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COMPARISON OF THE BINDING OF [2,4,6,7-^{3}H] ESTRADIOL-17β [$E_2{}^3H$] TO THE IMMATURE RAT UTERUS UNDER *IN VIVO*, *IN VITRO* AND "CELL-FREE" CONDITIONS—II. NUCLEAR BINDING

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SUMMARY

A study of nuclear-binding of cytosol estradiol–receptor complex in immature rat uteri under *in vivo*, *in vitro* and "cell-free" experimental conditions showed that nuclear-binding occurred under different forms which were related to the state and molecular form of the receptor present in the cytosol fraction.

Analysis of the KCl soluble nuclear extracts on 0·3 M KCl containing sucrose gradients showed that under *in vivo* conditions a distinct "5S" peak was present unlike under *in vitro* and "cell-free" conditions where both "4S" and "5S" peaks were simultaneously present, incubation at higher temperature favoring a higher proportion of "5S". Under "cell-free" conditions, in the presence of EDTA, the KCl soluble nuclear extract sedimented as a "4S" peak only.

On treatment with Dextran-coated charcoal no significant difference in the relative stabilities of the KCl soluble extracts obtained under the three experimental conditions was observed. Addition of excess unlabeled estradiol under *in vitro* and "cell-free" conditions resulted in a disappearance of the bound $E_2{}^3H$ both in the "4S" and "5S" forms, both in the cytosol and the nuclear extracts, demonstrating saturability of these bindings.

These data would suggest that nuclear binding of estradiol under *in vitro* and "cell-free" conditions involve components other than that observed *in vivo*.

Although *in vitro* and "cell-free" nuclear bindings differ from that observed *in vivo*, yet activation of the cytosol by temperature promotes nuclear binding in a form approaching that observed under *in vivo* conditions.

INTRODUCTION

The binding of estradiol to the "receptor" protein in the cytosol fraction of the uterine cell and a subsequent translocation and binding of the estradiol–receptor complex to the nucleus are believed to be mediating steps, leading to the manifestation of the biological activity, exerted by the hormone at the cellular level[1–3].

We have previously reported[4] on some of the physicochemical parameters of the cytosol receptor, formed under varying experimental conditions. In order to establish if any direct and close relationship existed between the experimentally induced status of the cytosol receptor and the status of the binding to the nucleus, the following study was undertaken.

Crude nuclear pellets from uteri of immature rats, either after *in vivo* infusion of the hormone or after *in vitro* incubation of whole uteri in the presence of $E_2{}^3H$ or after "cell-free" incubation of nuclei with cytosol preincubated with $E_2{}^3H$, were extracted with buffer containing 0·3 M KCl, and the sedimentation patterns of the specifically bound $E_2{}^3H$ were compared on sucrose density gradients. These data were then compared in parallel with respective data of the corresponding cytosol–receptor complex to assess the degree of influence exerted by the state of the cytosol receptor on the binding characteristics of estradiol to the nucleus.

MATERIALS AND METHODS

I. The chemicals and animals utilized were reported earlier[4].

Buffers (a) Tris–HCl pH 7·4 (Tris, 10 mM + HCl)
(b) Tris–HCl–KCl pH 8·5 (Tris, 10 mM + HCl + KCl 0·3M)
(c) Tris–EDTA pH 7·4 (Tris, 10 mM + EDTA, 1·5 mM)
(d) Tris–EDTA–KCl pH 8·5 (Tris, 10 mM + EDTA, 1·5 mM + KCl 0·3M)

II. Preparation of the nucleus bound $E_2{}^3H$ under *in vivo*, *in vitro* and "cell-free" experimental conditions. (a) After *in vivo* infusion to 6 animals of 26 ng/h of $E_2{}^3H$ for 3 h, the animals were sacrificed and the uteri were cleaned of fat and homogenized in Tris–HCl buffer. The homogenate was centrifuged at 800 ***g*** for 10 min, and the pellet remaining after removal of the supernatant cytosol was washed in the homogenizing buffer and centrifuged again at 800 ***g*** for 10 min. This procedure was repeated twice. The washed nuclear pellet was then extracted with 0·5 ml of Tris–HCl buffer containing 0·3 M KCl. The low-speed supernatant of the tissue homogenate was centrifuged

at 135,000 *g* for 15 min to obtain the high-speed cytosol fraction.

(b) After *in vitro* incubation of whole uteri from 10 animals as described earlier[5], in the presence of 3×10^{-9} M $E_2{}^3H$ for 1–2 h either at 0–4°C or at 22°C, the uteri were washed in the homogenizing buffer and blotted with filter paper thoroughly. This procedure was repeated three times in order to remove excess $E_2{}^3H$. The washed uteri were then homogenized in adequate buffer and homogenate treated as in IIa, to obtain the crude nuclear pellet, which was extracted with 0·3 M KCl containing adequate buffer as in IIa.

(c) Uteri from 10 animals were homogenized in adequate buffer and after subcellular fractionation of the homogenate into cytosol and nuclear fractions, the nuclear pellet was washed three times in the homogenizing buffer and conserved in a small volume of the same buffer at 0–4°C. Cytosol was incubated in the presence of 3×10^{-9} M $E_2{}^3H$ either at 0–4°C or at 22°C. The nuclear pellet was then incubated under "cell-free" condition at 0–4°C, under constant agitation, in the presence of preincubated cytosol for 1 h. At the conclusion of the incubation, the mixture was centrifuged at 800 *g* for 10 min and the nuclear pellet was isolated. It was washed in the homogenizing buffer and centrifuged at 800 *g* twice and extracted with adequate buffer containing 0·3 M KCl.

III. *Assay of specific binding to the nuclear pellet.* For whole tissue and "cell-free" incubations, the non-specific binding to the nuclear fraction was determined in the following manner:

(a) Non-specific binding to cytosol was determined by incubating 2 aliquots in parallel, one in the presence of 3×10^{-9} M $E_2{}^3H$, and the other in the presence of $E_2{}^3H$ in which the addition of an excess molar (100-fold) unlabeled estradiol prevented $E_2{}^3H$ binding to specific receptor proteins with low capacity but not to the non-specific high capacity components. Specific binding due to the receptor in the cytosol was then obtained by subtracting the radioactivity remaining in the supernate after charcoal treatment[4] from that obtained from cytosol containing only $E_2{}^3H$, after similar charcoal treatment.

(b) Two aliquots of the suspension of the nuclear pellet were incubated in parallel with either cytosol preincubated with $E_2{}^3H$ alone or with $E_2{}^3H$ containing 100 fold excess of unlabeled estradiol. After separating the supernate from the nuclear pellets at the conclusion of the incubation time, the nuclear pellets were washed and extracted with buffer containing 0·3 M KCl. The total radioactivity in the KCl soluble nuclear extracts was counted. The percent of non-specific binding in the KCl extract was calculated and the specific binding was computed as described in the appendix. Similar corrections for non-specific binding were done for the Dextran-coated charcoal treated[4] KCl extract and the residual precipitate (see appendix).

IV. *Sucrose density gradient analysis.* 0·2 ml samples of either the charcoal treated cytosol or the charcoal treated or untreated nuclear KCl extract was layered on top of a 5–20% linear sucrose density gradient containing 0·3 M KCl. The gradient was centrifuged at 135,000 *g* for 16 h at 0–4°C. At the conclusion of the centrifugation, the centrifuge tubes were pierced at the bottom and 27 fractions were collected directly into counting vials and the radioactivity was determined after addition of 5 ml "Instagel" (Packard).

RESULTS

I. *Sucrose density gradients analysis of the KCl soluble nuclear extract and the corresponding cytosol estradiol–receptor.* Some typical sedimentation patterns of the KCl soluble nuclear extracts obtained under *in vivo*, *in vitro* and "cell-free" experimental conditions are shown in Figs. 1, 2 and 3. The sedimentation profiles of the corresponding cytosol estradiol–receptor complexes are shown in parallel. Figure 1 shows that under *in vivo* conditions a well-defined and single "4S" peak was present in the cytosol fraction. The KCl soluble nuclear extract sedimented as a single and distinct "5S" peak. Figure 2 shows that under *in vitro* conditions, cytosol obtained from uteri incubated at 0–4°C in the presence of $E_2{}^3H$ showed mainly a "4S" peak and a minor "5S" peak; the KCl soluble nuclear extract on the contrary showed a major "5S" peak with a shoulder in the "4S" region. Figure 3 shows that under "cell-free" conditions, cytosol incubated at 0–4°C in the presence of $E_2{}^3H$ showed a major "4S" peak and a shoulder in the "5S"

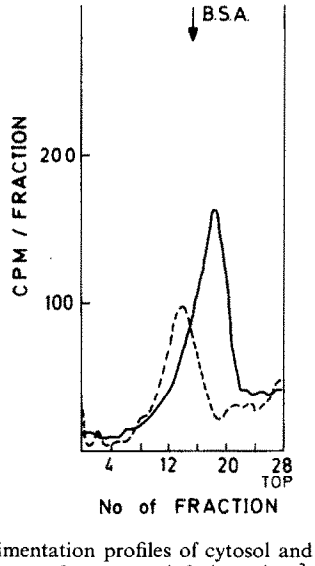

Fig. 1. Sedimentation profiles of cytosol and KCl soluble nuclear extracts after *in vivo* infusion of $E_2{}^3H$. Six immature rats were infused for 3 h with $E_2{}^3H$ at a rate of 26 ng/h. After subcellular fractionation in Tris–HCl buffer, 0·2 ml aliquots of the charcoal treated cytosol and the KCl soluble nuclear extracts were layered on top of a 5–20%, 0·3 KCl containing linear sucrose gradient in Tris–HCl buffer and centrifuged for 16 h at 135,000 *g*. ——— charcoal treated cytosol, – – – – charcoal treated KCl soluble nuclear extract.

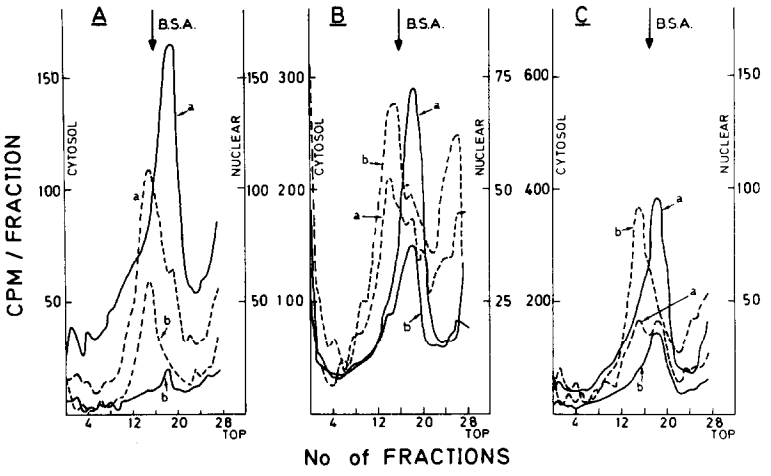

Fig. 2. Effect of duration and temperature of *in vitro* incubation of whole uteri on the subcellular bindings. Uteri from 10 immature rats were incubated in the presence of 3×10^{-9} M $E_2{}^3H$ for varying times and temperatures. After subcellular fractionation in Tris–HCl buffer, 0·2 ml aliquots of the charcoal treated cytosol fraction and of the charcoal treated KCl nuclear extract were layered on top of 5–20% 0·3 M KCl containing linear sucrose gradients, and centrifuged for 16 h at 135,000 *g*. A. Uteri incubated at 0–4°C for 2 h 15 min (a), or at 0–4°C for 2 h, followed by 15 min at 30°C (b). B. Uteri incubated at 0–4°C for 1 h 30 min (a), or at 0–4°C for 1 h, followed by 30 min at 22°C (b). C. Uteri incubated at 0–4°C for 2 h 5 min (a), or at 0–4°C for 2 h followed by 5 min at 30°C (b). ——— charcoal treated cytosol, – – – – charcoal treated nuclear KCl extract.

region. The KCl soluble nuclear extract of nuclei incubated with the above cytosol showed similar double peaks in the "4S" and "5S" regions. Dextran-coated charcoal treatment of both the cytosol and the KCl soluble nuclear extracts did not modify qualitatively the aspects of the sedimentation profiles, except for the unbound $E_2{}^3H$ on top of the gradient which was removed by charcoal.

II. *Effect of activation of the cytosol estradiol–receptor complex by temperature on the subsequent nuclear binding.* After *in vitro* incubations of whole uteri with $E_2{}^3H$ at temperature of 22°C or 30°C, similar but reduced "4S" peaks were observed in the cytosol as after incubation at 0–4°C. The KCl soluble nuclear extract however sedimented as a "5S" peak and was more important than that observed at 0–4°C incubation (Fig. 2). After "cell-free" incubation of nuclei at 0–4°C, with cytosol prepared in Tris–HCl buffer and preincubated with $E_2{}^3H$ at 22°C, the KCl soluble nuclear extract showed a diminution of the "4S" peak, and a slight increase of the "5S" peak. The corresponding cytosol showed also a shift of the peak from the "4S" to the "5S" region (Fig. 3).

III. *Influence of Tris–EDTA on the sedimentation profile of the KCl soluble nuclear extract under "cell-free" condition.* Figure 4 shows that when Tris–EDTA buffer was utilized instead of Tris–HCl for cytosol preparation, the KCl soluble nuclear extract sedimented as a distinct "4S" peak only and there was a complete absence of the "5S" peak.

IV. *Effect of excess unlabeled estradiol in the cytosol on the nuclear binding under "cell-free" condition.* Figure 5 demonstrates an absence of specific binding in the presence of excess (100-fold) unlabeled estradiol, both in the charcoal treated cytosol and the 0·3 M KCl soluble nuclear extract. Similar observations were made when cytosol was prepared either in Tris–HCl or in Tris–EDTA buffer.

V. *Stability of the specifically bound $E_2{}^3H$ in the KCl soluble nuclear extracts in the presence of Dextran-coated charcoal at 4°C.* In order to compare the relative stabilities of the $E_2{}^3H$ binding in the KCl soluble nuclear extracts obtained under the three experimental conditions, 0·2 ml aliquots of the various nuclear extracts were subjected to the Dextran-coated charcoal technique at 4°C, as described previously[4]. Table 1 shows that there was no significant difference in the relative stabilities of the $E_2{}^3H$ binding in the KCl soluble nuclear extracts under the three experimental conditions.

DISCUSSION

These comparative experimental data on the KCl soluble nuclear receptors from rat uteri, obtained under *in vivo*, *in vitro* and "cell-free" experimental conditions show that specific nuclear binding of $E_2{}^3H$ may appear under different forms which are related to the state and molecular form of the receptor present in the cytosol fraction. Nuclear binding under *in vivo* condition which is most physiological of the three conditions, gave rise to a single and well defined "5S" peak in the KCl soluble nuclear extract, irrespective of the buffer utilized to process the subcellular fractionation[5]. Under these conditions, the cytosol receptor appeared as a single "4S" peak[4,5]. Under *in vitro* experimental conditions, the presence of both "4S" and "5S" peaks were observed in the KCl soluble extract; the relative proportion of the two peaks depended on the activation by temperature, which increased the "5S" at the expense of the "4S" forms.

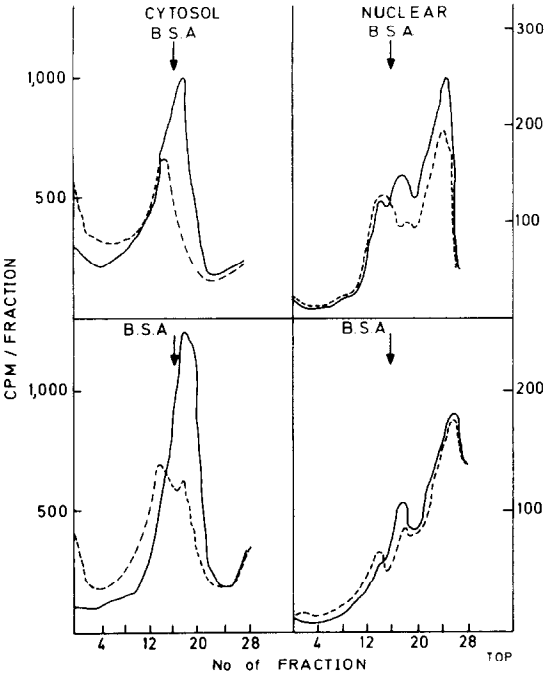

Fig. 3. Effect of activation by temperature on "cell-free" cytosol and nuclear binding. Uteri from 10 immature rats were homogenized in Tris–HCl buffer, and after subcellular fractionation, cytosol was incubated either at 0–4°C (——) or at 22°C (– – –) for 1 h in the presence of 3×10^{-9} M $E_2{}^3H$. The cytosol was then incubated at 0–4°C for 1 h in the presence of the nuclear pellet. After the incubation time, cytosol and nuclear fractions were separated and the nuclear pellet extracted with 0·3 M KCl containing Tris–HCl buffer. 0·2 ml aliquots of the charcoal treated cytosol and charcoal untreated KCl soluble nuclear extract were layered on top of 5–20% 0·3 M KCl containing linear sucrose gradients and centrifuged for 16 h at 135,000 *g*. The figure shows the results from three such experiments. Upper part left, charcoal treated cytosol and right, KCl soluble extract of the corresponding experiment. Lower part left, charcoal treated cytosol and right, KCl soluble extract of separate experiments.

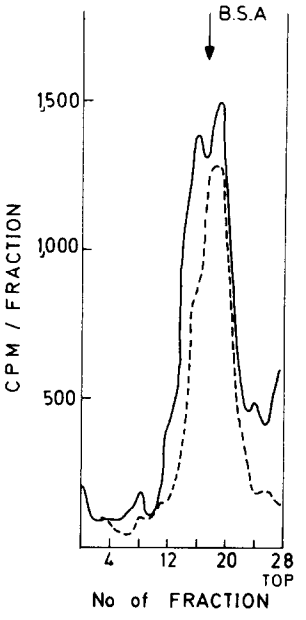

Fig. 4. Influence of EDTA on the nuclear binding. Uteri from 6 immature rats were homogenized either in Tris–HCl, or Tris–EDTA buffer, and after subcellular fractionation, the cytosols were incubated in the presence of 3×10^{-9} M $E_2{}^3H$ at 0–4°C for 2 h followed by incubation at 22°C for 1 h. The nuclear pellets were then incubated for 1 h at 0–4°C in the presence of corresponding labeled cytosols. At the conclusion of the incubation time, nuclear fractions were isolated, and extracted with the corresponding buffers containing 0·3 M KCl. After Dextran-coated charcoal treatment 0·2 ml aliquots were layered on top of 5–20% 0·3 M KCl containing linear sucrose gradients, and centrifuged for 16 h at 135,000 *g*. ——— charcoal treated KCl soluble nuclear extract: Tris–HCl buffer, – – – – charcoal treated KCl soluble nuclear extract: Tris–EDTA–buffer.

Under *in vitro* conditions "4S" and a minor "5S" peak were found in the cytosol at both temperatures[4]. After "cell-free" incubation at 4°C of nuclei with cytosol labeled with $E_2{}^3H$ at 4°C, both "4S" and "5S" peaks were present in the nuclear KCl soluble extracts. The temperature activation of the cytosol induced the expected shift of the "4S" to the "5S" form of the cytosol receptor, both of them being then present in the incubation medium[1,4,6,7]. Similarly, in the extracts of nuclei incubated with temperature activated cytosol the "4S" peak was decreased and there was a net although not equivalent increase in the "5S"

Table 1. Binding stability of $E_2{}^3H$ to the nuclear KCl extract

EXPERIMENTAL CONDITION	BUFFER UTILIZED	TIME OR TEMP. OF PREINCUBATION OF CYTOSOL OR WHOLE TISSUE	N° OF EXPTS.	% REMAINING IN SUPERNATANT AFTER DEXTRAN-COATED CHARCOAL TREATMENT AT 4°C. OF NUCLEAR KCl EXTRACT	PROT. CONC. OF CYTOSOL
In-Vivo	TRIS-HCl		2	34 - 22	1.2 mg/ml
In-Vitro	TRIS-HCl	2 h 15 min at 4°C	3	22 - 20 - 18	3.2 mg/ml
In-Vitro	TRIS-HCl	2 h at 4°C + 15' at 22°C	1	24	3.2 mg/ml
In-Vitro	TRIS-HCl	2 h at 4°C + 15' at 30°C	1	14	2.4 mg/ml
In-Vitro	TRIS-HCl	2 h at 4°C + 5' at 30°C	1	21	2.4 mg/ml
* Cell-Free	TRIS-EDTA	1 h 5' at 4°C	2	22 - 25	5.6 mg/ml
Cell-Free	TRIS-EDTA	1 h at 4°C + 5' at 22°C	2	18 - 28	4.8 mg/ml
Cell-Free	TRIS-EDTA	1 h at 4°C + 30' at 22°C	1	31	5.2 mg/ml
Cell-Free	TRIS-EDTA	2 h at 4°C + 1 h at 22°C	1	32	6.0 mg/ml
Cell-Free	TRIS-HCl	2 h at 4°C + 1 h at 22°C	2	41 - 26	5.8 mg/ml

* Under "cell-free" conditions, preincubated cytosol was incubated with the nuclear pellet for 1 h at 4°C.

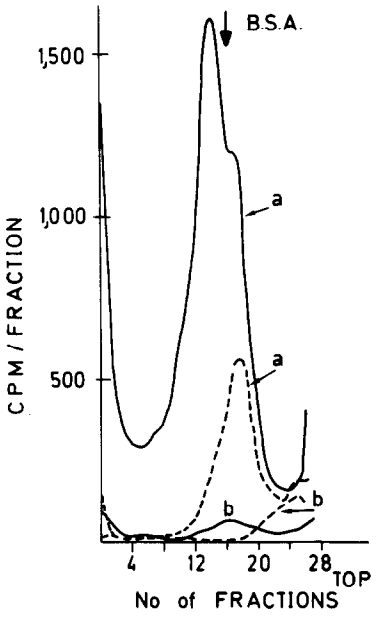

Fig. 5. "Cell-free" incubation of nuclear pellet with cytosol preincubated with $E_2{}^3H$ alone or with $E_2{}^3H$ containing excess unlabeled estradiol. Cytosol prepared in Tris–EDTA buffer, from 8 immature rat uteri were incubated with either 2×10^{-9} M $E_2{}^3H$ (a) or 2×10^{-9} M $E_2{}^3H$ containing 100-fold excess of unlabeled estradiol (b) for 1 h at 22°C. These cytosols were then incubated with the nuclear pellets for 1 h at 0–4°C. At the conclusion of the incubation, the nuclear pellets were isolated and extracted with 0·3 M KCl containing Tris–EDTA buffer. 0·2 ml aliquots of the cytosols and of the KCl extracts were layered on top of 5–20% 0·3 M KCl containing linear sucrose gradients and centrifuged for 16 h at 135,000 *g*. ——— charcoal treated cytosol, – – – – charcoal treated KCl soluble nuclear extract.

peak. Hence, under "cell-free" conditions some parallelism was found between the physical state of the receptor in the cytosol and in the KCl nuclear extract, depending on the experimental conditions of "activation" of the cytosol receptor. The importance of these conditions were further stressed by the observation that temperature activated cytosol, prepared in EDTA containing buffer, gave rise to only a "4S" peak in the KCl extractable nuclear fraction after "cell-free" incubation. EDTA has been shown to inhibit the temperature activation of the cytosol receptor[7] and the binding of the cytosol estradiol–receptor complex to uterine chromatin[8].

On treatment by Dextran-coated charcoal, no significant difference was observed in the relative stabilities of the KCl extracts under the three experimental conditions. Chase experiments *in vivo* had shown earlier that bound estradiol in cytosol and nucleus was exchangeable[5,9]. Similarly addition of excess unlabeled estradiol under *in vitro* (unpublished) and "cell-free" conditions resulted in a disappearance of the bound $E_2{}^3H$ both in the "4S" and "5S" form in the cytosol and nuclear extracts. Hence, both the "4S" and the "5S" nuclear KCl soluble binding components, which are present in "cell-free" or *in vitro* experimental situations, disclosed similar characteristics of low capacity and high affinity. The exact status of the nuclear binding under these conditions would not appear either from a simple KCl extract, eventually corrected for non-specific binding, as described in the appendix, or after removal of the loosely bound ligand with charcoal. Sucrose density gradient however would permit to characterise the form of the nuclear binding under a particular experimental situation.

These data indeed suggest that nuclear binding of estradiol under *in vitro* and "cell-free" experimental conditions involve components other than the ones observed *in vivo*. Musliner *et al.*[10] who also observed a simultaneous presence of "4S" and "5S" components in the nuclear extracts after "cell-free" incubation and Rochefort *et al.*[11] have advanced that in a "cell-free" system, part of the hormone accumulated by the nuclear fraction may be bound in a fashion different from that observed in nuclei of uteri exposed to estrogens *in vivo*. The experiments reported by Shepherd *et al.*[12] show that the amount of estradiol–receptor complex bound *in vitro* after preliminary *in vivo* binding is not affected by the latter, suggesting a large excess of nuclear acceptor sites over the cytosol receptor sites. However, the acceptor sites could be different in these two experimental conditions, as proposed by Higgins *et al.*[13].

It can be concluded that although "cell-free" and *in vitro* nuclear bindings differ from that observed *in vivo*, nevertheless, activation of the cytosol receptor by temperature promotes conformational modification of the receptor, and enhances binding to the nuclear component(s) in a form which approaches that observed under physiological conditions of *in vivo* infusion of the hormone.

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APPENDIX

Correction for non-specific binding.

	$E_2{}^3H$		$E_2{}^3H + E_2$	
Cytosol	d.p.m.	Numerical example	d.p.m.	Numerical example
Total	A	189839	A′	189839
Charcoal treated supernate	B	83298	B′	9520
Specific Binding	B − B′	73778	—	—
Non-specific Binding	[A − (B − B′)]	116061	—	—
Nuclear				
KCl Total	C	26688	C′	14449
% Non-specific Binding	$D = \frac{C' \times 100}{A'}$	7.6	—	—
Non-specific Binding	$E = \frac{D \times [A - (B - B')]}{100}$	8834	—	—
Specific Binding	F = C − E	17855	—	—
Charcoal treated KCl extract				
Total	G	4810	G′	389
% Non-specific Binding	$H = \frac{G' \times 100}{C'}$	2.69	—	—
Non-specific Binding	$I = \frac{H \times E}{100}$	237		
Specific Binding	$J = G - I$	4572		
Nuclear precipitate				
Total	L	9455	L′	7932
% Non-specific Binding	$M = \frac{L' \times 100}{A'}$	4.18	—	—
Non-specific Binding	$N = \frac{M \times [A - (B - B')]}{100}$	4851		
Specific Binding	O = L − N	4603		