight with catalase as standard.10 Therefore, in the rabbit uterus minces and in the cell-free state, it was clear that E23H was bound principally to a substance whose state of aggregation depended on the KCl concentration of the density gradient in which it was centrifuged.

Since previous reports that distinct “nuclear” and cytosol estrogen binders existed were based on studies of rat uteri,7,8 it was of importance to extend these observations to that species. Figure 5 compares the sedimentation patterns in ordinary and KCl gradients of the complex formed upon incubation of rat uterine cytosol with E23H. The sedimentation coefficients were 10S and 5S in the two
gradients, respectively. The minor 5S binder found in the ordinary gradient has been previously noted and discussed.7

Because it was stated that the “nuclear receptor” does not bind E2H in the cell-free state,9 we studied cytosol and nuclear preparations obtained from uteri after injecting rats with E2H intraperitoneally. As illustrated in Figure 6a, cytosol preparations containing E2H bound in vivo sedimented identically to those found in the in vitro studies. Treatment of the cytosol with charcoal prior to centrifugation increased the proportion of total counts in each peak by adsorbing some free E2H. The E2H-binder complex obtained by homogenization of the 2000 X g pellet with 0.3 M KCl had similar sedimentation properties in the two gradients to the E2H-cytosol complex (Fig. 6b). A substantial degree of aggregation and loss of binding activity accompanied the desalting process. It was found that 89 percent of total uterine radioactivity was found in the cytosol or was solubilized from the “nuclear” pellet by two KCl extractions.

Therefore it can be concluded that in both rabbit and rat uterus, the 10S estrogen-binding activity found in the high-speed supernatant undergoes a reversible alteration in buoyant density in the presence of 0.3 M KCl. Furthermore, most of the binding activity found after treating the whole rat or rabbit uterine minces with E2H sedimented similarly to the cytosol binder in the ordinary and KCl gradients. For that reason and because of the possible presence of the “cytosol” estrogen binder in the “nuclear” binder preparation, which would interfere with identification of a distinct nuclear binder in KCl-containing sucrose gradients, we find it difficult to conclude that a distinct “nuclear” estrogen binder has been demonstrated.

We have used the terms “cytosol” and “nuclear” for the estrogen-binding activities as operationally defined by the manner in which they were prepared. Their chemical characteristics, intracellular location, and state of aggregation in vivo are not known.

If it is assumed that the bulk of uterine estrogen-binding activity consists of the reversibly disaggregable “cytosol” binder, then we may ask about the possible relation of this binding activity to hormone action. The kinetic data previously obtained (Table 1) suggest the presence of a single type of binding site of high affinity which was largely unsaturated in both the rat6 and rabbit10 uterus. If the data suggesting action of the hormone at the level of transcription are correct,11,12 we may ask whether the uterine estrogen-binding protein could act as a repressor. It has certain similarities to the recently described Lac repressor13 in that both are proteins and both discriminate qualitatively and quantitatively among ligands. However, the number of binding sites per cell was estimated to

![Fig. 5.—Sedimentation patterns of rat uterine E2H-cytosol complex in KCl-containing (---) and ordinary (-----) gradients.](image-url)
Fig. 6.—Sedimentation patterns of cytosol and "nuclear" estrogen binders after administration of E$_2$H in vivo.

Twenty immature female Sprague-Dawley rats were injected with 2.5 µc of E$_2$H intraperitoneally. After 4 hr, they were sacrificed and the uteri homogenized in 3 vol of 0.01 M Tris-HCl buffer, pH 7.4, containing 0.001 M EDTA and 0.25 M sucrose. Cytosol preparations and KCl extracts of the nuclear pellet were prepared as described in the text. In (a), sedimentation patterns of charcoal-treated (to remove unbound counts) and untreated aliquots of cytosol are compared to an untreated aliquot centrifuged in KCl-containing gradient. In (b), sedimentation patterns of KCl extracts of the nuclear pellet are shown. Because of their density, the KCl extracts run on ordinary gradients required either dialysis or dilution in order to layer well on the sucrose gradient.

be substantially under 100 for the Lac repressor, whereas the presence of 70,000 cytosol binding sites per cell was calculated for the pregnant rabbit uterus, of which only 16 per cent were saturated. Even allowing for multiple binding sites per molecule and derepression at several chromosomal sites, each uterine cell would still contain far too many unsaturated binding sites under conditions of maximal stimulation to be a thermodynamically sound repressor.

The cytosol binder, through its high association constant for estradiol, can extract and retain circulating estradiol. No further role for the material has been elucidated. It is conceivable that estradiol binding precedes activation of a second message within the nucleus perhaps by transport of the steroid to a more avid nuclear binder, by transfer of the whole complex to the nucleus with or without alteration, or by activating a specific adenyl cyclase.

**Table 1. Kinetic and thermodynamic parameters of the reaction of E$_2$H with the specific binder of rabbit uterine cytosol.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium constants of dissociation</td>
<td></td>
</tr>
<tr>
<td>0°C</td>
<td>0.13 picomolar</td>
</tr>
<tr>
<td>10°C</td>
<td>0.26 picomolar</td>
</tr>
<tr>
<td>23°C</td>
<td>0.98 picomolar</td>
</tr>
<tr>
<td>37°C</td>
<td>5.8 picomolar</td>
</tr>
<tr>
<td>Free energy of association</td>
<td>-16,000 cal mole$^{-1}$</td>
</tr>
<tr>
<td>Enthalpy of association</td>
<td>-20,200 cal mole$^{-1}$</td>
</tr>
<tr>
<td>Entropy of association</td>
<td>-14 cal deg$^{-1}$ mole$^{-1}$</td>
</tr>
<tr>
<td>Energy of activation</td>
<td>+14,000 cal mole$^{-1}$</td>
</tr>
</tbody>
</table>
Summary.—Sucrose density gradient analysis of estrogen-binding activity in the cytosol fraction of rabbit and rat uterus has shown that in both species the specific binding protein underwent reversible change in buoyant density in 0.3 M KCl. In the presence of the cytosol binder, a distinct nuclear binder would not have been observed in KCl gradients. Even after injection of $E_2^3$H in vivo, the high-molecular-weight binder tended to predominate in KCl extracts of the rat uterus "nuclear" fraction when centrifuged in sucrose gradients free of KCl.

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5 Toft, D., and J. Gorski, these PROCEEDINGS, 55, 1574 (1967).

6 Toft, D., G. Shymala, and J. Gorski, these PROCEEDINGS, 57, 1740 (1967).


