Oestrogen and Nuclear Binding Sites

DETERMINATION OF SPECIFIC SITES BY [^3H]OESTRADIOL EXCHANGE

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A method was developed for the determination of the number of specific oestradiol-binding sites in the nuclear fraction of oestrogen-sensitive tissues. The method is based on the exchange of [^3H]oestradiol with non-labelled oestradiol that is bound to nuclear binding sites. The number of specific nuclear binding sites after the injection of $2.5\text{ }\mu\text{g}$ of oestradiol, an amount sufficient to saturate all binding sites, is $1.6\text{--}1.7\text{ pmol}$ per immature uterus. The number of sites occupied after an injection of physiological amounts of oestradiol ($0.1\text{ }\mu\text{g}$) was 0.46 pmol . The injection of oestradiol results in an increased number of nuclear binding sites in uterus and vagina, but has no effect on kidney or muscle. Injections of testosterone or progesterone failed to increase the number of uterine nuclear binding sites. This method permits an evaluation of the number of oestradiol-binding sites in the nuclear fraction of various tissues as a function of either endogenous oestradiol or non-labelled oestradiol administered by injection.

Oestrogenic compounds are preferentially retained by oestrogen target tissues. This phenomenon has been attributed to oestrogen-binding proteins. Numerous investigations have revealed that the interaction of oestradiol with the rat uterus is probably a two-step process in which oestrogen first binds with a cytoplasmic component to form a complex, which subsequently moves to the nucleus (Jensen *et al.*, 1968, 1969; Shyamala & Gorski, 1969). As a result of this translocation, the depletion of cytoplasmic binding sites is accompanied by a parallel increase in the quantity of nuclear binding sites (Giannopoulos & Gorski, 1971). The association of oestradiol with nuclear binding sites is considered to be of fundamental importance in the mechanism of action of oestrogen. Further, the determination of specific nuclear binding sites may be the only accurate reflection of the amount of oestradiol bound in the uterus (Williams & Gorski, 1971; J. H. Clark & E. J. Peck, unpublished work).

Previous methods for evaluating the quantity of nuclear binding sites have utilized gel filtration or density-gradient ultracentrifugation of nuclear extracts previously solubilized from the uterine nuclear fraction with buffered $0.3\text{--}0.4\text{ M}$ -potassium chloride solutions (Puca & Bresciani, 1968, 1969). These procedures are laborious, difficult to use quantitatively, and require the injection of [^3H]oestradiol for studies *in vivo*. We have developed a method for the determination of the number of oestradiol-binding sites in the nuclear fraction of oestrogen-target tissues that circumvents these difficulties. The method relies on the exchange of [^3H]oestradiol with oestradiol bound to the nuclear fraction and permits an evaluation of the concentration of specific oestradiol-binding sites under conditions *in vivo*.

Materials and Methods

Preparation of animals and materials

Immature female rats (21-23 days old) of the Purdue-Wistar strain were used in this study. The animals, which weighed between 50 and 65 g, were maintained in air-conditioned quarters and given food and water *ad lib*. Oestradiol dissolved in 0.5 ml of 0.9% NaCl was injected subcutaneously between 08.00 and 11.00 a.m. Where indicated, testosterone, progesterone, stilboestrol or 0.9% NaCl were used in place of the oestradiol. [$6,7\text{-}^3\text{H}$]Oestradiol (New England Nuclear Corp., Boston, Mass., U.S.A.; specific radioactivity 40 Ci/mmol) was examined for purity by t.l.c.

Determination of nuclear binding sites by the [^3H]oestradiol-exchange assay

Animals were killed by cervical dislocation; the uteri were stripped of adhering fat and mesentery and placed in cold 0.9% NaCl. All subsequent procedures were performed at 4°C unless otherwise indicated. The uteri were washed in buffer containing 10 mM -tris-HCl and 1.5 mM -EDTA, pH 7.4, and homogenized in all-glass Kontes homogenizers with a motor-driven pestle. The nuclear myofibrillar fraction was obtained by centrifugation of the homogenate at $800g$ for 10 min. This fraction was washed three times with 3 ml of buffer and each wash was followed by centrifugation at $800g$ for 10 min. The washed pellet was suspended in buffer to a concentration equivalent to 0.5 uterus/ml and gently rehomogenized. Portions (0.5 ml) of this suspension were dispensed into two series of tubes, A and B, containing 0.2 ml of buffer. Series A contained various

concentrations of [^3H]oestradiol and was used to determine the total amount of [^3H]oestradiol exchange. Series B contained the same concentrations of [^3H]oestradiol as in A plus a 100-fold excess of stilboestrol and was used to determine the specific [^3H]oestradiol exchange. The nuclear fractions were incubated with shaking at 37°C for 1 h. After the incubation, 3 ml of buffer was added to the incubation tubes, which were subsequently centrifuged at 800g for 10 min. Pellets were washed twice with buffer and extracted with 3 ml of ethanol. The ethanol extract was added to 10 ml of scintillation fluid [99.5% toluene–0.45% 2,5-diphenyloxazole–0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene]. Radioactivity was determined with a Packard 3375 liquid-scintillation counter at 33% efficiency. Automatic external standardization was employed to determine the degree of quenching in each sample.

Incubation of whole uteri in vitro

[6,7- ^3H]Oestradiol and non-radioactive oestradiol were prepared in ethanol and dried under nitrogen in 10 ml glass vials before use in the incubation experiments. Each incubation vial contained two uteri in 2 ml of Eagle's (1955) HeLa medium (Difco Inc., Detroit, Mich., U.S.A.). The incubations were conducted for 1 h at 37°C under air. After incubation, the uteri were washed twice with 3 ml of buffer and the nuclear fraction was isolated and treated as described above.

Results and Discussion

Validation of the nuclear exchange assay

The assumptions made to calculate the quantity of specific nuclear binding sites by the nuclear exchange assay method are as follows.

(a) The exposure of uteri to oestradiol leads to an increase in the number of specific nuclear binding sites.

(b) The quantity of specific binding sites found within the nuclear fraction is directly proportional to exchange during the assay period.

(c) Binding sites are not degraded in the nuclear fraction during the incubation period.

(d) The non-labelled oestradiol that is released from the nuclear fraction during the exchange process does not significantly decrease the specific radioactivity of the [^3H]oestradiol in the assay buffer, and therefore does not significantly affect the amount of [^3H]oestradiol bound by the nuclear fraction.

(e) Oestradiol, previously in a complex with nuclear binding sites as a result of hormone injection or the incubation of intact uteri with oestradiol, is freely exchangeable with [^3H]oestradiol during the incubation of the nuclear fraction at 37°C.

(f) The exchange of [^3H]oestradiol is stereospecific for oestrogen-binding sites.

To evaluate the above assumptions, incubations of intact uteri were conducted *in vitro* (Tables 1 and 2). In these studies intact uteri were incubated for 1 h at 37°C without oestradiol, with non-labelled oestradiol or with [^3H]oestradiol. After the incubation period the nuclear fraction was prepared and the nuclear exchange assay was performed as described in the Materials and Methods section. The first series of experiments (Table 1, A–D) demonstrates that the presence of oestradiol in the incubation medium promotes a large increase in the quantity of specific nuclear binding sites (assumption a). Comparison of (A) and (C), Table 1, shows this increase in nuclear binding sites and (B) and (D), Table 1, give a measure of the non-specific binding sites present in either case. The small but significant difference between (A) and

Table 1. Analysis of the nuclear exchange reaction after incubation of intact uteri *in vitro*

Intact uteri were incubated for 1 h at 37°C without oestradiol, with non-radioactive oestradiol or with [^3H]oestradiol and the nuclear fraction was prepared and assayed by the [^3H]oestradiol-exchange method. Each value is the mean $\pm$ S.E.M. of four to six determinations with two uteri/determination.

Conditions of incubation of intact uteri	Conditions for assay of nuclear fraction	[^3H]Oestradiol/uterus $\pm$ S.E.M. (pmol)
No oestradiol	(A) [^3H]Oestradiol (13 nM)	0.56 $\pm$ 0.04
	(B) [^3H]Oestradiol (13 nM) + stilboestrol (1.5 μM)	0.24 $\pm$ 0.02
Non-radioactive oestradiol (37 nM)	(C) [^3H]Oestradiol (13 nM)	1.58 $\pm$ 0.07
	(D) [^3H]Oestradiol (13 nM) + stilboestrol (1.5 μM)	0.26 $\pm$ 0.02
[^3H]Oestradiol (37 nM)	(E) Not assayed*	1.55 $\pm$ 0.11
	(F) No oestradiol added	0.94 $\pm$ 0.05
	(G) [^3H]Oestradiol (13 nM)	1.46 $\pm$ 0.05
	(H) Oestradiol (13 nM)	0.21 $\pm$ 0.02
	(I) Stilboestrol (1.5 μM)	0.14 $\pm$ 0.01

* The concentration of [^3H]oestradiol in the nuclear fraction was determined after incubation of intact uteri.

Table 2. *Effects of incubation temperature and time on total, non-specific and specific exchange of [³H]oestradiol by the nuclear fraction*

Rats were injected with 2.5 µg of oestradiol and their uteri were removed 1 h later. The nuclear fraction was incubated with [³H]oestradiol (13 nM) with and without a 100-fold excess of stilboestrol. The values for specifically bound [³H]oestradiol were obtained by difference from columns 3 and 4. Each value represents the mean ± S.E.M. of three or four determinations.

	Temperature of incubation (°C)	Time of incubation (min)	[³ H]Oestradiol (pmol)	[³ H]Oestradiol+100-fold stilboestrol (pmol)	Specifically bound [³ H]oestradiol (pmol)
(A)	0-4	60	0.52 ± 0.02	0.24 ± 0.01	0.28 ± 0.02
	25	60	1.15 ± 0.12	0.25 ± 0.01	0.90 ± 0.07
	37	60	1.82 ± 0.18	0.30 ± 0.02	1.52 ± 0.07
	55	60	0.57 ± 0.05	0.31 ± 0.03	0.26 ± 0.07
(B)	37	5	1.42 ± 0.05	0.24 ± 0.02	1.20 ± 0.06
	37	30	1.83 ± 0.10	0.28 ± 0.04	1.55 ± 0.14
	37	60	1.80 ± 0.04	0.28 ± 0.01	1.52 ± 0.04

either (B) or (D), Table 1, is a measure of nuclear sites that result from endogenous oestradiol. These results suggest that exposure of the uterus to oestradiol increases the number of specific nuclear binding sites *in vitro* (A versus C).

Fig. 1 demonstrates that assumption (b) is true; that is, the quantity of [³H]oestradiol specifically exchanged in the assay procedure is a linear function of the concentration of the uterine nuclear fraction. In this experiment, four concentrations of nuclei from oestradiol-treated uteri were examined with the exchange method. Fig. 1 is a plot of specifically bound [³H]oestradiol as a function of the concentration of nuclei. It is apparent that [³H]oestradiol exchange is proportional to the quantity of specific binding sites in the nuclear fraction.

To demonstrate the absence of significant degradation or loss of binding sites from the nuclear fraction during the assay procedure (assumption c), experiments were performed as described below and outlined in (E)–(G), Table 1: (E) gives a measure of total nuclear binding by uteri that have been exposed for 1 h to labelled oestradiol; in (F) the nuclear fraction was exposed for 1 h to buffer without oestradiol and the quantity of nuclear [³H]oestradiol was determined; (G) shows the quantity of [³H]oestradiol bound to nuclear fractions exposed to [³H]oestradiol for 1 h. A comparison of (E) and (F), Table 1, indicates that during the assay interval approx. 40% of [³H]oestradiol was lost to the assay buffer; this difference represents the dissociation of [³H]oestradiol from the nuclear fraction in the absence of oestradiol. However, the binding capacity of the nuclear fraction is not altered during the assay period, as shown by a comparison of (E) and (G), Table 1; the addition of [³H]oestradiol to the assay buffer results in a quantity of [³H]oestradiol (G) that is not significantly different from the control (E).

Fig. 1. *Relationship between specific [³H]oestradiol exchange and the amount of uterine nuclear fraction*

Rats were injected with 0.25 µg of oestradiol and their uteri were removed 1 h later. Uteri were homogenized and the uterine nuclear fraction was diluted to one uterine equivalent/ml. Portions of the uterine nuclear fraction containing 13 nM [³H]oestradiol with and without a 100-fold excess of stilboestrol at a final volume of 0.7 ml were incubated for 1 h at 37°C. The quantity of [³H]oestradiol bound in the presence of a 100-fold excess of stilboestrol has been subtracted from the amount of total nuclear binding with [³H]oestradiol alone to yield the quantity of specifically bound [³H]oestradiol, which is shown on the ordinate. Each point represents the mean ± S.E.M. of three determinations.

During the 1 h assay period the quantity of specific binding sites did not change.

In Table 1, (C) and (G) show that oestradiol bound to nuclear sites during the incubation of intact uteri

does not interfere with the subsequent exchange of [^3H]oestradiol (assumption *d*). In this experiment uteri were incubated with either [^3H]oestradiol or oestradiol. The nuclear fractions obtained from these incubations were subsequently incubated with [^3H]oestradiol. The amount of [^3H]oestradiol extracted from the nuclear fraction of the uteri that were incubated with oestradiol (1.58 ± 0.07 pmol/uterus) is not significantly different from that obtained from uteri that were incubated in [^3H]oestradiol (1.46 ± 0.05 pmol/uterus). In addition, these results suggest that oestradiol that is bound to nuclear sites during the incubation of intact uteri is replaced by the [^3H]oestradiol during the incubation of the nuclear fraction (see below).

To determine the exchangeability of oestradiol bound to nuclear sites (assumption *e*), intact uteri were incubated with [^3H]oestradiol. The addition of non-labelled oestradiol to the nuclear fractions from the above incubation decreased the specific nuclear binding of [^3H]oestradiol by 90–95% (see Table 1: E, H and I). Therefore oestradiol bound to nuclear binding sites is readily exchangeable with oestradiol in the assay buffer at 37°C.

To determine the effects of various oestrogenic and non-oestrogenic hormones on the quantity of specific oestrogen-binding sites in the uterine nuclear fraction, rats were injected with 2.5 µg of testosterone, progesterone, oestradiol, stilboestrol or 0.9% NaCl. At 1 h after injection, the uterine nuclear fraction was prepared and used in the [^3H]oestradiol nuclear exchange assay as described in the Materials and Methods section (Fig. 2). The administration of the potent oestrogens oestradiol and stilboestrol resulted in a 10-fold increase of specific nuclear binding over control values. In contrast, the non-oestrogenic steroids, progesterone and testosterone, failed to show any significant effect on the number of nuclear receptors in the rat uterus. Thus Fig. 2 conclusively demonstrates the specific nature of receptor translocation by the oestrogenic hormones.

Because nuclear sites involved in the [^3H]oestradiol-exchange assay are expected to exhibit a high specificity for oestrogenic compounds, various hormones were added to [^3H]oestradiol in the assay buffer (Fig. 3). The addition of progesterone or testosterone failed to alter significantly the quantity of [^3H]oestradiol bound ($P > 0.05$). However, at all concentrations stilboestrol markedly competes with [^3H]oestradiol for the nuclear sites. Binding to nuclear sites is not a result of the non-specific interaction of hydrophobic steroids with nuclear proteins, but rather is a stereospecific process.

The exchange between specifically bound oestradiol and [^3H]oestradiol in the assay buffer appears to be a highly temperature-dependent process (Table 2). The specific nuclear exchange of [^3H]oestradiol increased from low values at 0–4°C to maximal values

Fig. 2. Effects of various hormones on the quantity of oestrogen-binding sites in the uterine nuclear fraction

Rats were injected with 2.5 µg of oestradiol, stilboestrol, testosterone, progesterone or saline and their uteri were removed 1 h later. The nuclear fraction was incubated with [^3H]oestradiol (13 nM) (■) or [^3H]oestradiol in the presence of a 100-fold excess of stilboestrol (□). Results are expressed as the mean $\pm$ S.E.M. of at least three samples/group.

Fig. 3. Specificity of the exchange assay: effects of various hormones on the exchange of [^3H]oestradiol in uterine nuclear fraction

Rats were injected with 2.5 µg of oestradiol and their uteri removed 1 h later. Various non-labelled hormones were mixed with [^3H]oestradiol (13 nM) at the ratios (non-labelled steroid/[^3H]oestradiol) given in parentheses and were incubated with the uterine nuclear fraction. Each mean $\pm$ S.E.M. was calculated from six determinations.

at 37°C. At 55°C the quantity of specific binding was drastically decreased whereas non-specific uptake of [^3H]oestradiol was not significantly altered. These

results at elevated temperatures have been observed by others and suggest denaturation of specific nuclear binding sites (Toft & Gorski, 1966; Jensen *et al.*, 1968; Puca & Bresciani, 1968). Table 2(B) shows the

Table 3. *Effects of oestradiol treatment on the quantity of specific nuclear binding sites in oestrogen target and non-target tissues*

Rats were injected with 0.9% NaCl or 2.5 µg of oestradiol and their uteri removed 1 h later. The nuclear fractions obtained from kidney, diaphragm, vagina and uterus were incubated with [³H]oestradiol (13 nM) with and without a 100-fold excess of stilboestrol. Each value represents the mean ± S.E.M. of at least three determinations.

Tissue	Specifically bound [³ H]oestradiol (pmol/100 mg wet wt.)	
	Saline-injected	Oestradiol-injected
Diaphragm	0.08 ± 0.04	0.06 ± 0.02
Kidney	0.24 ± 0.05	0.20 ± 0.06
Vagina	0.41 ± 0.05	2.15 ± 0.01
Uterus	0.61 ± 0.05	5.92 ± 0.41

time-course of nuclear exchange at 37°C as a function of incubation time. Maximum specific exchange occurred by 30 min and no significant decrease was observed at 1 h. The time-course of exchange supports the observations in Table 1 that no significant degradation occurs during the exchange reaction.

Influence of oestradiol on the number of nuclear binding sites of target and non-target tissues

It has been suggested that the translocation process of the cytoplasmic receptor-oestrogen complex to the nuclear fraction is responsible for the characteristic retention of oestrogenic hormones in target tissues (King *et al.*, 1965; Jensen *et al.*, 1968; Shyamala & Gorski, 1969). This suggestion predicts that oestradiol treatment would promote an increase in the number of binding sites in the nuclear fraction exclusively in oestrogen-sensitive tissues. Table 3 supports this prediction. Oestradiol treatment results in a marked increase in specific nuclear binding sites in both the uterus and the vagina. If a value of 100 is assigned to the number of sites in the uterine nuclear fraction after the injection of 2.5 µg of oestradiol, the respective number of nuclear sites in the vagina, kidney and muscle are 36, 4 and 1 respectively (Table 3). These results parallel the

Fig. 4. *Determination of the number of specific nuclear binding sites in the nuclear fraction of rat uteri by the [³H]oestradiol-exchange assay*

(a) Rats were injected with 2.5 µg of oestradiol. At 1 h after hormone administration the rats were killed and the uterine nuclear fraction was prepared as described in the Materials and Methods section. This fraction was incubated for 1 h at 37°C with either [³H]oestradiol alone (series A, ●—●) or [³H]oestradiol plus stilboestrol at 100 times the concentration of [³H]oestradiol (series B, ▲—▲). Series C (○—○) was obtained by subtracting the amount of [³H]oestradiol found with the nuclear fraction incubated with stilboestrol from the total nuclear binding in the absence of stilboestrol. Each point represents the mean of six determinations, with three rats per determination. (b) Lineweaver-Burk plot of the data represented by series C (○—○) in part (a). The apparent K_d^{app} is 1.3 nM and the number of binding sites per uterus is 1.8 pmol.

quantities of radioactivity found within these tissues after an injection of [^3H]oestradiol (Jensen & Jacobson, 1962).

Measurement of nuclear binding sites after the injection of oestradiol

The [^3H]oestradiol-exchange assay as described in the Materials and Methods section was performed 1 h after an injection of $2.5\mu\text{g}$ of oestradiol (Fig. 4a). The concentration of [^3H]oestradiol found within the nuclear fraction in the presence of a 100-fold excess of stilboestrol is a linear function of the concentration of [^3H]oestradiol initially present in the assay buffer. Because a 100-fold excess of stilboestrol results in maximal competition for nuclear binding of [^3H]oestradiol (Fig. 3), the [^3H]oestradiol present in nuclear fractions that were incubated with stilboestrol is non-specifically bound. The quantity of oestradiol specifically bound to the nucleus can be determined by subtracting the non-specific fraction from the total nuclear binding, as shown in Fig. 4(a) (J. H. Clark & E. J. Peck, unpublished work). Analysis of these results by a double-reciprocal plot, Fig. 4(b), gives an apparent dissociation constant, K_d^R , of $1.3 \times 10^{-9}\text{M}$, which is in agreement with that obtained by others for both cytoplasmic and nuclear oestrogen-binding sites (Toft & Gorski, 1966; Toft *et al.*, 1967; Clark & Gorski, 1969; Erdos *et al.*,

1969; Puca & Bresciani, 1969; Shyamala & Gorski, 1969; Baulieu & Raynaud, 1970). The number of specific nuclear binding sites per immature rat uterus (wet wt. 28–36mg) that are present 1 h after the injection of $2.5\mu\text{g}$ of oestradiol is approx. 1.7–1.8 pmol. To determine the number of pmol of specific binding site present in the nuclear fraction under any experimental situation, either a saturation curve such as Fig. 4(a) or a concentration of oestradiol equivalent to $10^3 \times K_d^R$ must be employed. Since either experiment is expensive in experimental animals or labelled oestradiol or both, we have chosen to operate at 94–95% saturation in subsequent experiments. Approx. 94% of the specific nuclear binding sites are occupied by [^3H]oestradiol at a concentration of 13 nM in the assay buffer (Fig. 4a). In subsequent experiments this concentration of [^3H]oestradiol with and without a 100-fold excess of stilboestrol was utilized.

The effect of oestradiol dosage on the increase of specific nuclear binding sites 1 h after the injection of hormone is shown in Fig. 5. The number of specific sites in the uterine nuclear fraction is a hyperbolic function of the dosage of oestradiol. The number of specific binding sites increases from the control value of 0.2 pmol/uterus to maximum values of 1.6–1.7 pmol/uterus in rats injected with 2.5 or $25\mu\text{g}$ of oestradiol. The small but significant number of nuclear binding sites in control animals also seen in Table 1, (A) versus (B) or (D), may reflect the small

Fig. 5. Measurement of the number of specific nuclear binding sites by the [^3H]oestradiol-exchange assay after the injection of various dosages of oestradiol

Rats which weighed 50–65 g were injected subcutaneously with various doses of oestradiol or 0.9% NaCl. At 1 h after the injections, the animals were killed and the uterine nuclear fraction was incubated with [^3H]oestradiol alone (13 nM) (—) or [^3H]oestradiol (13 nM) plus a 100-fold excess of stilboestrol (---). Each point on the graph is the mean $\pm$ S.E.M. of at least four determinations, with two rats per determination.

quantity of endogenous oestrogen that is present in the prepubertal rat (Presl *et al.*, 1967).

Conclusion

The [³H]oestradiol-exchange assay can be used to determine the number of specific nuclear binding sites in oestrogen-sensitive tissues. This assay is of special significance in view of the demonstrations that the amount of oestrogen that is specifically bound in the uterus is that present in the nuclear fraction (Williams & Gorski, 1971; J. H. Clark & E. J. Peck, unpublished work). It has been suggested that the interaction of oestradiol with target tissues and the responses that are produced are directly related to the number of oestrogen-binding sites in the nucleus. The method given in the present paper will enable the determination of the number of nuclear binding sites and the comparison of the quantitative relationships between the number of sites and the extent of response. In addition, the exchange assay will permit the examination of the quantity of nuclear binding sites during the oestrous cycle and under experimental conditions that heretofore could be performed only with [³H]oestradiol.

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