

## NUCLEAR BINDING OF THE ESTROGEN RECEPTOR: HETEROGENEITY OF SITES AND UTEROTROPIC RESPONSE

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### SUMMARY

Two kinds of estradiol binding sites are present in purified nuclei from the rat uterus following estradiol injection. One of these sites (type I) corresponds to the well-known estrogen receptor which undergoes translocation from the cytoplasm to the nucleus. The second site (type II) is not translocated from the cytoplasm to the nucleus, however, estradiol treatment does stimulate an increased number of these sites. Type II sites are observed in purified nuclei and chromatin isolated from the uterus but not from non-target tissues such as the spleen and diaphragm. Thus an elevation in the levels of type II sites appear to be a specific nuclear response of the rat uterus to estradiol. Saturation analysis over a wide range of [<sup>3</sup>H]-estradiol concentrations produces a binding curve for type II sites which is sigmoidal and hence no accurate estimation of the dissociation constant is possible. The binding of [<sup>3</sup>H]-estradiol to nuclear type II sites is inhibited by estradiol and diethylstilbestrol but not by progesterone, testosterone, or corticosterone.

Extraction of nuclei isolated from estrogen treated rat uteri with KCl provides a complex picture. Direct labeling of nuclear estrogen receptors either by in vivo injection or in vitro incubation of intact uteri with [<sup>3</sup>H]-estradiol measures only a fraction of the specific estrogen binding sites associated with the nuclear pellet following 0.4 M KCl extraction. These sites are more accurately determined by performing saturation analysis over a wide range of [<sup>3</sup>H]-estradiol concentrations by exchange which measures specific estrogen binding sites, not [<sup>3</sup>H]-steroid.

Saturation analysis of estradiol binding to KCl extracted nuclei when performed by exchange, with appropriate corrections for type II binding, reveals that approximately 1000-2000 receptors per nucleus are resistant to KCl extraction 1 hr after administration. The same numbers of type I sites display long-term nuclear retention.

A single injection of estradiol results in long term (>6 h) retention of type I sites, rapid and sustained elevations (1-72h) in type II sites and true uterine growth (uterine wet weight at 24-48 h). Estriol injection caused a rapid increase in nuclear type I sites which was not accompanied by an increase in type II sites and no true uterine growth occurred. Administration of estriol or estradiol as a pellet implant, which causes continuous occupancy of type I sites, increases the quantity of nuclear type II sites and stimulates true uterine growth. Therefore, we conclude that elevated levels of nuclear type II sites correlate with the long term uterotrophic response to estrogenic hormones.

Although we do not understand the function of this second class of binding sites it is possible that the type II sites represent a major component in the mechanism by which estrogens stimulate growth of the uterus.

#### INTRODUCTION

The interaction of estrogen receptor hormone complexes with the nuclei of target tissues appears to be important in the ultimate expression of the action of the hormone. It is generally felt that the binding or receptor hormone complexes to the nucleus occurs through some acceptor mechanism which is probably associated with chromatin (see 1-4 for review). We have suggested that the numbers of acceptor sites in uterine nuclei are limited, approximately 1000-2000 sites/nucleus, and that receptor estradiol complexes must remain associated with these acceptor sites for an extended period of time in order to cause true uterine growth (5-7). This concept is supported by a previous report from our laboratory in which a single salt extraction of uterine nuclei and the [<sup>3</sup>H]-estradiol exchange assay were used to measure tightly bound receptor estrogen complexes. Our initial observations using this technique indicated that the number of hormone receptor sites which were not solubilized by high salt was equal to that number of sites which exhibit long term nuclear retention and stimulate true growth of the uterus. It was proposed that these sites represent receptor hormone complexes bound to nuclear acceptor sites (7).

Recently, several reports have both confirmed and contradicted our earlier results. For instance, Juliano and Stancel (8) found no evidence for a class of receptors which were specifically bound to acceptors and thus preferentially retained in uterine

nuclei following estradiol administration. Similarly, Traish et al.(9,10) suggested that salt extraction could not discriminate between receptor hormone complexes bound to nuclear acceptor or non-acceptor sites. Their data was interpreted to indicate that the salt insoluble sites may represent receptor hormone complexes physically entrapped in the gelatinous salt-extracted nuclear pellets. In contrast, Ruh and Baudendistal (11) have found the presence of specific salt-resistant sites in the rat uterus following estrogen administration. This work has also been confirmed by Barrack et al. (12) who point out the differences in these studies may be largely accounted for by differences in methodology, particularly the use of the [<sup>3</sup>H]-estradiol exchange assay to measure nuclear associated receptors as compared to direct injections of [<sup>3</sup>H]-estradiol.

The forementioned results are further complicated by recent reports from this laboratory which described a second class of estrogen specific binding sites in the nuclear fraction of uteri from estrogen treated rats (13,14). These sites, which we refer to as nuclear type II, are not translocated from the cytoplasm as in the case of the estrogen receptor (type I). However, the quantity of type II sites increases following estrogen administration. This second class of sites remains elevated for extended periods of time following estrogen administration and are present in much larger quantities than are type I sites (13), and consequently make interpretation of nuclear binding data extremely difficult. In this paper we have extended our observations on secondary estrogen binding sites in uterine nuclei and have examined their influence on the interpretation of results obtained by salt extraction. In addition, the relationships between nuclear retention of type I sites, elevations in nuclear type II sites and the long term uterotrophic response to estrogenic hormones have been examined.

#### MATERIALS AND METHODS

Animals: Immature (21-25 days old) or mature-ovariectomized (50-60 days old) Sprague Dawley rats were utilized in all experiments. Immature rats were injected subcutaneously with 2.5 µg estradiol which was dissolved in 0.9% NaCl-2% ethanol. Mature-ovariectomized rats received single injections (10 µg) of estradiol or estriol in the same vehicle. Paraffin pellets containing 10% (w/w) estradiol or estriol were prepared and administered to the mature-ovariectomized animals as previously described (15,16). The hormone containing pellets weighed 8-10 mg. The rats were sacrificed by cervical dislocation at indicated times following hormone administration and the uteri were rapidly removed, stripped of adhering fat, weighed and placed into ice-cold 0.9% NaCl.

Nuclear isolation:

Crude nuclei were isolated by homogenization of uteri in an all glass Duaall homogenizer (Kontes Glass) equipped with a motor driven pestle. Uteri were homogenized in TE buffer (0.01 M Tris-HCl, 0.0015 M EDTA, pH 7.4 at room temperature) at a concentration of 2 uteri/ml. The nuclear pellet was collected by centrifugation at 800 x g for 20 min. and washed in the same buffer 3 times by resuspension and centrifugation. The final pellet was suspended in TE buffer to give a uterine equivalent of 25 mgs fresh weight/ml. Purified nuclei were isolated by one of three procedures. Most experiments employed nuclei isolated by the glycerol-Triton X-100 procedure of Schibler and Weber (17) with the following modifications: the glycerol concentration in the homogenization buffer was increased from 30% to 50% and the temperature of samples was maintained at 0° rather than at -20°. The other procedures employed were the hexylene glycol procedure as previously described (18) or the sucrose method of Blobel and Potter (19). In all cases uteri were homogenized in approximately 10 mls of homogenization medium/gram tissue with the use of a polytron PT-10 (Brinkman Instruments) at a medium speed for three 15 sec bursts with intermittent cooling. All subsequent resuspensions of nuclear pellets were accomplished with the use of a Dounce homogenizer (Kontes Glass) equipped with a loose-fitting pestle. Final nuclear pellets were resuspended in TGM (0.01 M Tris-HCl, 0.002 M MgCl<sub>2</sub>, 25% glycerol, v/v, pH 7.4 at room temperature) buffer to a final concentration equivalent to 25 mgs of fresh tissue/ml of buffer. Diaphragm and spleen nuclei were purified by the glycerol-Triton X-100 procedure as described above.

Chromatin isolation:

All steps of the isolation procedure were performed at 0-4°C. A modified version of the procedure of Spelsberg *et al.*, (20) was used for chromatin isolation. Uteri were drained, weighed and minced finely with iris scissors. Ten to twelve volumes of 0.5 M Sucrose-TKM (0.01 M Tris-HCl pH 7.4, 0.025 M KC1, 0.002 M MgCl<sub>2</sub>) were added to the minced tissue. The tissue was homogenized with a Polytron PT-10 at a medium rheostat setting until no tissue clumps were visible. The homogenate was filtered through 2 layers of organza (100 mesh) into a pre-cooled glass homogenizer equipped with a tight-fitting teflon pestle. The filtrate was further homogenized by 6-10 strokes of the motor-driven pestle. Nuclei were collected by centrifugation at 10,000 x g for 10 min. Nuclear pellets were resuspended in 1.8 M sucrose-TKM and centrifuged at 13,000 x g for 45 min. The resultant pellet was resuspended in 0.5 M Sucrose-TKM + 0.2% (v/v) Triton X-100. The pellet was collected by centrifugation at 10,000 x g for 10 min. The final nuclear pellet was washed three times by resuspension in 80 mM NaCl, 20 mM Na<sub>2</sub>EDTA

(pH 6.3) followed by centrifugation at 2000 x g for 10 min. The washed nuclear pellet was resuspended in 50 ml of 0.01 x SSC (1 X SSC = 0.15 M NaCl, 0.015 M NaCitrate pH 7.0) and held on ice until >95% of nuclei had lysed as judged by phase contrast microscopy. The chromatin was collected by centrifugation at 10,000 x g for 10 min. and resuspended in TE buffer to a final concentration equivalent to 25 mg tissue fresh weight/ml.

#### KCl extraction:

The volume of the nuclear pellet to be extracted was visually estimated by comparison with known volumes in identical tubes and an equal volume of 0.8 M KCl-TE was added. The nuclear pellet was dispersed by vortexing and 0.4 M KCl-TE was added to give a uterine concentration equivalent to 125 mgs uterine fresh weight/ml. Extraction was carried out at 0° for 30 min. with vortexing every 5 mins. At the end of the extraction, the suspension was centrifuged at 800 g for 10 mins. The supernatant was removed and centrifuged at 25,000 g for 20 mins. while the pellet was washed 3 times with TE buffer by resuspension and centrifugation. The washed pellet and the supernatant were both resuspended in TE buffer in a volume equivalent to 25 mgs tissue fresh weight/ml.

#### In vivo injection of [<sup>3</sup>H]-estradiol:

Immature female rats were injected with 0.1 µg of [<sup>3</sup>H]-estradiol. Four hrs. after injection, animals were sacrificed, uteri removed and crude nuclear pellets prepared as described above. Aliquots of nuclear pellets were either extracted at 30° for 30 mins. with 1 ml of absolute ethanol, subjected to [<sup>3</sup>H]-estradiol exchange at a single concentration of 10 nM [<sup>3</sup>H]-estradiol or extracted with KCl as described above. Aliquots of the KCl extracted pellets were either subjected to direct ethanol extraction or to [<sup>3</sup>H]-estradiol exchange as described above.

#### In vitro labeling of intact uteri:

Untreated uteri were removed from animals and placed into warmed, oxygenated Eagle's minimal essential medium at a concentration of 2 uteri/ml. The medium contained a final concentration of 20 nM [<sup>3</sup>H]-estradiol plus 2 µM DES<sup>1</sup>. Uteri were incubated with shaking at 37° for 120 min. The uteri were rinsed with cold saline and crude nuclear pellets prepared. The nuclei were either subjected to [<sup>3</sup>H]-estradiol exchange, ethanol extraction, or KCl extraction as described above.

#### Saturation analysis:

Saturation analysis of nuclear estrogen binding sites was

performed by the [<sup>3</sup>H]-estradiol exchange assay in the presence of [<sup>3</sup>H]-estradiol in final concentrations ranging from 0.4-40.0 nM. 250  $\mu$ ls of resuspended nuclei or chromatin were added to two series of tubes, one of which contained [<sup>3</sup>H]-estradiol in 100  $\mu$ ls of TE buffer; the other was identical except it contained a 100-fold molar excess of DES. In competition experiments the DES was replaced with other steroid hormones as indicated. Tubes were generally incubated at 37° for 30 min. At the end of the incubation period, 1.0 ml of ice-cold TE buffer was added and the nuclei or chromatin pelleted by centrifugation at 2000 x g for 10 mins. Nuclear or chromatin pellets were washed 3 times with cold TE by resuspension and centrifugation. The final washed pellet was drained well and extracted at 30° for 30 min. with 1.0 ml of absolute ethanol. The extract was centrifuged at 2000 x g for 10 mins. and the ethanol extract counted in 5.0 mls of toluene based scintillation fluid at 35% efficiency.

#### Hydroxylapatite assay:

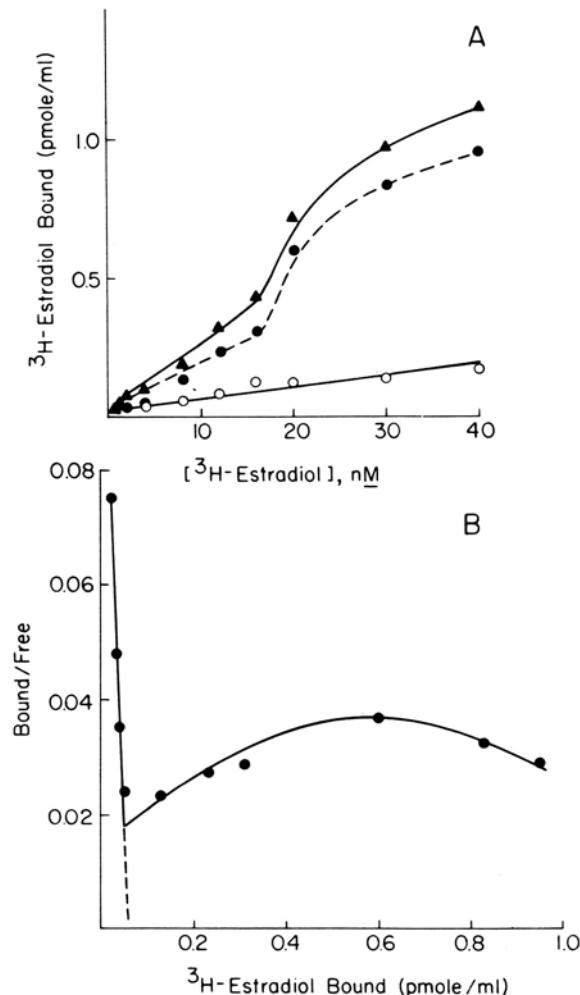
Saturation assays on the KCl extracted material was performed using the hydroxylapatite assay as previously described (14).

#### Materials:

DES was obtained from Sigma and 2,4,6,7[<sup>3</sup>H]-estradiol (Sp. Act. 90-115 Ci/mmol) from New England Nuclear. Biogel HT hydroxylapatite was from BioRad Labs; Eagle's minimal essential medium was from Gibco, and Triton X-100 was from Eastman. All other reagents were of highest available grade.

#### RESULTS

Nuclear Type I and Type II Estrogen Binding Sites. Previous results from this laboratory have demonstrated the presence of two specific classes of estrogen binding sites in crude rat uterine nuclei (3). One of these, which we refer to as nuclear type I, is the estrogen receptor while the other class, nuclear type II, is a entity distinct both from the receptor and cytoplasmic type II sites (13,14). Since our initial experiments were performed on crude nuclei the possibility existed that nuclear type II sites might be a result of cytoplasmic or serum contamination of the nuclear preparations. To circumvent this problem, saturation analysis was performed by the [<sup>3</sup>H]-estradiol exchange assay on nuclei purified by three different procedures. A representative result of these studies is presented in Fig. 1. In this study, nuclei were purified by the glycerol-Triton X-100 method of Schibler and Weber (17) with the modifications described above. The saturation curve is very similar to the one obtained when crude uterine nuclei are used in the same type of study. The

Figure 1

Saturation analysis of estrogen binding in purified rat uterine nuclei isolated by the glycerol-Triton X-100 method as described in Methods. The data presented as specific binding (●—●) which is determined by subtracting the quantity of [ $^3\text{H}$ ]-estradiol bound in the presence of DES (nonspecific binding, ○—○) from that quantity bound in its absence (total binding, ▲—▲). Scatchard analysis of the data in Fig. 1A.

two components which make up this curve are clearly resolved by Scatchard analysis (Fig. 1B). The first component, which is defined by the straight line, represents the type I receptor which has been translocated from the cytoplasm to the nucleus. The second component, which is defined by the curved line, represents type II sites. Similar results are obtained with uterine nuclei purified by either of the three methods employed in this study.

Although it is not possible to make an accurate determination of the number of type I sites from these data, an estimate can be made by extrapolating the straight line in Fig. 1B to the X-axis (dashed line, Fig. 1B). This number corresponds to approximately 2000 sites/cell and actually represents a slight overestimate due to the influence of type II sites on the measurement of type I sites. Type II sites may be estimated from the saturation curve, however, the estimation of the number and binding parameters of type II sites are necessarily only approximate. In this case the analysis yields values of  $n = 13,000$  sites/cell with a half saturation value of  $\sim 30\text{nM}$ . (For more details concerning the estimation of these parameters see Eriksson *et al.*, (13) and Discussion).

The results presented above, and the observation that two binding components were observed by saturation analysis of purified uterine chromatin (Fig. 2), strongly suggest the elevations in type II sites are a specific nuclear response to estrogen rather than the result of cytoplasmic or serum contamination of nuclear preparations.

To further examine the role of estrogen administration on accumulation of nuclear type II sites, nuclei were purified at various times after estrogen administration and saturation analysis performed. Nuclei isolated from uteri of control animals do not contain either nuclear type I or II sites in significant quantities (Fig. 3a). One hour after an estradiol injection translocation of the estrogen receptor from the cytosol to the nucleus was measurable (0.5 pmoles type I). In addition, a large quantity of nuclear type II sites could be measured (1.5 pmole type II, Fig. 3B); six hours after the hormone injection approximately 0.2 and 0.9 pmoles of type I and type II sites respectively are present (Fig. 3C); while twenty-four hours after a single estradiol injection, the estrogen receptor is no longer present in the nucleus, while nuclear type II sites remain elevated (0.5 pmoles, Fig. 3D).

The possibility existed that nuclear type II sites were a general mechanism to bind steroid hormones and inactivate them. If this were the case, type II sites would be expected to bind all steroid hormones equally well. To examine this question, nuclei were prepared from uteri of estrogen treated animals. These nuclei

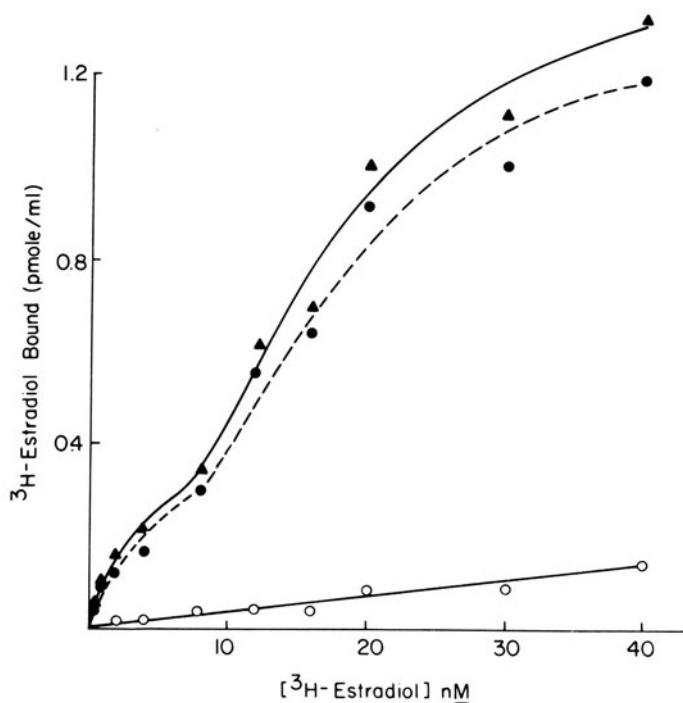


Figure 2

Saturation analysis of estrogen binding in isolated immature rat uterine chromatin. Total binding  $\blacktriangle$ — $\blacktriangle$ ; nonspecific binding  $\circ$ — $\circ$ ; specific binding,  $\bullet$ — $\bullet$ .

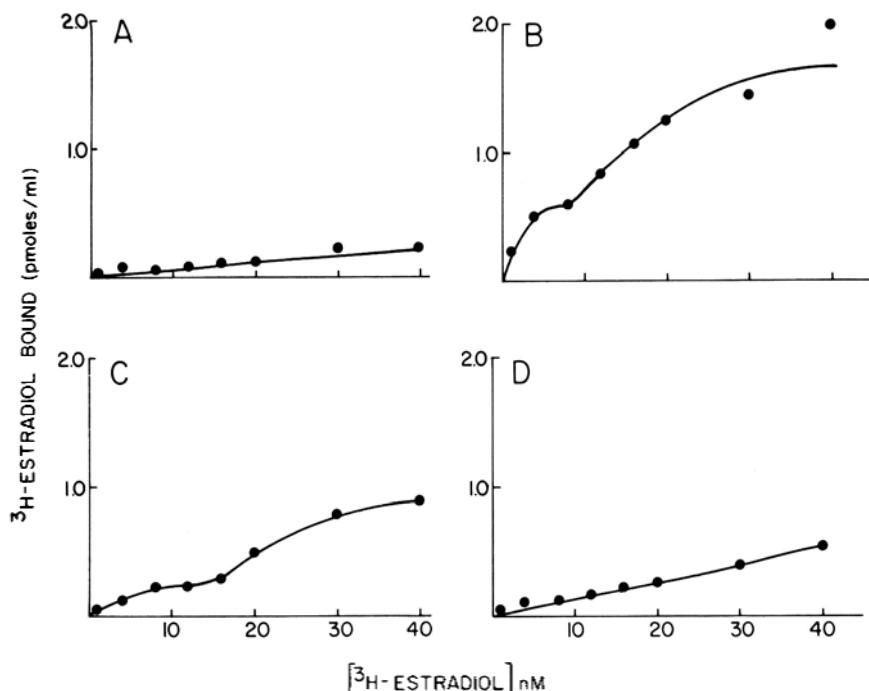


Figure 3

Effects of time administration of estrogen on saturation analysis of specific estradiol binding in purified nuclei. Immature rats were sacrificed at various times following hormone administration. A. Saline control; B. Estradiol, one hr.; C. Estradiol 6 hr.; D. Estradiol, 24 hr. All data represent specific binding.

were used in [<sup>3</sup>H]-estradiol exchange assays using a 100-fold molar excess of various unlabeled steroid hormones. The results presented in Table I demonstrate that only diethylstilbestrol competed for [<sup>3</sup>H]-estradiol binding while progesterone, corticosterone, and testosterone failed to compete.

If nuclear type II sites are involved in the mechanism of action of estrogen, they should be found only in estrogen sensitive cells and not in non-target cells. To examine this possibility saturation analysis was performed by the [<sup>3</sup>H]-estradiol exchange assay on nuclei isolated from various estrogen target or non-target tissues of rats injected with estradiol prior to sacrifice. A typical result of such studies are shown in Fig. 4. As can be seen no specific estrogen binding is observed in nuclei isolated from spleen, diaphragm or in serum over a wide range of [<sup>3</sup>H]-estradiol concentrations. However, nuclear type II sites are present in other estrogen target cells such as in the mammary gland and hormone dependent mammary tumors (Watson and Clark, unpublished). These results demonstrate that the nuclear type II sites are specific for estrogen responsive tissues.

TABLE I

EFFECT OF VARIOUS STEROID HORMONES ON [<sup>3</sup>H]-ESTRADIOL BINDING TO SPECIFIC NUCLEAR BINDING SITES.

Purified uterine nuclei were incubated at 37° for 30 min in the presence of 20 or 40 nM [<sup>3</sup>H]-estradiol + a 100-fold molar excess of the indicated unlabeled compounds. At the end of the incubation nuclear pellets were washed 3 times with ice-cold TE buffer and radioactivity associated with the pellets was determined by extraction with absolute ethanol followed by scintillation counting.

Competitor	20 nM [ <sup>3</sup> H]-estradiol, pmoles/ml	40 nM [ <sup>3</sup> H]-estradiol, pmole/ml
Buffer	2.60	4.10
Diethylstilbestrol	0.75	1.60
Progesterone	2.15	4.00
Corticosterone	2.85	4.15
Testosterone	2.85	4.15

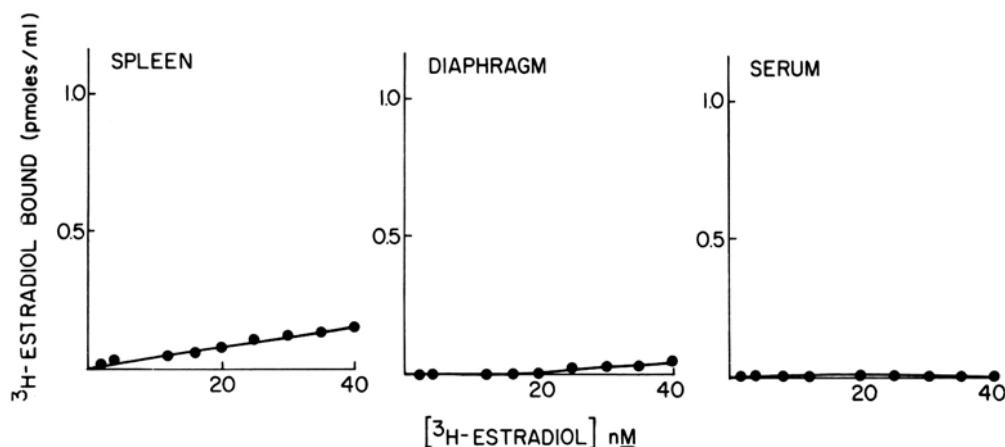


Figure 4

Saturation analysis of estrogen binding to non-target nuclei or to serum. Nuclei were purified from the spleen and diaphragm of immature rats injected with 2.5  $\mu\text{g}$  of estradiol 1 hr. prior to sacrifice. Saturation analysis for estradiol binding was performed by the HAP absorption assay as described in Methods. All data represent specific binding.

Effects of Salt Extraction on the Levels of Nuclear Type I and Type II Estrogen Binding Sites. The presence of at least two specific binding sites for estradiol in uterine nuclei of the immature rat following hormone administration is important at a number of levels and obviously complicates interpretation of data obtained by salt extraction. Since we had previously used salt extraction to measure tightly bound type I sites, it was necessary to re-evaluate this method. In addition, since the validity of this procedure has been questioned, we reinvestigated the entire concept of salt-insoluble receptors.

Uterine nuclei isolated from animals injected *in vivo* (Table II) or incubated *in vitro* (Table III) contain specific estrogen binding sites which may be measured by either direct extraction of nuclei with ethanol and counting the extract or by the [<sup>3</sup>H]-estradiol exchange assay. Extraction of the nuclear pellets with 0.4 M KCl removes a large portion of the specifically bound [<sup>3</sup>H]-estradiol (Table II and III, 0.4 M KCl extracted nuclei, direct counting). The radioactivity released by this extraction appears to be both free hormone as well as receptor-hormone complexes (Table II and III, 0.4 M KCl extract, HAP assays). Only a portion of the [<sup>3</sup>H]-estradiol bound to nuclei following either *in vivo* or *in vitro* labeling remains bound following 0.4 M KCl extraction. However, when the KCl insoluble fraction is subjected to [<sup>3</sup>H]-estradiol exchange assays it is found that a number of specific estrogen binding sites are still associated with the residual nuclear fraction which were not measured by direct labeling. The most likely explanation of these results is that exposure of nuclei to KCl strips the hormone from the receptor while leaving the receptor in an active configuration still capable of binding hormone. An alternative possibility is that exposure to high salt causes the receptor to undergo a conformational change which causes the release of the hormone even though the receptor remains bound in the nucleus. Washing of the extracted nuclear pellet with low salt buffer might then result in an altered conformation of the receptor which is then capable of rebinding hormone while the receptor site still remains bound in the nucleus.

To investigate the influence of nuclear type II sites and salt extraction on the patterns of estradiol binding to type I sites in uterine nuclei, we measured the levels of [<sup>3</sup>H]-estradiol binding under exchange conditions in KCl soluble and insoluble fractions. These studies were performed on nuclei isolated from immature rats treated with either a physiological (0.1 µg/rat) or pharmacological (2.5 µg/rat) dose of estradiol. Animals were sacrificed at either 1 or 6 hrs following hormone administration and specific estrogen binding was determined (Fig. 5).

TABLE II

COMPARISON OF DIRECT [<sup>3</sup>H]-ESTRADIOL LABELING AND [<sup>3</sup>H]-ESTRADIOL EXCHANGE FOR MEASURING RECEPTOR COMPLEXES ASSOCIATED WITH UTERINE NUCLEI FOLLOWING [<sup>3</sup>H]-ESTRADIOL ADMINISTRATION IN VIVO.

Immature female rats were injected *in vivo* with 0.1 µg of [<sup>3</sup>H]-estradiol. Crude nuclei were isolated and either directly extracted with ethanol, subjected to [<sup>3</sup>H]-estradiol exchange (1) or extracted with 0.4 M KCl-TE. No significant differences were observed between direct counting and [<sup>3</sup>H]-estradiol exchange. The KCl extract was counted directly (2) while the KCl-extracted pellet was either directly extracted with ethanol and counted (3) or subjected to [<sup>3</sup>H]-estradiol exchange followed by counting (4).

<u>In vivo</u> injection of [ <sup>3</sup> H]-estradiol	pmoles [ <sup>3</sup> H]-estradiol bound/uterus	sites/cell
1) Nuclei, direct counting or [ <sup>3</sup> H]-E <sub>2</sub> exchange	0.17	2000
2) 0.4 M KCl extract, direct counting	0.15	1800
3) 0.4 M KCl extracted nuclei, direct counting	0.02	250
4) 0.4 M KCl extracted nuclei, [ <sup>3</sup> H]-E <sub>2</sub> exchange	0.30	3600

The results presented in Fig. 5A are very similar to previously reported data (9) and indicate that the injection of a pharmacological dose of hormone results in the translocation of 4-5 times more estrogen receptor from the cytoplasm to the nucleus than does an injection of a physiological dose of estradiol. The major difference in this study from previous ones is the data reported here were obtained by performing saturation analysis over a much wider range of [<sup>3</sup>H]-estradiol concentrations. One hr after injection of 2.5 µgs of estradiol a large quantity of type II sites were present in the nuclear fraction, and the quantity of type I sites which were extracted from the high dose group appeared to be greater than that of the low dose group (Fig. 5,B). This also appears to be true for the quantity of type I sites which are resistant to salt extraction (Fig. 5,C). However, it is obvious from these data that the quantity of type II sites is elevated and results in an artifactual over-estimate of type I sites. The quantity of type I sites in either Fig. 5,B

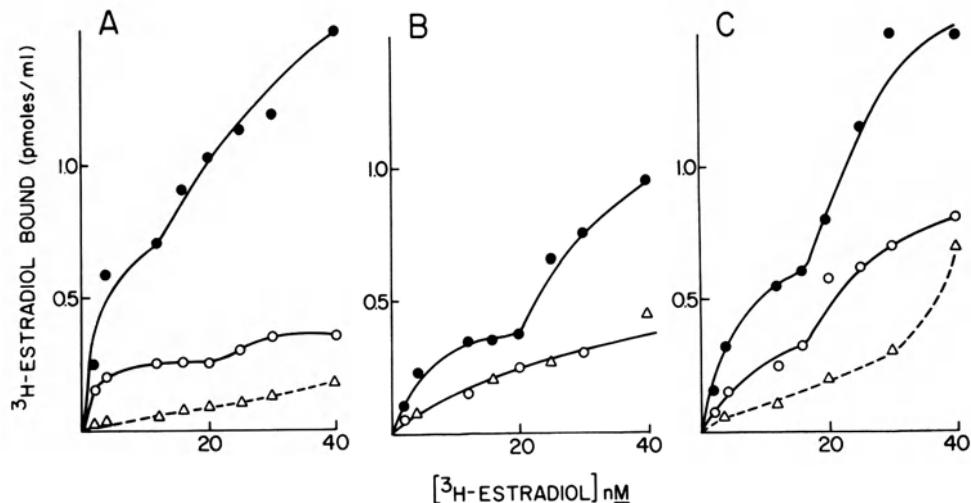


Figure 5

Effects of KCl extraction and hormone dose on saturation analysis of estradiol binding one hr following estradiol administration. Immature female rats were injected with either saline ( $\Delta$ ), 0.1  $\mu$ g of estradiol ( $\circ$ ), or 2.5  $\mu$ g of estradiol ( $\bullet$ ) and sacrificed 1 hr later. Crude nuclear pellets were prepared and saturation analysis performed by exchange (A). The remaining nuclei were extracted with 0.4 M KCl soluble and insoluble fractions were analyzed by exchange and the HAP absorption assay as described in Methods. All data represent specific binding.

TABLE III

COMPARISON OF DIRECT [ $^3\text{H}$ ]-ESTRADIOL LABELING AND [ $^3\text{H}$ ]-ESTRADIOL EXCHANGE FOR MEASURING RECEPTOR COMPLEXES ASSOCIATED WITH UTERINE NUCLEI FOLLOWING [ $^3\text{H}$ ]-ESTRADIOL TREATMENT IN VITRO.

Immature rat uteri were incubated in vitro in the presence of 20 nM [ $^3\text{H}$ ]-estradiol. Crude nuclei were prepared and either directly extracted with ethanol or subjected to [ $^3\text{H}$ ]-estradiol exchange (1). No significant differences were observed between direct counting and [ $^3\text{H}$ ]-estradiol exchange. The remaining nuclei were extracted with 0.4 M KCl. The levels of receptor-hormone complexes in the KCl extract were determined by HAP assays either before (2) or after [ $^3\text{H}$ ]-estradiol (4). KCl-extracted pellets were either counted directly following ethanol extraction (3) or subjected to [ $^3\text{H}$ ]-estradiol exchange and counting (5).

<u>In vitro</u> incubation	pmoles [ $^3\text{H}$ ]-estradiol bound/uterus	sites/cell
1) Nuclei, direct counting or [ $^3\text{H}$ ]-E <sub>2</sub> exchange	1.20	14,500
2) 0.4 M KCl extract, <u>in vitro</u> labeled, HAP assay	0.20	2400
3) 0.4 M KCl extracted nuclei, direct counting	0.5	6000
4) 0.4 M KCl extract, [ $^3\text{H}$ ]-E <sub>2</sub> exchange, HAP assay	0.35	4200
5) 0.4 M KCl extracted nuclei, [ $^3\text{H}$ ]-E <sub>2</sub> exchange	0.9	10,800

or C are not significantly different for the low and high dose groups if the contribution of type II sites is taken into account. An example of this correction is presented in Fig. 6 in which the data from Fig. 5,C has been replotted. The saturation curve for high and low dose groups has been resolved by geometric fitting into two components: type I which is represented as a rectangular hyperbola and type II which is represented as a sigmoid curve. These results point out the importance of correcting for the contribution of type II levels in receptor assays. One hour after hormone administration, the high dose of hormone induces much greater quantities of nuclear type II sites than does the low dose. Therefore, accurate determinations of type I sites can be made only when complete saturation analysis is performed.

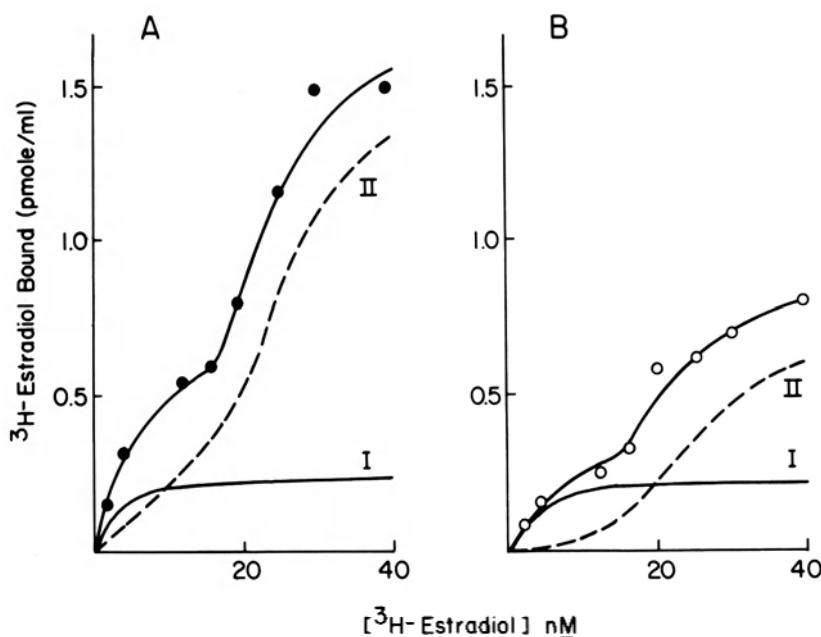


Figure 6

Resolution of two components in a mixed binding system. The data from Fig. 5,C was replotted for the high dose (A) and low dose (B) of the hormone. Each curve was resolved into two components by geometric fitting similar to that proposed by Rosenthal (24). The sum of the rectangular hyperbola (type I) and the sigmoid curve (type II) is equal to the total amount of bound hormone (● or ○).

Six hours after hormone administration the levels of type I and II sites are equivalent in non-extracted and in salt extracted nuclei (Fig. 7A,C). The quantity of type I sites which is extracted from the low and high dose group is not different from the saline treated animals. However, it should be noted that exposure of the control nuclei to KCl has caused an increase in the anticipated number of both classes of sites, i.e., the number of sites extracted exceeds that observed by exchange in non-extracted nuclei (Fig. 7A,B). The reason for this increase is not known at this time. Exposure to KCl increases the quantity of type II sites above that measured in nuclei not exposed to salt, especially in the KCl insoluble fraction (Fig. 7,C).

The levels of type I sites are approximately equal in the KCl insoluble fraction from both treatment groups (Fig. 7,C). The total quantity of type II sites also appear to be equivalent; however, the absolute shape of the binding curve is somewhat different. This appears to be due to a shift in the apparent  $K_d$  for type II sites. The factors which influence this shift are not clear and are still being investigated.

#### Temporal Relationship Between Nuclear Type I and Type II Estrogen Binding Sites and Uterotrophic Response to Estrogenic Hormones.

In order to examine the physiological relationship between the levels of nuclear type I and type II estradiol binding sites and the long uterotrophic response to estrogenic hormones, mature ovariectomized rats were treated with 10  $\mu$ g of estradiol or estriol and sacrificed at various times following injection. The nuclear levels of type I and type II estrogen binding sites were determined by saturation analysis. The data demonstrated that the nuclear retention patterns of type I sites and elevations in nuclear type II estradiol binding sites were very similar (Fig. 8A). Maximal levels of type I and type II sites were observed one hour after an injection of estradiol. The quantity of type I sites then declined gradually to control levels by 72 h. The levels of type II sites also declined gradually but were maintained at 2-3 fold the control levels at 24, 48, and 72 hours. Estradiol and estriol treatment elevated the quantity of type I sites at one hour-post-injection, (compare Fig. 8A + 8B) and caused a corresponding increase in uterine wet weight at 4 h (Fig. 8C). Conversely, only estradiol induced long term nuclear retention of the type I sites (>4-6 h), sustained elevations in nuclear type II sites (4-48 h), and stimulated true uterine growth (uterine wet weight at 24-48 h). Failure of an injection of estriol to stimulate true uterine growth (Fig. 8C) correlated with the inability of this estrogen to induce long term (4-6 h) nuclear retention of type I sites or increase the levels of nuclear type II estrogen binding sites above control levels (Fig. 8,B).

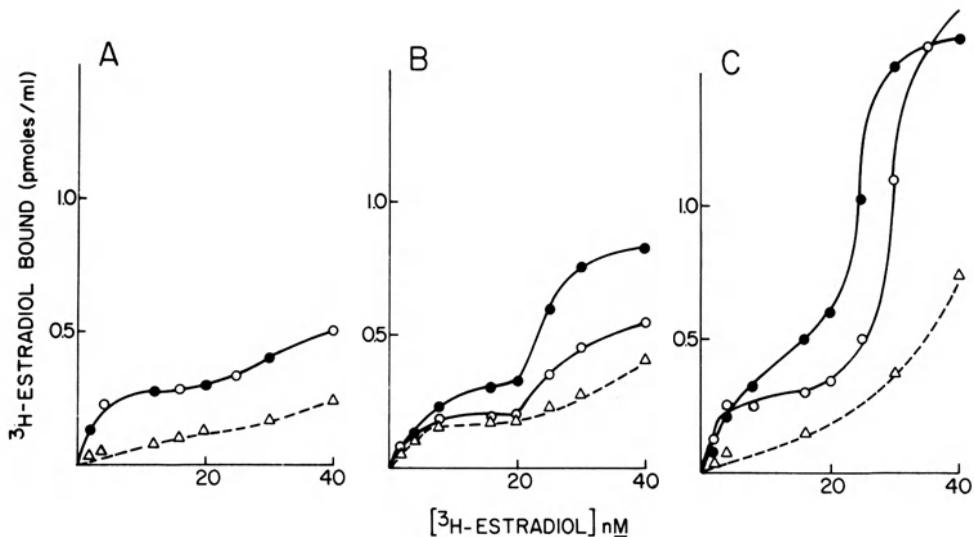


Figure 7

Effects of KCl extraction and hormone dose on saturation analysis of estradiol binding 6 hr following estradiol administration. The experiments were performed exactly as described in Fig. 5 except animals were sacrificed 6 hrs after hormone treatment. Symbols are the same as in Fig. 5.

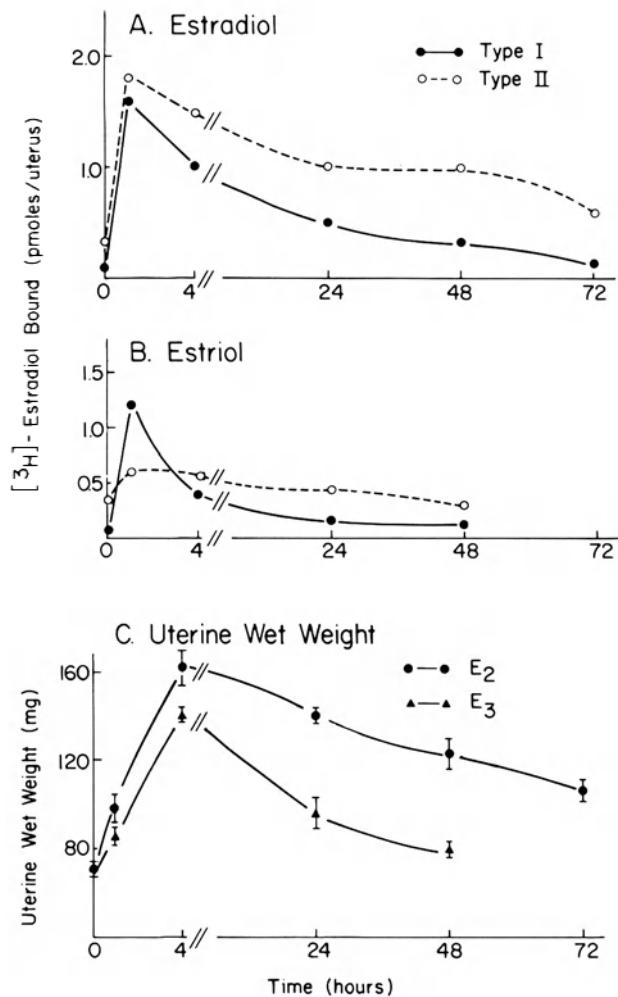


Figure 3

Temporal effects of estradiol (A) and estriol (B) injection on uterine wet weight (C) and nuclear type I ( $\bullet$ — $\circ$ ) and type II ( $\circ$ --- $\circ$ ) estrogen binding sites. Mature-ovariectomized rats were treated with 10  $\mu$ g of estradiol or estriol and sacrificed at various times following injection. The quantity of specifically bound [<sup>3</sup>H]-estradiol was determined by saturation analysis of uterine nuclear fractions at 37° for 30'.

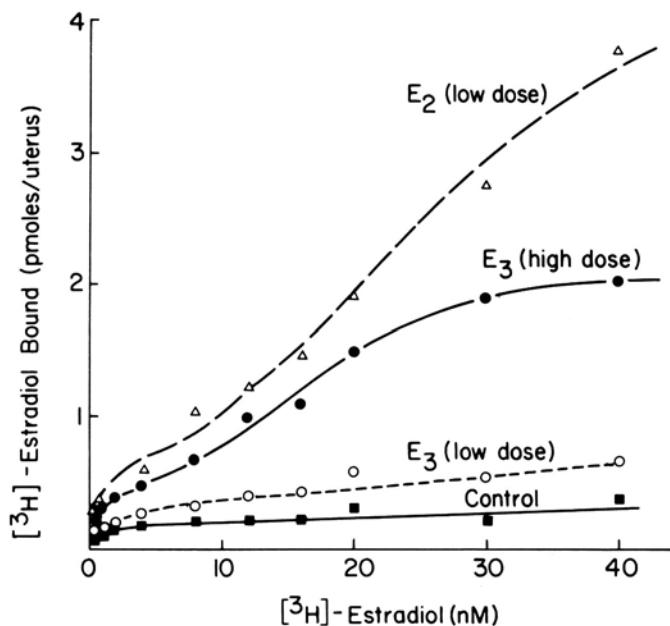


Figure 9

Saturation analysis of estrogen binding sites in the nuclear fraction of the rat uterus. Mature-ovariectomized rats were implanted with a high (4 pellets) or low (1 pellet) dose of estradiol or estriol and were sacrificed 48 hours following hormone administration. All data represent specific binding.

Relationship Between Nuclear Type II Estrogen Binding Sites and Long Term Uterotrophic Response to Estrogen. To further examine the relationship between nuclear type II sites and estrogen stimulation of true uterine growth, mature-ovariectomized rats were treated with paraffin pellets containing either estradiol (1 pellet) or estriol (1 or 4 pellets) and were sacrificed 48 h following hormone administration. Under these experimental conditions estriol treatment sustains elevations in nuclear type I sites and stimulates true uterine growth in the immature rat (15,16). If elevated nuclear type II sites are related to estrogen stimulation of true uterine growth, then increased quantities of this second nuclear estrogen binding component should be observed in animals treated with an estriol implant. The data presented in Fig. 9 and Table IV support this hypothesis. Saturation analysis of nuclear fractions by the [<sup>3</sup>H]-estradiol exchange assay demonstrates that, while not as effective as the estradiol implant, the high dose estriol implant resulted in sustained elevations in occupied type I sites (0.4 pmoles/uterus) and a 6-8 fold increase in the numbers of nuclear type II sites (>4.0 pmoles/uterus) as compared to the low dose estriol treated animals or paraffin controls. These elevations in nuclear type II sites also correlated with estradiol or estriol stimulation of true uterine growth (Table I). Estriol, when administered at the high dose level caused the uterus to grow and the numbers of both sites were increased. As expected, this was also true for the animals which were implanted with estradiol. Uterine growth was not observed in the animals treated with the low dose implants of estriol and this is correlated with the low level of both sites in uterine nuclei. These data contrast sharply to the results obtained following a single injection of estriol where this estrogen failed to increase either the numbers of nuclear type II estrogen binding sites or stimulate true uterine growth (Fig. 8B,C).

#### DISCUSSION

The presence of at least two specific binding sites for estrogen in the nuclei of immature rat uteri following hormone administration is of importance at a number of levels. Of obvious importance, as pointed out previously (13,14), is the interference of the type II sites in the accurate estimation of the levels of nuclear associated estrogen receptor (for example, see Fig. 5C). This complication is better illustrated if one examines the data from Fig. 5C which are redrawn in Fig. 6. The total quantity of specific binding is the sum of two components; type I which is represented by the rectangular hyperbola and type II which is represented as a sigmoid function of the concentration of free hormone. It can be seen that the quantity of type I sites is identical in uterine nuclei from either the high or low dose group. However, the amount of type II binding is significantly elevated in the high dose group. In order to make accurate

determinations of the quantity of either component, the contribution of one component must be subtracted from the other. In Fig. 6 the resolution of these components was done by a geometric fitting procedure similar to that proposed by Rosenthal (21). We originally suggested that this could be accomplished by performing exchange assays at both 4° and 37° (13). Under these conditions total sites are measured at 37° and only type II sites are measured at 4°. This method is both costly and time consuming and is not practical when one has either a number of samples or a limited quantity of any particular sample. This method also is inaccurate if one or both of the sites display differential lability at one of the temperatures used in the assay.

TABLE IV

## EFFECT OF ESTRADIOL AND ESTRIOL CONTAINING PARAFFIN IMPLANTS ON UTERINE WET WEIGHT AND NUCLEAR TYPE II SITES.

Mature-ovariectomized rats were implanted with paraffin pellets containing 10% by weight of estradiol or estriol and sacrificed 48 hours following treatment. The pellets weighed approximately 10 mg.

<sup>a</sup> number in parenthesis is the number of pellets implanted in each animal.

<sup>b</sup> values represent the MEAN  $\pm$  SEM for uteri obtained for 6-8 animals.

Treatment		Uterine Wet Weight (mg)	Type II Site (pMoles/uterus)
Control	(4) <sup>a</sup>	87.3 $\pm$ 3.4 <sup>b</sup>	0.4
Estradiol	(1)	212.0 $\pm$ 11.7	8.0
Estriol	(1)	82.3 $\pm$ 3.9	0.5
Estriol	(4)	180.0 $\pm$ 8.8	4.2

The ultimate solution to this problem appears to be the development of an appropriate computer program which can resolve the two components. Until such capabilities are developed, rough estimates can be made under certain circumstances by extrapolation of the number of type I sites as shown in Fig. 1B. Only a very small error is incurred in this particular case because the influence of type II below 10 nM free [<sup>3</sup>H]-estradiol is slight. However, as shown in Fig. 7C, we have observed the shape and half saturation point of type II sites may shift due to unknown factors. Therefore,

it is absolutely necessary to determine the influence of these various sites on one another by performing saturation analysis over a wide concentration range of labeled steroid. The quantity of each binding site can be estimated by fitting a rectangular hyperbola and a sigmoid curve as shown in Fig. 6.

The time course of appearance and the nuclear retention of type II sites in uterine nuclei following a single injection of estradiol are intriguing. Prior to hormone injection, uterine nuclei contain extremely low levels of type I and II sites (Fig.3). Within 1 hr of hormone administration, large quantities of both sites are present in the nucleus. For type I sites this represents the classically described translocation of the estrogen receptor from the cytoplasm to the nucleus. Since the type II sites do not translocate to the nucleus, it is not clear whether the appearance of these sites represent *de novo* synthesis of a new class of binding molecules or activation of a pre-existing molecule or a combination of these possibilities. Once type II sites are present they appear to remain for at least 24 hrs. In contrast, type I sites have declined to non-detectable levels by this time and have been recycled or replenished in the cytoplasm (4).

The validity of the use of salt extraction of uterine nuclei to measure specific estrogen binding sites associated with nuclei has recently been questioned (8-10). The results presented in Tables II and III indicate that KCl extraction of uterine nuclei followed by [<sup>3</sup>H]-estradiol exchange assays presents a more accurate picture of specific estrogen binding sites associated with nuclei than does direct labeling by either injection or *in vitro* incubation with [<sup>3</sup>H]-estradiol and subsequent isolation of target cell nuclei followed by salt extraction of the nuclear fraction as a function of time after hormone injection (10,11). In these studies, 90-95% of the estradiol is extracted from the nuclear fraction at all times examined. The residual 5-10% of hormone is thought to result from trapping of receptor-hormone complexes in the gelatinous pellets resulting from the exposure of chromatin to high salt.

Although salt-resistant sites may be fortuitous, the experiments described above are not adequate to test this hypothesis. This is clear from the results shown in Tables II and III. Our experiments have involved the injection of non-labeled estradiol with subsequent measurement of specific nuclear bound receptor sites by the [<sup>3</sup>H]-estradiol exchange assay. In this manner, the presence of high affinity, specific estrogen binding sites are measured, not simply radioactivity counts due to the presence of [<sup>3</sup>H]-steroid.

An additional point concerning disparities in these various experiments should be made. In our experiments a single 0.4 M KCl extraction was performed while in the experiments of others several extractions with 0.6 M KCl plus sonication of the nuclear fraction were conducted. This continued extraction with elevated salt, particularly when coupled with shearing of DNA, would be expected to solubilize all receptor and hormone even if the receptor were bound to high affinity acceptor sites. Such techniques are commonly used to solubilize nuclear components, such as RNA polymerase, which is bound to chromatin with a very high affinity (22). The observation that repeated high salt extraction removes the majority of nuclear associated [<sup>3</sup>H]-estradiol does not discount the fact that following a single 0.4 M KCl extraction 10-15% of the receptor sites remain tightly bound in the nuclear fractions (7).

This latter observation is partially accounted for by the observations of Barrack *et al.* (12) as well as results in Table II. Virtually all of the [<sup>3</sup>H]-estradiol associated with uterine nuclei following an injection of labeled hormone is extracted by a single exposure to 0.4 M KCl; however, 2000-3000 receptor sites are measurable in these nuclei by [<sup>3</sup>H]-estradiol exchange. The simplest explanation of these results is that hormone is readily extracted while receptors which are tightly associated with the nuclear fraction are resistant to extraction. This is particularly attractive if the major role of the hormone is in receptor transport; i.e., to allow the nuclear accumulation of receptors and their subsequent binding to acceptor sites. Once bound to acceptor sites the receptor might then stimulate the biochemical reactions associated with the hormone's action regardless of whether hormone continues to be associated with the receptor or not.

Our present results on salt insoluble nuclear receptor sites are completely consistent with our previous results when the contribution of type II sites is taken into account. That is, the quantity of type I sites which are resistant to a single salt extraction are similar at one and six hours after either a high or low dose of injected estradiol. The surprising result of this study is the apparent large increases in type II sites following KCl extraction of uterine nuclei. It is not clear why this occurs, however, several obvious possibilities are apparent. The KCl extraction may remove some component which is preventing the labeling of these sites during the exchange reaction in non-extracted nuclei. This could be something as complicated as some undetermined factor necessary for activation. Another possibility is that the extraction process causes a conformational or activational change in the binding site which allows previously unavailable sites to bind hormone.

The present studies also suggest there is a good correlation between elevated levels of nuclear type II sites and the long term (24-48 h) uterotrophic response to estrogen. This relationship was demonstrated by the ability of a single injection of estradiol to increase the levels of nuclear type II sites and uterine wet weight 24-48h after injection (Fig. 8), whereas a single injection of estriol failed to cause significant elevations of type II sites and little true growth was observed. In contrast, implants of estradiol or estriol (high dose) stimulated type II sites and uterine growth (Fig. 9; Table IV) in the mature-ovariectomized rat. Thus while nuclear levels of type I sites following estradiol or estriol administration to immature (15,23) or mature-ovariectomized rats (Fig. 8), correlate well with early uterine responses (1-4h), elevations in nuclear type II sites are more highly correlated with true uterine growth. True uterine growth consists of nucleic acid and protein synthetic events which result in cellular hypertrophy and hyperplasia. We have shown previously that a single injection of estradiol stimulated DNA synthesis (18) RNA polymerase activity (18) and template activity (24) while an injection of estriol failed to do so.

Thus, estrogen stimulation of true uterine growth appears to be the result of both long term (>6 h) nuclear retention of type I sites (5,6,23) and rapid and sustained elevations in the levels of nuclear type II estrogen binding sites. Elevations in nuclear type II sites do not correlate with the ability of estradiol or estriol to stimulate "early" responses (1-4 h) in the rat uterus (fig. 8). A single injection of estriol was equivalent to estradiol in stimulating uterine wet weight at 1-4 h, even though estriol treatment failed to elevate nuclear levels of type II sites.

The elevated levels of nuclear type II sites observed in this study do not appear to result from a nuclear translocation process as is seen with the type I sites (13,14). These type II sites may represent nuclear components which are always present and are activated by estradiol and/or the receptor estradiol complex. The ability of estradiol to increase the quantities of nuclear type II sites appears to be a specific response since both purified nuclei (Fig. 1) and chromatin (Fig. 2) from uteri, but not diaphragm, spleen or serum contain these sites (Fig. 4). Thus, it is unlikely that these type II sites occur in the nucleus as a result of cytoplasmic or serum contamination.

The precise requirements for estrogenic stimulation of nuclear type II sites remain to be resolved. The ability of estriol when administered by multiple injection (23) or paraffin implant (15,16 and Fig. 9) to increase nuclear type II sites and to stimulate true uterine growth (Table IV) suggests one requirement for these elevations in nuclear type II sites may be sustained nuclear occupancy by receptor hormone complexes. However,

the specificity of the interaction between receptor hormone complexes and nuclear sites which result in the increase in type II sites may also be involved. This conclusion is supported by the observation that while a single injection of either estradiol or estriol resulted in an equivalent accumulation of receptor hormone estrogen complexes at 1-4 h post-injection, only the receptor estradiol complexes were associated with rapid and sustained elevations in nuclear type II sites between 1 and 48 h following treatment. Whether the increases in nuclear type II sites in estradiol-implanted animals were due to long term nuclear occupancy by receptor-estriol complexes or to saturation of specific nuclear (receptor-estradiol complex)-binding sites through a lower affinity interaction with receptor-estriol complexes remains to be established.

One of the important questions which is not answered in this study is: do type II sites bind estradiol *in vivo*? Since these sites display "cooperative-like" binding it is not possible to calculate a dissociation constant, and hence, it is not possible to predict whether *in vivo* binding occurs. It is likely that these sites do not bind estradiol alone *in vivo*, but instead, they bind the receptor estradiol complex. If this were true it would not be possible to detect type II sites by an injection of [<sup>3</sup>H]-estradiol *in vivo*. Our measurements reflect only the ability of type II sites to bind [<sup>3</sup>H]-estradiol in the exchange assay and not their ability to bind the type I complexes.

In conclusion, these data indicate that two estrogen binding entities may be involved in the response of the rat uterus to E<sub>2</sub>. Whereas early responses may be mediated through estrogen interaction with type I sites (23,25), estrogen stimulation of nuclear events associated with true uterine growth requires not only long term nuclear retention of type I sites (5,6) but rapid and sustained elevations in the levels of nuclear type II sites (Fig.8). The function of these sites is not known; however, the type II sites appear to be "stimulated or activated" by the translocation of type I sites, and remain elevated for 24 hrs in the immature rat or for longer periods in the mature rat. This is much longer than either type I sites or injected [<sup>3</sup>H]-estradiol remain in the nucleus and correlates well with time of major stimulation of DNA, RNA and protein synthesis (26). Thus, the presence of nuclear type II sites correlate better with uterotrophic stimulation than does the nuclear binding of type I sites. It is possible that these sites represent estrogen specific binding sites associated with chromatin that are involved in the control of DNA synthesis and sustained RNA synthesis.

It is also possible that type II sites are a part of the nuclear binding-processing mechanism. We have proposed that the binding of receptor steroid complex to nuclear sites involves a nuclear retention mechanism and is not a simple function of mass

action (4,7). Type II sites might bind the receptor estrogen complex and form a new complex which would be the functional unit of gene activation and receptor processing. This processing unit could be involved not only in gene activation but also in the recycling or replenishment of the receptor to the cytoplasm. Thus, it is possible that nuclear type II sites could be precursors of cytosol type I sites. Nuclear processing could also involve the metabolism of the hormone as a part of the mechanism by which uterine cells eliminate estrogens from their local environment.

Another possibility which has not been fully explored is the cellular distribution of these specific hormone binding molecules within the target tissue. For instance, we have recently demonstrated that triphenylethylene derivatives primarily stimulate the luminal epithelium of the uterus (27). Likewise, estrogens cause the growth and differentiation of the tubular gland cells of the chick oviduct preferentially over other oviductal cell types (28). This raises the possibility that these two classes of specific estrogen binding sites might have specific cellular distribution which is important in specific estrogen induced growth patterns.

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