Estrophilic Binding Sites of the Uterus. Relation to Uptake and Retention of Estradiol *in Vitro*†

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ABSTRACT: The uptake and retention of [^{3}H]estradiol by uterine and diaphragm tissue have been examined under initial velocity and equilibrium conditions, respectively. Evidence is presented to demonstrate that the estrogen receptor is not involved in the movement of estradiol into uterine tissue. Thus the rate of uptake of estradiol by the nontarget tissue, diaphragm, which possesses no estrogen receptor is the same as that for the uterus. In addition, inhibitors of the estrogen receptor fail to alter the rate of uptake of estradiol in either tissue. Retention of estradiol under equilibrium conditions involves primarily two types of binding sites. One type is of limited capacity but high affinity and specificity for estradiol while the second has a low affinity but a very large capacity for estradiol. Data are presented to suggest that the high affinity sites are the estrogen receptor while the "unsaturable" sites are primarily on serum binding proteins.

Data on the uptake and retention of estradiol by the uterus have been reported by many investigators (for a review see Gorski *et al.*, 1968). It is generally held that a two-step mechanism exists in which the estrogen receptor (R)[1] serves both an uptake function, *i.e.*, the receptor recognizes and binds estrogen which results in the increased uptake of estradiol in the uterus, and a retention function, *i.e.*, the receptor–estrogen complex (RE) once formed acts to retain estrogen within uterine cells as a result of translocation of RE to the nucleus (Shyamala and Gorski, 1969; Jensen *et al.*, 1968). Unfortunately previous investigations have failed to differentiate clearly between uptake and retention so that it is difficult to evaluate these phenomena separately. Uptake is defined in this report as the initial rate of movement of estradiol into tissues whereas retention is defined by the amount of estradiol found in the tissue under equilibrium conditions.

The quantity of estrogen receptor in the uterus is in excess of that required to produce a physiological response to estrogen (Jensen *et al.*, 1968). An uptake function has been attributed to this excess receptor (Jensen *et al.*, 1968). Studies in our laboratory also indicate that the receptor is in considerable excess over that required for physiological responses (Anderson *et al.*, 1972a,b); however, we view the function of excess receptor as one of retention. Other investigators have suggested that the entry of estradiol into uterine cells occurs by an active process (Milgrom *et al.*, 1972). The present report suggests that R is not involved in the process of uptake or transport, but rather functions to retain estrogen within the uterus. Evidence is presented which suggests that estrogen is not actively transported nor does the receptor enhance the rate of uptake. Rather there appears to be a partitioning of estradiol between the medium and the tissue in a nonspecific, passive manner.

In addition, the present report demonstrates that the subsequent retention of estradiol consists primarily of two types of binding sites: a limited number of sites with high affinity and specificity for estradiol and a second with low affinity and a very large capacity. The existence of two types of sites has been reported previously (Gorski *et al.*, 1968; Rochefort and Baulieu, 1969; Erdos *et al.*, 1969). Calculations are presented to suggest that the low affinity and large capacity sites are probably a mixture of serum binding proteins and other nonspecific sites such as intrauterine lipids. Finally, the interaction of estradiol with the uterus is modeled in terms of these two classes of binding sites and data from equilibrium studies are compared with predictions from the model.

Experimental Procedure

Materials. Immature female Purdue-Wistar rats (21–23 days old) were used in all experiments. Animals were kept in a controlled environment of 70–72°F, with a relative humidity of 45–55%. The light–dark cycle consisted of 13 hr of light and 11 hr of darkness, with the light cycle starting at 7 a.m. Food and water were given *ad lib.* All experiments were started between 9:00 and 11:00 a.m.

[6,7-^{3}H]Estradiol-17β (New England Nuclear, 40 Ci/mmol) was examined for purity *via* thin layer chromatography. Eagle's HeLa medium (Difco) was used in all experiments. Solutions of crystalline bovine serum albumin (Pentex, Kankakee, Ill.) in Eagle's HeLa medium were prepared fresh daily.

Tissue Preparation and Cell Fractionation. Rats were killed by decapitation and their uteri and diaphragms removed. Uteri were cleaned of mesentery and fat. Diaphragms were cut into strips of tissue which resembled the uterus in both surface area and weight. Tissues were quickly weighed on a torsion balance to the nearest 0.1 mg and after *in vitro* incubation (see below) they were homogenized in cold TE buffer (10 mM Tris–1.5 mM EDTA, pH 7.4) in all-glass Kontes homogenizers using a motor driven pestle. For the determination of total tissue estradiol, multiple aliquots of the

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[1] Abbreviations used are: R, estrogen binding protein or receptor; E, estradiol; RE, the complex of estradiol with the estrogen receptor; SA, bovine serum albumin; SAE, the complex of estradiol with serum albumin; MalNEt, *N*-ethylmaleimide; N_2ph, 2,4-dinitrophenol; TE, a buffer containing 10 mM Tris and 1.5 mM EDTA at pH 7.4.

homogenate were suspended in 3 ml of 100% ethanol and added to 10 ml of scintillaton fluid [99.5% toluene; 0.45% 2,5-diphenyloxazole; 0.05% 1,4-bis(5-phenyloxazol-2-yl)benzene] for counting. The nuclear–myofibrillar fraction was prepared by centrifugation of the uterine homogenate at 800*g* for 10 min, washed three times in 3 ml of cold TE buffer by centrifugation at 800*g* for 10 min, and subsequently extracted with 3 ml of 100% ethanol for analysis. Aliquots of the supernatant of the first centrifugation were added to 100% ethanol for the determination of radioactivity in the "cytosol" fraction.

Rate of Uptake. Uteri and diaphragm strips were placed in 10-ml glass vials containing 2 ml of Eagles medium with or without bovine serum albumin at 37°. The vials contained various concentrations of [^{3}H]estradiol with or without diethylstilbestrol (100 × the concentration of [^{3}H]estradiol). The tissues were incubated with shaking for 15 and 30 sec. Incubations were terminated by decanting the incubation medium and washing the tissues two times with 5 ml of cold TE buffer. The tissues were homogenized in 2 ml of TE buffer and 0.1-ml aliquots were taken for the determination of radioactivity. The influence of *N*-ethylmaleimide (MalNEt) and 2,4-dinitrophenol (N_2ph) on the rate of uptake was examined by preincubating tissue in MalNEt (millimolar) or N_2ph (millimolar) for 30 min at 37°. Following preincubation the tissues were incubated with [^{3}H]estradiol with or without diethylstilbestrol as above. The data were expressed as picomoles of [^{3}H]estradiol/g of tissue/sec.

Retention at Equilibrium. Uteri and diaphragm strips were placed in 10-ml glass vials containing 2 ml of Eagle's medium with or without bovine serum albumin. All procedures were carried out at 4° except for the incubation periods. The tissues were incubated for 60 min at 37° in the absence of bovine serum albumin and for 120 min at 37° when bovine serum albumin was present. Tissues were incubated under air in a Dubnoff shaker in media containing various concentrations of [^{3}H]estradiol with or without diethylstilbestrol (100 × the concentration of [^{3}H]estradiol). In some experiments tissues were preincubated in MalNEt (millimolar) for 30 min before incubation in [^{3}H]estradiol. Following incubation, the tissues were washed two times with 5 ml of cold TE buffer and homogenized in 2 ml of TE buffer. Aliquots of the incubation medium were taken for radiometric analysis. The homogenate was fractionated by centrifugation as above and the radioactivity in the nuclear and cytoplasmic fractions was determined. Data were expressed as picomoles of [^{3}H]-estradiol/g of tissue.

Determination of the Level of Serum Albumin in the Rat Uterus. Uterine soluble proteins were prepared by homogenization of the tissue, one uterus/ml of TE buffer, and subsequent centrifugation of the homogenate at 20,000*g* for 10 min. Samples (10, 20, and 50 μl) of the 20,000*g* supernatant were added to 30% sucrose and applied to 6-mm diameter gels containing 7.5% acrylamide. Alternatively homogenization and centrifugation were performed using 30% sucrose instead of TE buffer. Solutions of crystalline bovine serum albumin were analyzed simultaneously with tissue extracts. Electrophoresis was done essentially according to Davis (1964). The gels consisted of a 0.5-cm stacking gel, pH 6.7, and a 6.0-cm running gel, pH 8.9. Electrophoresis was conducted at 3 mA/gel for 1.5–2.0 hr at 5° in a 5 mM Tris–38 mM glycine buffer, pH 8.4,

Gels were stained with either Buffalo Black (0.5% w/v) or Fast Green (1% w/v) in 7% acetic acid for either 2 hr or overnight at room temperature. Destaining was accomplished in one of two ways: by bathing the gels in repeated changes of 7% acetic acid until clear or by rapid destaining in a horizontal electrophoretic destaining apparatus (Gelman Co.).

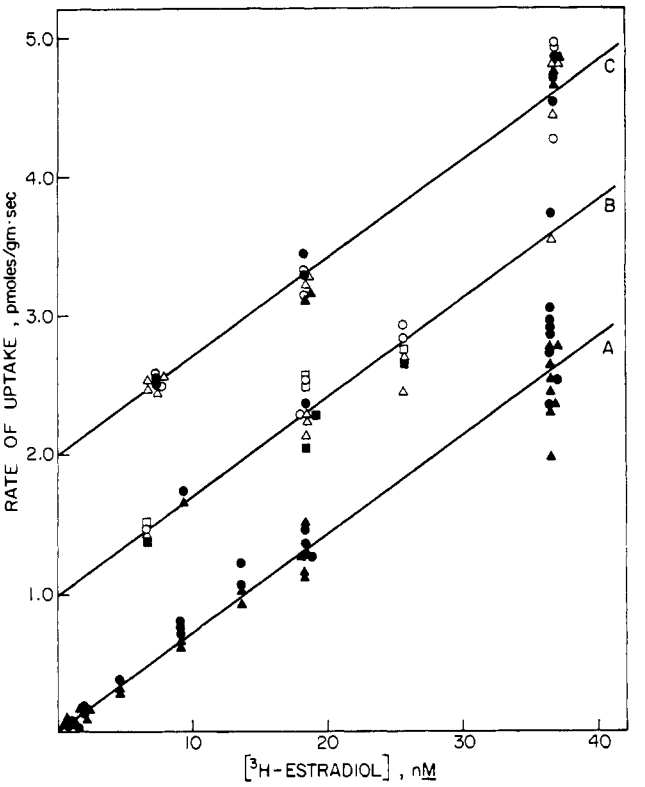

FIGURE 1: Initial rate of uptake of [^{3}H]estradiol by uterine and diaphragm tissue. Tissues were incubated with shaking for 15 and 30 sec and washed, and [^{3}H]estradiol in the tissue was determined as outlined in the Experimental Procedure. Note that in this figure the data for B and C have been displaced along the vertical axis by 1 and 2 pmol/g per sec, respectively: (A) (●) normal uterine tissue; (▲) normal diaphragm tissue; (B) initial rates were determined for control uteri (●), uteri plus 1 mM MalNEt (○), uteri plus 1 mM N_2ph (□), control diaphragm strips (▲), diaphragm strips plus mM MalNEt (△), and diaphragm strips plus 1 mM N_2ph (■); (C) initial rates were determined for uteri (circles) and strips of diaphragm (triangles) in the presence (open symbols) and absence (closed symbols) of diethylstilbestrol.

Densitometry of stained gels was performed with a Gilford Gel Scanner using either 650 mμ (Buffalo Black) or 625 mμ (Fast Green). Quantitative analysis was achieved by cutting out the peaks of the densitometer tracings and weighing them on an analytical balance.

General Methods. Radioactivity was measured with a Packard 3375 liquid scintillation spectrometer. The scintillation cocktail consisted of 3 ml of 100% ethanol and 10 ml of scintillation fluid [99.5% toluene–0.45% 2,5-diphenyloxazole–0.05% 1,4-bis[2-(5-phenyloxazolyl)]benzene]. All samples were examined for quenching by automatic external standardization and analyzed at 32% efficiency for tritium. All computations were done on a CDC 6500 computer. Nonlinear least-squares analyses were performed according to Cleland (1963).

Results

Uptake of Estradiol by the Uterus and Diaphragm. To determine whether the rate of uptake of estradiol into the uterus is dependent on a saturable component, a comparison of the initial rate of uptake of [^{3}H]estradiol by the uterus (○) and diaphragm (△) as a function of the initial concentration of estradiol in the medium is shown in Figure 1. Diaphragm was

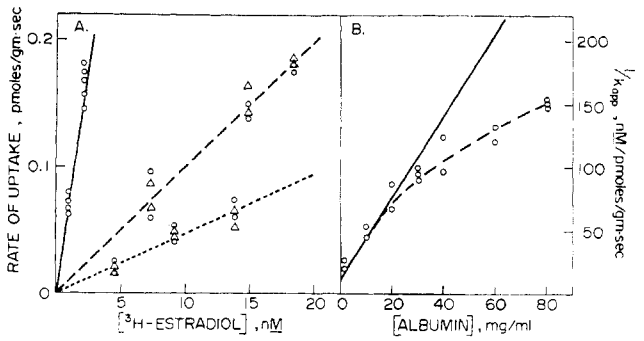

FIGURE 2: Influence of exogenous serum albumin on the initial rate of uptake of estradiol. (A) Initial rate of uptake by uterine and diaphragm tissue. Initial rates were determined as in Figure 1 for uterus (○) and diaphragm (△) at 0 (—), 4 (- - -), and 6% (· · ·) bovine serum albumin. (B) Replot of slopes (k_{app}) as in Figure 4A *vs.* serum albumin concentration. The solid line was calculated from the rate constant for the uptake of estradiol in the absence of serum albumin and the dissociation constant, 5×10^{-4} M, for the serum albumin–estrogen complex. The open circles represent individual experimental values.

chosen as a control tissue because it was possible to cut diaphragm into strips which resemble the uterus in the surface area/weight ratio. Preliminary experiments with 30-mg pieces of body wall indicated that the surface area/weight ratio was critical for uptake studies. The initial rate of uptake of [^{3}H]estradiol is a linear function of the initial concentration of estradiol in the medium and is independent of the tissue source. The slope of the line in Figure 1A is 0.070 ± 0.002 pmol/g per sec per nM with a vertical intercept of 0.037 ± 0.040 pmol/g per sec when based on 48 pieces of data.

To further evaluate the possibility that the receptor or some active transport process might function in estradiol uptake, tissues were exposed to several potential inhibitors of the receptor or active transport. The initial velocity of uptake of [^{3}H]estradiol by uterine and diaphragm tissues was examined in the presence of a 100-fold excess of diethylstilbestrol (Figure 1C) or after preincubation for 30 minutes or 1 hr with *N*-ethylmaleimide (MalNEt) or 2,4-dinitrophenol (N_2ph) (Figure 1B). The initial velocity of uptake of [^{3}H]-estradiol is unaffected by these reagents. This indicates that the uptake of estradiol is not dependent on the estradiol receptor because the receptor would be adversely affected by MalNEt (Jensen *et al.*, 1967) and competitively blocked by diethylstilbestrol (Toft and Gorski, 1966). While an active transport process is not ruled out with N_2ph, strict linearity over a wide range of estradiol concentrations (Figure 1A) together with the N_2ph studies (Figure 1B) makes the existence of an active transport process doubtful.

Effect of Serum Albumin on [^{3}H]Estradiol Uptake. Under physiological conditions, the uterus must absorb estradiol from circulating plasma and retain it. Plasma is a source of many proteins that are potential estrogen binding sites. The effect of one such protein, serum albumin, has been examined and the results are shown in Figure 2A. Figure 2A is a plot of the initial rate of uptake of [^{3}H]estradiol by uterine and diaphragm tissue from Eagles medium with or without bovine serum albumin.

The initial rate of uptake of estradiol in the presence and absence of serum albumin is a linear function of estradiol concentration in the incubation medium and first order with respect to estradiol in the medium over the concentration range investigated. However, the presence of serum albumin in the medium decreased the rate of uptake of estradiol by uterine (○) and diaphragm (△) tissue. Since estradiol uptake is first order and not saturable with respect to estradiol in the presence or absence of serum albumin, it can be assumed that the uptake of estradiol into the uterus or diaphragm is a partition phenomena and that serum albumin reduces the concentration of *free* estradiol present in the medium. This reduction in turn leads to the decreased rates of uptake in both tissues as seen in Figure 2A.

If the serum albumin binding sites for estradiol are equal and independent, then by analogy with enzyme systems in which inhibitors interact with the substrate, the initial velocity of estradiol uptake in the presence of serum albumin (SA) can be expressed as a function of an apparent first-order rate constant, k_{app}, which is defined as

$$k_{app} = k[K_{SAE}^{eff}/(K_{SAE}^{eff} + [SA_T])] \quad (1)$$

where k is the rate constant for estradiol uptake in the absence of serum albumin, K_{SAE}^{eff} is an apparent or effective dissociation constant for the serum albumin–estrogen complex, and $[SA_T]$ is the concentration of total serum albumin. The rate constant, k, can be determined by double reciprocal analysis of eq 1 in a standard manner as shown in Figure 2B.

The solid line in Figure 2B was calculated from the rate constant, k, for the uptake of estradiol in the absence of serum albumin and the calculated effective dissociation constant, 5×10^{-4}, for the serum albumin–estrogen complex which was obtained from equilibrium data (see below). Although the solid line appears to describe the data reasonably well at low albumin concentrations, the data at higher albumin concentrations are clearly nonlinear and suggest that the model does not hold for higher concentrations of serum albumin. Although many possibilities exist to explain the hyperbolic nature of Figure 2B, one possibility is that serum albumin might bind to uterine membranes and modify them in such a way as to alter the rate of transfer of estradiol across the membrane. Analogous properties have been reported for the sensitization of L cells to streptomycin by serum albumin and suggest that serum albumin can alter the morphology of cell membranes (Casciano, 1971). In addition, the diffusion of glucose across phospholipid model membrane systems is enhanced by serum albumin (Sweet and Zull, 1969).

Retention of [^{3}H]Estradiol by Uterus and Diaphragm. When uterine or diaphragm tissue is incubated at 37° for 1 hr in the presence of varying concentrations of [^{3}H]estradiol, an equilibrium is established between medium and tissue bound label (Giannapoulos and Gorski, 1971).[2] Uteri exposed to [^{3}H]estradiol plus excess diethylstilbestrol behave just as the control tissue, diaphragm, whereas uteri exposed to [^{3}H]-estradiol alone retain additional label at equilibrium (data not shown). These results demonstrate that diaphragm tissue is capable of retaining [^{3}H]estradiol to the same extent as uteri with nonfunctional receptor, *i.e.*, receptor saturated with diethylstilbestrol.

Following homogenization and centrifugation (see Experimental Procedure) the subcellular localization of [^{3}H]-estradiol at equilibrium was determined for the uterus (Figure 3A) and diaphragm (Figure 3B). The cytoplasmic compartments of uterus and diaphragm are identical in their retention

[2] In the present study adequate controls were performed to assure that equilibrium is established by 1 hr at 37°. However, we have not presented data to support this contention in view of the large number of reports to that effect.

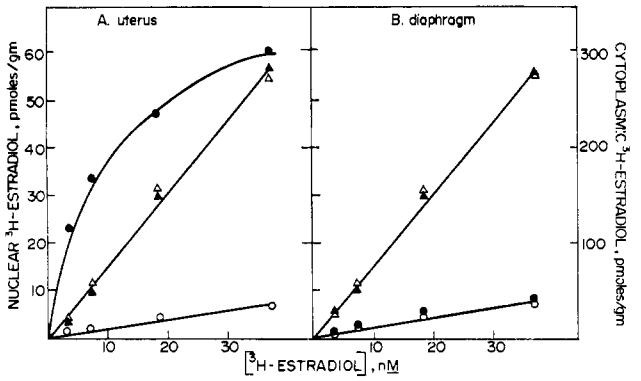

FIGURE 3: Subcellular localization of [^{3}H]estradiol at equilibrium in the uterus and diaphragm. Uteri (A) and diaphragm strips (B) were incubated for 1 hr at 37° in the presence of varying concentrations of [^{3}H]estradiol and the presence or absence of excess diethylstilbestrol. The nuclear (circles) and cytoplasmic (triangles) fractions were prepared and analyzed for [^{3}H]estradiol as outlined in the Experimental Procedure: closed symbols, no exposure to diethylstilbestrol; open symbols, excess diethylstilbestrol in the incubation medium.

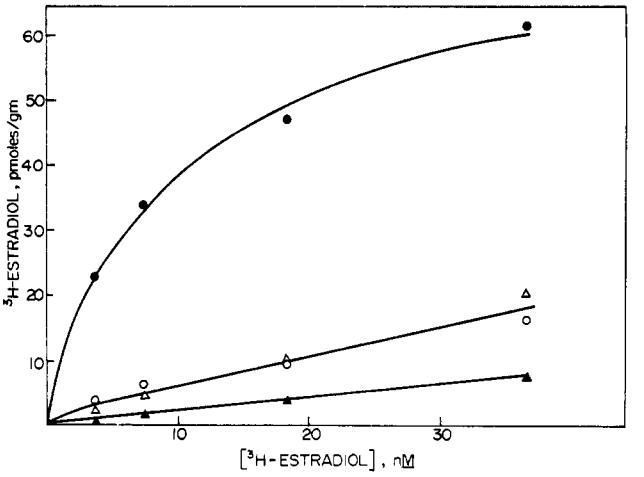

FIGURE 4: Effect of *N*-ethylamaleimide on nuclear retention of [^{3}H]-estradiol by uterine and diaphragm tissue. Uteri or strips of diphragm were preincubated 1 in mM MalNEt for 30 min prior to incubation in various concentrations of [^{3}H]estradiol. The nuclear fraction was prepared and [^{3}H]estradiol was determined as described in the Experimental Procedure: (○) uterine nuclei plus MalMEt; (●) uterine nuclei, no MalNEt; (△) diaphragm nuclei plus MalNEt; (▲) diaphragm nuclei or uterine nuclei plus diethylstilbestrol.

of [^{3}H]estradiol in the presence or absence of an excess of diethylstilbestrol (△,▲, A and B). In addition, the retention of [^{3}H]estradiol by the nuclear fraction of uteri exposed to excess diethylstilbestrol (○; A) is identical with that of diaphragm nuclei with or without diethylstilbestrol (○,●; B). Only the nuclear fraction of uteri which have not been exposed to excess diethylstilbestrol exhibit an increased retention (●; A) relative to the coresponding fraction from diaphragm.

Because the amount of estradiol retained by the nuclear fraction of the uterus is saturable and a hyperbolic function of the concentration of label in the medium, the data from Figure 3A were fitted by a nonlinear least-squares analysis of eq 2 (see Experimental Procedure)

$$[RE] = [RE_{max}][E_{free}]/([E_{free}] + K_d^{RE}) \quad (2)$$

In this calculation the assumption was made that the cell membrane acts in a semipermeable manner with respect to estradiol (see above) and thus the concentration of *free* estradiol outside the cell is equal to the concentration of *free* estradiol inside the cell. The stoichiometry, $[RE_{max}]$, was found to be 0.064 ± 0.003 pmol/mg of tissue and the dissociation constant, K_d^{RE}, was found to be 4.62 ± 0.6 nM. Since errors in $[RE_{max}]$ and K_d^{RE} are small, it can be suggested that a simple noncooperative model (eq 3) will adequately fit the binding data. A simple noncooperative model has also been

$$R + E \underset{}{\overset{K_d^{RE}}{\rightleftharpoons}} RE \quad (3)$$

demonstrated by Gorski and coworkers (Giannopoulos and Gorski, 1971; Williams and Gorski, 1972). While the present results and those of Gorski and coworkers do not agree with the cooperative scheme suggested by others (Erdos *et al.*, 1971; Puca *et al.*, 1971), it should be pointed out that the apparent cooperativity in those studies might result from a consistent underestimate of bound ligand or a failure to reach equilibrium. Both possibilities exist when working with extremely low concentrations of ligand.

Effect of MalNEt on the Retention of Estradiol. In order to assess the effect of sulfhydryl reagents on the retention process, uterine and diaphragm tissues were preincubated with MalNEt and subsequently exposed to various concentrations of [^{3}H]-estradiol for 1 hr. Figure 4 shows the result of pretreating uterine or diaphragm tissues with MalNEt on the subsequent retention of [^{3}H]estradiol by the nuclear fraction. Exposure to MalNEt (○) abolishes essentially all of the specific binding of estradiol (●) by uterine nuclei whereas MalNEt exposure (△) appears to increase nonspecific binding in diaphragm nuclei (compare △ with ▲).[3]

Effect of Serum Albumin on Retention of Estradiol. As pointed out earlier the uterus must adsorb and retain estradiol in the presence of extrauterine steroid binding proteins such as serum albumin. In addition, it has been suggested the serum binding proteins may be involved in increasing the selectivity of target tissues which possess protein-permeable capillary beds (Keller *et al.*, 1969). In order to determine the effect of serum albumin on the equilibrium binding (retention) of estradiol, we have incubated uterine and diaphragm tissue in the presence of various concentrations of [^{3}H]estradiol plus 4% serum albumin. Figure 5 is analogous to Figure 3 and shows the intrauterine and intradiaphragm distributions of [^{3}H]estradiol following equilibration at various concentrations of [^{3}H]estradiol and 4% serum albumin. The retention of estradiol by the uterus (Figure 5A) and diaphragm (Figure 5B) is plotted against the equilibrium concentration of total estradiol in the medium in the presence of 4% serum albumin. The linearity of the two data sets for diaphragm (nuclear, circles; cytoplasmic, triangles) in Figures 3B and 5B suggests that there are no tight binding sites for estradiol in the diaphragm. Since serum albumin competes with the diaphragm tissue for estradiol, it is possible to estimate an apparent dissociaton constant for the serum albumin–estradiol complex from the distribution of estradiol inside and outside of the cell. If, as we have previously stated, there is no active estrogen transport in the diaphragm the total estradiol outside the diaphragm cells, $E_{T,out}$ is a function, eq 4, of total estrogen

[3] While no extensive examination of this apparent increase has been made, it might be suggested that MalNEt alters the nuclear membrane in such a way as to increase nonspecific binding.

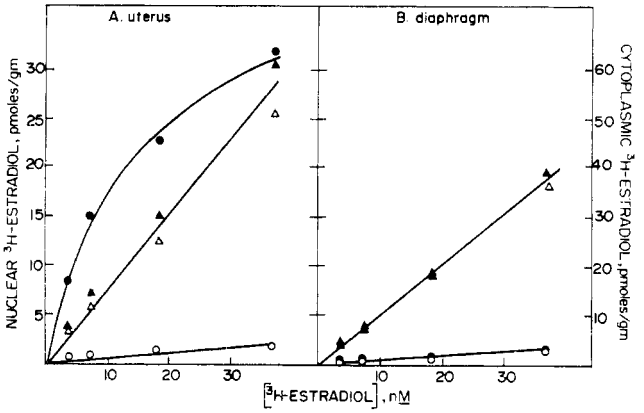

FIGURE 5: Effect of serum albumin on the subcellular distribution of [^{3}H]estradiol at equilibrium in uterus and diaphragm. Data were collected as in Figure 4 in the presence of 4% bovine serum albumin. Nuclear (circles) and cytoplasmic (triangles) levels of [^{3}H]estradiol were analyzed for uterine (A) and diaphragm (B) tissues which were exposed (open symbols) or not exposed (closed symbols) to excess diethylstilbestrol in the incubation medium.

TABLE I: Endogenous Levels of Serum Albumin in the Rat Uterus with and without Luminal Fluid.[a]

Tissue	Concn of Serum Albumin	
	mg/g of Uterus	μmol/1000 g of Uterus
Uterus		
Intact and unwashed (5)	6.2 ± 0.5	91 ± 7.4
Intact and washed (5)	4.7 ± 0.5	69 ± 7.4
Slit and washed (6)	3.7 ± 0.3	54 ± 4.4
Diaphragm		
Intact and washed (4)	1.3 ± 0.17	19 ± 2.5

[a] Serum albumin was determined as described in the Experimental Procedure. Numbers in parentheses refer to the number of determinations. Each determination represents a pool of two or three uteri. Molar quantities were calculated by assuming a mol wt of 68,000 for serum albumin.

inside the diaphragm, $E_{T,in}$, and the serum albumin concentration, $[SA_T]$.

$$E_{T,out} = E_{T,in}[(K_{SAE}^{eff} + [SA_T])/(1 + K_x)(K_{SAE}^{eff})] \quad (4)$$

The constant K_x defines additional nonspecific interaction of estradiol with the diaphragm and is equal to the ratio of the bound estrogen to the *free* estrogen. In the absence of serum albumin, eq 4 reduces to

$$E_{T,in}/E_{T,out} = 1 + K_x \quad (4A)$$

It is possible to determine a value for the apparent or effective dissociation constant for the serum albumin–estradiol complex from the slope of a plot of $E_{T,in}$ *vs.* $E_{T,out}$ in the presence and absence of serum albumin (not shown). The apparent dissociation constant for the serum albumin–estradiol complex, K_{SAE}^{eff}, is 5×10^{-4} M. The dissociation constant was calculated with the assumption that the number of binding sites per serum albumin molecule was equal to unity. If the number of equal and independent binding sites is equal to ten then the dissociation constant would be increased by a factor of ten.

A value for the nonspecific binding constant can also be calculated from the concentration of serum albumin in the uterus (see below) and the dissociation constant of the serum albumin–estradiol complex. This value, 1.1 M, for K_x agrees reasonaby well with that for K_x (0.54 M) determined from the cytoplasmic data of Figure 3A and may represent the dissociation constant for the interaction of estradiol with cellular lipids.

The dissociation constant of the RE complex, K_d^{RE}, in the presence of serum albumin can be calculated from the titration of the uterus with estradiol and the apparent dissociation constant for the serum albumin–estradiol complex. The medium surrounding the uterus consists of free estradiol and the serum albumin–estradiol complex. Since serum albumin $\gg$ estradiol, the ratio of serum albumin–estradiol to free estradiol is equal to the ratio of total serum albumin and the dissociation constant for the serum albumin–estradiol complex. The concentration of free estradiol can be determined from eq 5. The concentrations of E_{free} in the medium

$$[E_{free}] = [E_{medium}]/(1 + [SA_T]/K_{SAE}^{eff}) \quad (5)$$

were calculated *via* eq 5 from initial concentrations of estradiol and serum albumin by using the calculated value of K_{SAE}^{eff}. The specific binding of [^{3}H]estradiol to the nuclear fraction was then fitted as a hyperbolic function of these calculated values of $[E_{free}]$. The calculated values for K_d^{RE} and the value R_{max} are, respectively, 1.3 ± 0.1 nM and 0.04 ± 0.008 pmol/mg and should be compared with the values calculated from Figure 3 (4.62 ± 0.6 nM and 0.064 ± 0.003 pmol/mg, respectively). The differences between the constants calculated in the presence (Figure 5) and absence (Figure 3) of serum albumin are probably not significant and reflect the error in the determination of $[E_{free}]$ from a calculated K_{SAE}^{eff}.

Levels of Serum Albumin in the Uterus and Diaphragm of Rats. In a search for additional estrogen binding sites within the uterus, the serum binding protein, albumin, was considered. If one homogenizes uteri and separates the soluble proteins by electrophoresis, the major protein peak is an albumin fraction which moves rapidly to the anode. Table I shows the result of quantitative electrophoretic analysis of tissue extracts of diaphragm and uterus. The concentration of albumin in the uterus, assuming 100% water, is between 5×10^{-5} and 1×10^{-4} M. The equivalent calculation for diaphragm gives a value of about 2×10^{-5} M. Estimates of the extracellular space of uteri range from 20 to 60% with 40% the most accepted value (Peterson and Spaziani, 1971). Assuming that the bulk of the albumin is located in the extracellular space, the concentration of albumin in the uterus is about 1×10^{-4} M. This concentration of albumin allows for a large number of additional estrogen binding sites in the uterus (see Discussion) and has been used in calculations described above.

Discussion

In determining the physical state of estradiol within the uterus it is important to determine the exact relationship between the receptor protein, R, and the uptake and retention of estradiol, E, by the uterus. Although previous investigators have examined "uptake" phenomena, as stated in the introductory statement, these studies have failed to differentiate clearly between uptake and retention. A particular difficulty

which arises in interpreting previous data on uptake is the long time interval usually employed in these studies. An investigation of uptake must employ short time intervals for two reasons: first, only initial velocity measurements are adequate for studying catalyzed processes such as active transport or facilitated diffusion; second, any deviation from short time periods will result in confusion because of the translocation of RE to the nucleus. In the following discussion uptake is defined as the initial velocity of movement of estradiol into the tissue and has the units picomoles/milligram of tissue per second. Retention, on the other hand, is defined by the equilibrium concentration of estradiol in subcellular fractions of tissues.

This investigation has examined the role of the receptor in uptake and retention. The present results indicate that R is involved only in the retention of estradiol by the uterus and is not important in the uptake mechanism. The experiments employing the specific irreversible sulfhydryl reagent, MalNEt, and the specific competitive inhibitor of the RE interaction, diethylstilbestrol, demonstrate that the receptor is not involved in the initial rate of estradiol uptake (see Figures 1B and 1C). Additional evidence which indicates that R plays no active role in estradiol uptake has been shown by comparing the kinetics of uptake in uterine tissue which possesses 1.2–1.8 pmol of R per uterus and an equivalent mass of diaphragm which possesses no measurable R. Figures 1A–C and 2 demonstrate that there exist no differences in the rate of uptake of estradiol by these tissues. Table II gives the rate constants for the uptake process in both tissues as calculated from Figures 1A–C; no significant differences can be found. In addition, investigations employing an uncoupler of oxidative phosphorylation, N_2ph (see Figure 1B), as well as the observation that the initial velocities of estradiol uptake by the uterus and diaphragm are not saturable processes suggest that no active transport system is involved. A previous examination of the partitioning of steroid hormones between various media has suggested that simple diffusion processes may account for estradiol uptake (Bischoff *et al.*, 1954). It would appear from the present results that estradiol enters the uterus *via* passive diffusion and, subsequent to the diffusion process, estradiol associates with R specifically in a manner which leads to the migration and binding of the RE complex to the nuclear fraction.

In the present investigation cytoplasmic and nuclear compartments were examined to demonstrate that retention of estradiol is the primary function of R and that this retention of estradiol as the RE complex is mainly limited to the nuclear compartment (see Figures 3–5 and below). A plot of total estradiol within the uterus or diaphragm at equilibrium as a function of the initial concentration of [^{3}H]estradiol (not shown) demonstrates that at saturating concentrations of estradiol the difference between diaphragm- or diethylstilbestrol-treated uteri and nontreated uteri accounts for the 1.2–1.8 pmol of cytoplasmic receptor measured in the uterus by other methods (Jensen *et al.*, 1968; Anderson *et al.*, 1972a,b; Toft and Gorski, 1966; Clark and Gorski, 1969; Notides, 1970). In addition, this saturable site is found at equilibrium predominantly in the nuclear fraction (Figures 3–5) (Shyamala and Gorski, 1969). The cytoplasmic compartments of uteri and diaphragms contain the same amount of estradiol at equilibrium *in the absence of serum albumin* and in the presence or absence of diethylstilbestrol (Figure 3). The observation by Williams and Gorski (1972) that equilibrium levels of cytoplasmic RE are very low with respect to nuclear levels of RE indicates that the interaction of the receptor–estradiol complex with nuclear acceptor sites is very favorable. The model (eq 3) must be expanded to include the step (eq 6)

TABLE II: First-Order Rate Constants for Estradiol Uptake in Uterine and Diaphragm Tissue.

Treatment	Uterus	Diaphragm
Control	75.0 ± 3.8[a]	68.0 ± 5.7
Excess diethylstilbestrol	69.0 ± 7.4	72.0 ± 4.9
1 mM MalNEt	73.0 ± 3.6	62.0 ± 6.3
1 mM N_2ph	76.0 ± 8.9	63.0 ± 5.5

[a] Values represent the slope (nmol/g per sec per nM) ± the standard error of the estimate. None of the values differ significantly.

$$R \cdot E \underset{}{\overset{K_1}{\rightleftharpoons}} R \cdot E_{cyt} \overset{K_2}{\rightleftharpoons} R \cdot E_{nuc} \qquad (6)$$

where the receptor–estradiol complex binds to the nucleus. The binding function (eq 7) describing the model for the binding of the RE complex to the nuclear fraction is more complex than that for the interaction of estradiol with cytoplasmic R (Williams and Gorski, 1972). However, the function is hyperbolic in nature since eq 7 can be reduced to the form $[RE_{nuc}] = [E]K_2/([E] + K_2)$.

$$[RE_{nuc}] = \frac{[E]K_2[RE_{max}]/(1 + K_2)}{[E] + [K_1/(1 + K_2)]} \qquad (7)$$

where $K_2 = [RE_{nuc}]/[RE_{cyt}]$ and $K_1 = [E][R]/[RE_{cyt}]$. The equilibrium between RE_{cyt} and RE_{nuc} greatly favors the formation of RE_{nuc}; thus $K_2 \gg 1$ and eq 7 simplifies to

$$[RE_{nuc}] = [RE_{max}][E]/(K_1/K_2) + [E] \qquad (8)$$

The above model does not allow for the possibility of dissociation of the RE_{nuc} complex. Since labeled estradiol in the RE_{nuc} complex will exchange with cold estradiol without loss of nuclear bound receptor (Anderson *et al.*, 1972a,b), it can be suggested that the estradiol does dissociate from the RE_{nuc} complex. A second compelling argument for suggesting another step (eq 9) in the model is the agreement between the dissociation constant determined using the [^{3}H]estradiol exchange assay and partially purified nuclei (Anderson *et al.*, 1972a,b) and dissociation constant calculated from data on whole uteri (see Results).

$$R_{cyt} + E \underset{}{\overset{K_1}{\rightleftharpoons}} RE_{cyt} \overset{K_2}{\rightleftharpoons} RE_{nuc} \overset{K_3}{\rightleftharpoons} R_{nuc} + E \qquad (9)$$

The general binding equation (eq 10) has also been derived for this model

$$[RE_{nuc}] = [RE_{max}][E]/\{[1 + (1/K_2)][E] + K_1/K_2 + K_3\} \qquad (10)$$

where $K_3 = [R_{nuc}][E]/[RE_{nuc}]$.

As before it can be assumed that K_2 is much greater than unity and thus the $1/K_2$ is negligible with respect to unity. Also, it is possible to further simplify eq 1 if K_3 is equal to or greater than K_1, thus yielding a simple hyperbolic function (eq 11).

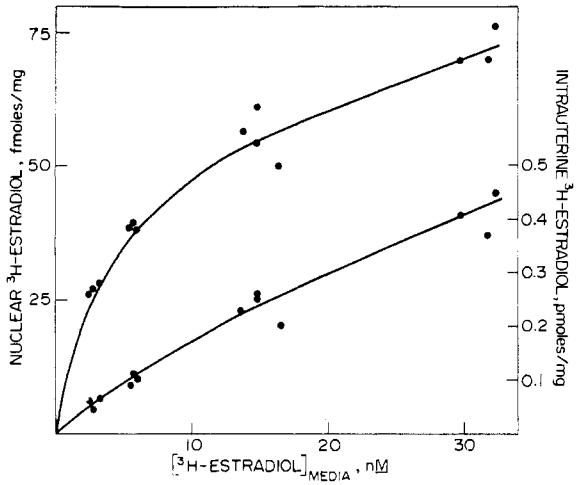

FIGURE 6: Nuclear and total intrauterine [^{3}H]estradiol retention as a function of the concentration of estradiol in the medium at equilibrium: upper curve, [^{3}H]estradiol in the nuclear fraction at equilibrium; lower curve, total intrauterine [^{3}H]estradiol at equilibrium. The closed circles represent individual data points while the lines are computer calculated from the constants derived in Results.

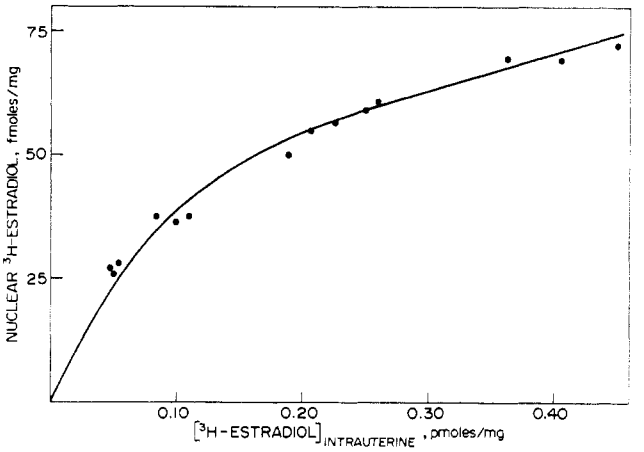

FIGURE 7: Nuclear [^{3}H]estradiol as a function of total intrauterine estradiol. Closed circles represent experimental points while the line was calculated *via* computer simulation employing the constants derived from the present results and utilized in Figure 6.

$$[RE_{nuc}] = [RE_{max}][E]/(K_3 + [E]) \quad (11)$$

Since the dissociation constant calculated from binding experiments involving the whole uterus is very similar to the dissociation constant determined for partially purified nuclei, it seems reasonable to assume that the absorption of the receptor protein to the nucleus does not affect the binding of estradiol to the receptor protein.

In investigating the uptake and retention of estradiol by the uterus one must consider the role of plasma binding proteins in the overall process. Figure 2 examines the kinetics of uptake of estradiol in the presence of varying concentrations of serum albumin. The rate of uptake of estradiol by uterus or diaphragm is reduced in the presence of serum albumin but first-order kinetics continue to apply. The decrease in the rate of uptake may be attributed to a decrease in the concentration of *free* estradiol in the external medium containing serum albumin. In fact, the effect of varying the concentration of serum albumin on uptake rate of estradiol has been used to calculate an effective dissociation constant for the serum albumin–estradiol complex (see Results).

The effect of serum albumin on the retention of estradiol in the uterus can be seen in Figure 5. In the presence of exogenous serum albumin, the concentration of estradiol required for the saturation of nuclear RE is dramatically increased, undoubtedly the result of competition for estradiol by serum albumin. This competition if applied to the interstitial and/or intracellular concentration of plasma binding proteins may explain the high levels of estradiol required to saturate R within the uterus. In fact, using the dissociation constants previously calculated for the RE complex, the concentration of serum albumin measured for uterine tissue (see Table I), and the dissociation constants calculated for serum albumin–estradiol complexes from kinetic and equilibrium studies, a computer simulation of RE formation in the uterus *in vitro* may be made. Figure 6 shows the results of such an analysis in which RE and total intrauterine E are plotted as a function of the concentration of estradiol [E_T] in the medium at equilibrium. The points on this plot are experimental data whereas the line was calculated by computer analysis (see Results) (Cleland, 1963). Figure 7 shows nuclear levels of RE as a function of total *intrauterine* estradiol. Again the points are experimental data while the line is a replot of RE *vs.* total intrauterine estradiol from the computer simulation of Figure 6. The agreement between the experimental results and the computer-simulated plots suggests that this analysis which employs only two types of sites essentially defines the interaction of estradiol with the uterus *in vitro*. Similar studies in media containing other steroid binding proteins and/or *in vivo* experiments should allow for a complete understanding of the physiologic interaction of estradiol with the uterus.

In summary, these studies demonstrate that the entry of estrogen into uterine cells is a passive process and that cytoplasmic R is not involved in the rate of estrogen uptake. Rather, the formation of the RE complex subsequent to the entry of E functions to retain E within the uterus and perhaps, more specifically, within the nuclei of uterine cells. The presence of serum steroid binding proteins in the incubation medium, and presumably in the blood, decreases the *rate* of entry of E into the uterus (see Figure 2B) but does not alter the passive nature of E entry *in vitro*. It has been suggested that serum binding proteins may act to direct hormonal signals toward organs with protein-permeable vascular beds (Keller *et al.*, 1969) and it has been shown that estradiol increases the accumulation and retention of albumin by the uterus while not affecting such nontarget tissues as psoas muscle, liver, or brain (Peterson and Spaziani, 1971). This accumulation and retention of albumin and thus presumably albumin-bound estrogen may be involved in establishing levels of *total* estrogen in the uterus that are greater than those of the systemic circulation and which may be maintained at higher levels for longer periods of time. It should be noted from the present results that cytoplasmic levels of estradiol at equilibrium are greater for the uterus than for diaphragm *in the presence of serum albumin* (Figure 5), whereas levels of estradiol at equilibrium are the same for both tissues *in the absence of serum albumin* (Figure 3). Thus, as suggested by Keller *et al.* (1969), steroid binding proteins, of which serum albumin is one example, may have a physiological role in increasing hormone concentration preferentially in target organs which possess protein-permeable vascular beds, thereby maintaining the concentration of hormone at a level which is required to produce hormonal stimulation.

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References

Anderson, J., Clark, J. H., and Peck, E. J., Jr. (1972a), *Biochem. J. 126*, 561.

Anderson, J. N., Clark, J. H., and Peck, E. J., Jr. (1972b), *Biochem. Biophys. Res. Commun. 48*, 1460.

Bischoff, F., Stauffer, R., and Gray, C. (1954), *Amer. J. Physiol. 177*, 65.

Casciano, D. A. (1971), Ph.D. Thesis, Purdue University, Lafayette, Ind.

Clark, J. H., and Gorski, J. (1969), *Biochim. Biophys. Acta 192*, 508.

Cleland, W. W. (1963), *Nature (London) 198*, 463.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Erdos, T., Bessada, R., Best-Belpomme, M., Fries, J., Gospodarowicz, D., Menahem, M., Reti, E., and Veron, A. (1971), *Advan. Biosci. 7*, 119.

Erdos, T., Bessada, R., and Fries, J. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 5*, 161.

Giannapoulos, G., and Gorski, J. (1971), *J. Biol. Chem. 246*, 2524.

Gorski, J., Toft, D., Shyamala, G., and Smith, D. (1968), *Recent Progr. Horm. Res. 24*, 25.

Jensen, E. V., Hurst, D. J., DeSombre, E. R., and Jungblut, P. W. (1967), *Science 158*, 385.

Jensen, E., Suzuki, T., Kawashima, T., Stumpf, D., Jungblut, P., and DeSombre, E. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 632.

Keller, N., Richardson, U. I., and Yates, F. E. (1969), *Endocrinology 84*, 49.

Milgrom, E., Atger, M., and Baulieu, E. E. (1972), *C. R. Acad. Sci. 274*, 2771.

Notides, A. C. (1970), *Endocrinology 87*, 987.

Peterson, R. P., and Spaziani, E. (1971), *Endocrinology 89*, 1280.

Puca, G. A., Nola, E., Sica, V., and Bresciani, F. (1971), *Advan. Biosci. 7*, 97.

Rochefort, H., and Baulieu, E. E. (1969), *Endocrinology 84*, 108.

Shyamala, G., and Gorski, J. (1969), *J. Biol. Chem. 244*, 1097.

Sweet, C., and Zull, J. E. (1969), *Biochim. Biophys. Acta 173*, 94.

Toft, D., and Gorski, J. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 1574.

Williams, D., and Gorski, J. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 3464.

Instability of the Uterine Estrogen Receptor under *in Vitro* Conditions†

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ABSTRACT: The stability of the estrogen receptor in excised uteri during *in vitro* incubation at various temperatures in a medium which is devoid of estrogen has been examined. The uterine estrogen receptor is very unstable in culture conditions with an 85% loss in diethylstilbestrol-competeable, high-affinity estrogen binding in the cytoplasmic fraction after 2 hr at 30–37°. Attempts to demonstrate the leakage of specific binding sites into the incubation medium or the translocation of receptor to the nuclear compartment of uterine tissue were without success. The presence of retinal in the incubation medium enhanced the rate of loss of receptor from uterine cytoplasm; however, hydrocortisone acetate did not prevent the loss of receptor under *in vitro* conditions. The present results suggest that the uterine estrogen receptor is very unstable at elevated temperatures in the absence of estrogen and that this instability may explain the failure of previous attempts to demonstrate physiologic responses of the uterus *in vitro*.

Since the recognition that estrogens exert their effects on target tissues *via* a specific protein, the estrogen receptor (R),[1] many attempts have been made to correlate target tissue responses with the levels of receptor–estrogen complex (RE) present in the target tissue. A great deal of effort has been expended on the development of an *in vitro* system for relating physiologic response to the level of RE in uterine cells. However, with the exception of the induction of an estrogen-dependent uterine protein (IP) by Gorski and coworkers (Katzenellenbogen and Gorski, 1972), all attempts at the development of an *in vitro* system which is responsive to physiologic concentrations of estrogens have failed (Mueller *et al.*, 1958; Hecter *et al.*, 1966; Rao and Talwar, 1972).

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[1] Abbreviations used are: R, the high affinity estrogen binding protein or receptor; E, estradiol; RE, the complex of estradiol with the estrogen receptor; TE, a buffer containing 10 mM Tris and 1.5 mM EDTA at pH 7.4; TKM, a buffer containing 40 mM Tris, 100 mM KCl, and 4 mM $MgCl_2$ at pH 7.2.