Intracellular Relationships of the Oestrogen Receptor in the Rat Uterus and Hypothalamus during the Oestrous Cycle

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Simultaneous measurements were made of the specific oestrogen receptor in the nuclear and cytosol fractions prepared from the uterus and hypothalamus of 50–81-day-old female rats undergoing a 4-day oestrous cycle. In the uterus, the content of nuclear receptor fluctuated in concert with known cyclic changes in the secretion of oestrogen, being maximal at pro-oestrus. Over the period of 50–81 days, the nuclear content at all phases increased with age, again corresponding to known age-related increases in ovarian secretion of oestrogen. This age-related increase in nuclear content, averaged from the values of the different phases in each age group, was related to equivalent increases in uterine wet weight, an increase of 1 pmol of receptor being accompanied by an increase of 80–90 mg. The concentration of cytosol receptor was maintained constant, with respect to wet weight, throughout the cycle and with age, irrespective of changes in nuclear content. In the uterus of normal mature females, translocation of receptor into the nucleus did not lead to depletion of cytosol receptor, suggesting a process of continuous replenishment/synthesis. In the hypothalamus, the nuclear content of oestrogen receptor was also maximal at pro-oestrus. In contrast with the uterus, the content of hypothalamic cytosol receptor was minimal at this phase and reflects depletion of the cytosol receptor, possibly as a result of translocation. The extent of translocation was low compared with that in the uterus and did not alter with age during the age-period studied. This low nuclear binding of the receptor *in vivo* is discussed in relation to the presence of a cytosol factor, present in limiting amounts, which *in vitro* mediates the binding of cytosol receptor to oligo(dT)–cellulose. The difference in the physiological response of the uterus and of the hypothalamus to oestrogens may be related to the extent of nuclear binding of receptor.

The ovarian secretion of oestrogens is regulated by gonadotropins released from the anterior pituitary. Variations in this secretion play a key role in the regulation and maintenance of the oestrous cycle in rats. There are cyclic changes in the physiology of the uterus accompanying these cyclic changes in plasma concentrations of oestrogen (Astwood, 1939; Brenner & West, 1975).

Within the uterus there are specific receptors for oestrogen. It is widely accepted that these receptors in the cytosol, on binding to oestradiol, are translocated to the nucleus, where they exert their effect on genetic activity (Buller & O'Malley, 1976; Gorski & Gannon, 1976). The content of nuclear receptor has been shown to change in concert with the changes in the concentration of circulating oestradiol, being highest at pro-oestrus (Clark *et al.*, 1972; Iacobelli, 1973). However, there are conflicting reports on the behaviour of the cytosol receptor during the oestrous cycle (see Brenner & West, 1975). For example, at pro-oestrus, Lee & Jacobson (1971) reported that

cytosol receptor content was lowest, whereas Feherty *et al.* (1970) observed that the content was maximal at this phase.

The hypothalamus of the adult female rat is directly involved in the maintenance of the oestrous cycle. It regulates the release of gonadotropins, which control periodic changes in uterine physiology. At pro-oestrus the large increase in the concentration of circulating oestrogens leads to an increased secretion of gonadotropins, which results in ovulation at oestrus (Barraclough, 1973; Schwartz & McCormack, 1972). It has been shown that oestradiol binds to neurons in the hypothalamic region of the brain (Pfaff, 1968; Stumpf, 1968) and that there are specific oestrogen receptors within the hypothalamus (Eisenfeld, 1970). As in other target organs for the hormone, the hypothalamic receptor is translocated from the cytosol into the nucleus in response to the administration of oestradiol (Anderson *et al.*, 1973). At pro-oestrus it has been observed that there is a depletion of the cytosol receptor (Ginsburg *et al.*, 1975), possibly as a result of translocation, although nuclear receptor content was not measured.

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In the present paper, we re-examine the intracellular relationships of the oestrogen receptor in the uterus and report that the increase in nuclear content at pro-oestrus is not accompanied by depletion of the cytosol receptor; further, we show that cytosol content is related to the wet weight throughout the cycle and with age. In addition, we have made simultaneous measurements on the hypothalamus, and observe that in this tissue nuclear content of receptor is highest at pro-oestrus and that translocation is accompanied by cytosol depletion. We have also measured changes in the binding of the hypothalamic cytosol receptor to oligo(dT)-cellulose; this binding *in vitro*, which mimics nuclear binding of receptor (Thrower *et al.*, 1976), is discussed in relation to the low extent of translocation in the hypothalamus.

Experimental

[2,4,6,7(n)-³H]Oestradiol-17 β (99 Ci/mmol) was supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Sephadex LH-20 was from Pharmacia (G.B.) Ltd., London W.5, U.K. Bio-Solv BBS 3 was from Beckman RIIC Ltd., Hitchin, Herts., U.K., and butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] from Ciba, Horsham, Sussex, U.K.

Monitoring the oestrous cycle

Groups of 40–42 mature female Wistar rats of identical age (but different groups had different ages in the range 45–75 days old) were purchased from Charles River (U.K.), Margate, Kent, U.K., and maintained in our laboratory for 1 week (12 h light/12 h dark schedule, food *ad libitum*) before use. Progress of the rats through the oestrous cycle was followed by means of vaginal smears, taken on the morning of several successive days, including the day of experiment.

After killing, the swollen uteri, characteristic of pro-oestrus, provided a further check. Care was taken to check that normal cycling was not upset by the handling of the animals; where there was any uncertainty or irregularity about the phase of a rat, it was not used. About 5% of the rats showed a 5-day oestrous cycle during the period of observation; the remaining rats were on a 4-day cycle. The sequence late dioestrus $\rightarrow$ pro-oestrus $\rightarrow$ oestrus $\rightarrow$ metoestrus/early dioestrus on successive days of the cycle was invariably observed, indicating that the phases of oestrus and pro-oestrus each lasted 24 h. On the same basis, metoestrus lasted considerably less than 24 h, and dioestrus rather more than 24 h; for convenience we grouped these animals as metoestrus/early dioestrus (24 h) and late dioestrus (24 h).

Preparation of tissue extracts

Animals were killed by decapitation at midday on the day of experiment. The brain and uterine horns,

from which any liquid in the lumen was expelled by running the back of the scalpel blade along the tissue, were removed into ice-cold TED buffer [10 mM Tris/HCl, (pH 7.6), 1–1.5 mM-EDTA/1 mM-dithiothreitol].

(a) *Uterus*. Uteri from animals of the same phase were pooled, weighed, chopped finely and homogenized in 3 vol. of TED buffer containing 5 nM-[³H]oestradiol by using a type X1020 homogenizer (The Scientific Instrument Centre, London W.C.1, U.K.) for two bursts of 10 s. A crude nuclear pellet was obtained by centrifugation of the homogenate at 1400 g for 10 min at 4°C. Further centrifugation of the supernatant at 200 000 g for 1–1.5 h at 4°C gave the cytosol fraction; the nuclear pellet was washed with 5 $\times$ 10 ml of ice-cold TED buffer, and resuspended in 10 vol. (relative to wet wt. of the tissue) of TED buffer.

(b) *Hypothalamus*. The brains were dissected into blocks of tissue containing the pre-optic area, hypothalamus, amygdala and part of the midbrain, i.e. the oestradiol-binding region described by McEwen & Pfaff (1970). This brain preparation is hereafter referred to as the hypothalamus. These hypothalamic blocks were collected in preweighed vessels containing TED buffer for weighing. The tissue was hand-homogenized in 4 vol. of TED buffer, by using a Tri-R Teflon pestle/glass homogenizer (Camlab, Cambridge, U.K.) with ten up-and-down strokes. A crude nuclear fraction and the cytosol were prepared from the homogenate by centrifugation as described for the uterus.

Assay of cytosol receptor

(a) *Uterus*. Cytosol (0.15–0.2 ml) prepared from the [³H]oestradiol-labelled homogenate was chromatographed in TED buffer on columns (10 cm $\times$ 0.8 cm) of Sephadex LH-20 (Ginsburg *et al.*, 1974; Thrower *et al.*, 1976); in each case the first 0.9 ml of eluate was discarded, and the breakthrough material, containing macromolecular-bound [³H]oestradiol, was collected in a further 0.8 ml and counted for radioactivity in 20 vol. of scintillation 'cocktail' containing Bio-Solv BBS 3 and butyl-PBD in toluene as previously described (Thrower *et al.*, 1976). All measurements were carried out in duplicate. Non-specific binding of [³H]oestradiol was determined in parallel experiments, after incubation of cytosol at 37°C for 30 min in the presence of a 200-fold excess of diethylstilboestrol to replace all the [³H]oestradiol bound to specific oestrogen receptor. This non-specific binding accounted for less than 10% of the total bound radioactivity. Duplicates differed by less than 5%. Estimates of cytosol receptor were corrected to correspond to the concentration of 5 nM-[³H]oestradiol used in the nuclear exchange assay.

(b) *Hypothalamus*. Cytosol (1 ml) was incubated with various concentrations of [³H]oestradiol, in the

absence and presence of a 200-fold excess of diethylstilboestrol, at 4°C for 18–24 h. Chromatography on Sephadex LH-20 was then performed as described for the uterus to obtain values for the cytosol oestrogen receptor.

Assay of nuclear receptors

(a) *Uterus*. Duplicate samples of the resuspended crude nuclear pellet (0.5 ml) were incubated at 37°C for 1 h in the presence of 5 nM-[³H]oestradiol, with or without 200-fold excess of diethylstilboestrol, to replace the endogenous oestradiol bound to the receptor (Anderson *et al.*, 1972). Under these conditions, exchange of receptor was virtually complete within 30 min (results not shown). After five washes (3.5 ml) with ice-cold TED buffer, the nuclear material was extracted with 2 × 1 ml of ethanol, and the extracted radioactivity counted in 10 ml of a scintillation 'cocktail' containing butyl-PBD in toluene. Duplicate estimates of specific receptor agreed to within 10–15% (non-specific binding accounted for 40–75% of the extracted radioactivity). Breakdown of receptor was at a rate of 14–15%/h and estimates of nuclear receptor were adjusted accordingly.

(b) *Hypothalamus*. The technique used was as described for the uterus with slight modifications. After incubation at 37°C for 1 h with different concentrations of [³H]oestradiol in the absence or presence of excess diethylstilboestrol, the nuclear preparation was washed seven times by centrifugation at 1000g for 10 min and resuspension in 5 ml of TED buffer at 4°C. During the last three washes, the nuclei were left at 4°C for 15–20 min between each round of resuspension and recentrifugation. Radioactivity in the final washed pellet was extracted into 6 ml (pooled 2 × 3 ml extracts) of scintillation 'cocktail' containing butyl-PBD in toluene.

Binding of hypothalamic cytosol oestrogen receptor to oligo(dT)-cellulose

From the cytosol sample to be used for Sephadex LH-20 chromatography, normally incubated with 5 nM-[³H]oestradiol, a further sample (0.5 ml) was removed and incubated with 150 mg of oligo(dT)-cellulose (Thrower *et al.*, 1976) in 5 ml centrifuge tubes, for 1 h at 4°C. Free [³H]oestradiol together with material not bound to the matrix was removed by successive washing with 3 ml of TED buffer. The final washings (3 × 0.5 ml) and bound material released from the matrix with 1.2 M-KCl/TED buffer (3 × 0.5 ml) were collected separately for radioactivity measurement.

Measurement of radioactivity

Nuclear fractions. Radioactivity extracted into the butyl-PBD 'cocktail' (4.5 g of butyl-PBD in 1 litre of

toluene) was measured in a final volume of 10 ml with an efficiency of 46% in a Beckman LS 330 liquid-scintillation spectrometer.

Cytosol. Material excluded from Sephadex LH-20 and samples from oligo(dT)-cellulose chromatography were monitored for radioactivity in 20 ml and 10 ml respectively of scintillation 'cocktail' containing Bio-Solv BBS-3 (7.5 g of butyl-PBD, 100 ml of BBS-3, 900 ml of toluene). The efficiency of counting was 43% in a Beckman LS 330 spectrometer.

The difference between the radioactivity measurements in samples incubated in the absence and presence of diethylstilboestrol was used to determine the content of specific oestrogen receptor.

Results

Distribution and content of oestrogen receptor in the uterus during the oestrous cycle

(a) *Assay for cytosol receptor*. It is widely accepted that the exchange method of Anderson *et al.* (1972) is a suitable one for measuring nuclear receptor content in uterine tissue. However, the conflicting reports on cyclic changes in the content of cytosol receptor could be due, in part, to deficiencies in the assay for cytosol receptor. We therefore set out to ensure that our assay for cytosol receptor was precise enough and that it took into account the problem of receptor instability and the possibility that endogenous oestradiol would mask vacant receptor sites, leading to variable results.

Our method for measuring cytosol receptor content involved homogenizing uterine tissue in buffer containing [³H]oestradiol, since oestradiol is known to have a stabilizing effect on the receptor (King & Mainwaring, 1974). The content of the [³H]oestrogen-receptor complex formed at 4°C was determined by chromatography on Sephadex LH-20.

When we attempted to assess the effects of endogenous oestradiol in the homogenate on the measurements of receptor obtained by this means, we found that the incubation of cytosol at 37°C for 30 min, to exchange the bound oestradiol for [³H]oestradiol of known specific radioactivity, led to breakdown of over 60% of the specific receptor. We therefore used the technique of Chamness *et al.* (1975), involving precipitation of the receptor from cytosol by addition of protamine sulphate. (The precipitated oestrogen-receptor complex when subjected to the 37°C exchange procedure was stable for up to 4 h.)

Measurements of cytosol receptor obtained by (a) chromatography on Sephadex LH-20 were compared with those obtained by protamine precipitation of the receptor either (b) without or (c) with an additional step involving exchange with fresh [³H]oestradiol (Table 1). All three methods gave similar results. The effect of endogenous oestradiol on measurements

Table 1. *Measurements of cytosol oestrogen receptor by Sephadex LH-20 chromatography and the effects of endogenous oestrogen*

Uteri pooled from 11 female rats were homogenized in buffer containing 5 nM-[³H]oestradiol, and cytosol was prepared as described in the Experimental section. A portion (0.1 ml) of the cytosol (a) was subjected to exclusion chromatography on Sephadex LH-20 and the amount of specific binding determined as described in the Experimental section. Other portions were treated with protamine sulphate (final concn. 0.6 mg/ml) to precipitate the oestrogen receptor (Chamness *et al.*, 1975). In (b) the amount of specific binding was estimated after exchange with excess diethylstilboestrol at 30°C for 3 h. In (c) samples were incubated at 30°C for 3 h in the presence of fresh [³H]oestradiol (5 nM), with or without excess of diethylstilboestrol, to determine the amount of specific receptor exchanged. All measurements are in duplicate, which differed by less than 7%.

Determination	Macromolecular binding of [³ H]oestradiol (c.p.m./0.1 ml)		
	Total	Non-specific	Specific
(a) Sephadex LH-20	32 700	2140	30 560
(b) Protamine precipitation	65 950	38 150	27 800
(c) Protamine precipitation + exchange with fresh [³ H]oestradiol	67 180	40 720	26 460

involving Sephadex LH-20 chromatography was thus negligible. In the chromatographic procedure the specific [³H]oestrogen-receptor complex contributed over 90% of the macromolecular-bound radioactivity, thus providing a suitable method for assaying cytosol receptor content.

(b) *Expression of results.* During the oestrous cycle, the DNA content of the tissue, and presumably the cell number, changes little (Clark *et al.*, 1972). Thus, though the weight of the uterus changes markedly during the cycle, the number of genetic units is relatively constant. For comparative purposes, therefore, all results have been expressed relative to uterus number.

A further consideration was the effect of the age of the animal on the parameters being measured. Fig. 1 shows the uterus weight, and the total, cytosol and nuclear receptor content at each phase for each experimental group of 40–42 rats, in order of age of the group at the time of use. Over the age group used (50–81 days), there were marked and progressive increases with age in all these parameters.

Within each experimental group of the same age there was a pattern of changes associated with the phasic changes of the oestrous cycle. This pattern was repeated throughout the various age groups. We expressed the data for the separate phases of the

cycle in each group in terms of the mean value of the four different phases for each particular age group (mean = 1.00; values of the individual means are given in Table 2). The data for each of the five age groups could then be combined as mean ± s.d. (Table 3). This allowed us to analyse the parameters in each phase in relation to the other three phases of the oestrous cycle, irrespective of age.

(c) *Changes in uterus weight and cytosol receptor content.* The wet weight of the uterus was elevated over the mean during pro-oestrus and oestrus and fell to a minimum during metoestrus/early dioestrus, increasing again during late dioestrus (Table 3). The amount of oestrogen receptor in the cytosol followed this pattern very closely, being highest in pro-oestrus, and lowest in metoestrus/early dioestrus. Fig. 2 shows that the correspondence between the wet weight of the tissue and the amount of oestrogen receptor in the cytosol existed not only between different phases of the same group, but also between different phases in different age groups regardless of age. Results obtained for miscellaneous groups of rats in this age range in other experiments in this laboratory also fit this pattern (results not shown). Regression analysis showed a linear correlation ($P < 0.001$) between wet weight of uterus and oestrogen receptor content of uterus cytosol over the age range 50–81 days.

(d) *Changes in nuclear receptor and total receptor content during the cycle.* The amount of oestrogen receptor in the nucleus was also highest at pro-oestrus, decreasing sharply to a minimum (one-quarter) in oestrus and metoestrus/early dioestrus, and beginning to increase again during late dioestrus (Table 3). This pattern differed markedly from those of changes in uterus weight, or cytosol content of oestrogen receptor. Total content of receptor in the uterus (cytosol + nuclear receptor) was again highest in pro-oestrus and lowest in metoestrus/early dioestrus.

Hypothalamic oestrogen receptors and the oestrous cycle

(a) *Assay of cytosol oestrogen receptor.* The concentration of oestrogen receptors in the hypothalamus is considerably lower than in the uterus. Chromatography of [³H]oestradiol-labelled cytosol on Sephadex LH-20, and the use of excess diethylstilboestrol in parallel (Ginsburg *et al.*, 1974), allowed us to distinguish the high-affinity receptor from other non-specific oestrogen-binding components, present in relatively higher concentration in brain homogenates. As with uterine preparations, incubation of the cytosol, containing [³H]oestradiol-receptor complexes, at 37°C for 30 min resulted in a loss of up to 50% of the receptor, compared with incubation at 4°C for 18–24 h. We eliminated the possibility that

Fig. 1. Cyclic changes in (a) wet weight, (b) total, (c) cytosol and (d) nuclear receptor content in the uterus of 50–81-day-old rats. Groups of 32–40 female rats were separated into the four individual phases of the oestrous cycle. There were five different groups of the following ages: (1) 50 days (○); (2) 65 days (●); (3) 67 days (□); (4) 67 days [but of a heavier body weight than those in group (3)] (■); (5) 81 days (▲). Wet weight and receptor content were measured as described in the Experimental section. The measurements in each individual phase were derived from a pool of six to ten rats. The broken lines indicate when results from one particular phase were not available. Abbreviations: M, metoestrus/early dioestrus; D₂, late dioestrus; P, pro-oestrus; O, oestrus.

Table 2. Age-group average of measurements of uterine weight and oestrogen receptor content in 50–81-day-old female rats

Each value is obtained from 32–40 rats, as the average of four measurements, each measurement representing the rats from one phase of the oestrous cycle. The variation ±s.d. seen thus represents the normal variations over the whole of the cycle, with values being minimal at metoestrus and maximal at pro-oestrus (see Fig. 1).

Age (days)	Uterus wet wt. (mg)	Receptor (pmol/uterus)		
		Total	Cytosol	Nuclear
50	207±53	2.16±0.72	1.94±0.62	0.22±0.19
65	239±48	2.43±0.85	1.85±0.54	0.58±0.49
67	255±53	3.30±1.10	2.54±0.60	0.76±0.65
67	265±45	—	2.85±0.71	—
81	289±48	3.73±0.93	2.44±0.33	1.29±0.83

endogenous oestrogen interfered with the assay procedure without resorting to exchange procedures at 37°C by administering [³H]oestradiol intraperitoneally. We found that preparations of the cytosol receptor contained no endogenous [³H]oestradiol.

Measurements of the cytosol receptor by using Sephadex LH-20 chromatography and incubation at 4°C showed that 90–95% saturation of the specific receptor sites resulted on incubation of cytosol in 5 nM-[³H]oestradiol (Fig. 3a). This concentration was used in subsequent determinations of cytosol receptor content. Scatchard (1949) analysis showed that the high-affinity receptor measured in this manner was similar to the high-affinity oestrogen receptor of other target tissues in having a dissociation constant (K_d) of 0.73 nM (Fig. 3b) (Buller & O'Malley, 1976; King & Mainwaring, 1974).

(b) Assay of nuclear oestrogen receptor. Clark *et al.* (1973) were able to determine the nuclear content of receptor in brain preparations by using the method, developed for uterine preparations (Anderson *et al.* 1972). We found that the method, as originally described, was not satisfactory because of the relatively high content of non-specific oestradiol-binding components present in brain preparation. Others (D. F. Salaman, personal communication; Westley & Salaman, 1976) overcame these problems by using purified nuclear preparations and extended washing periods after the exchange with [³H]oestradiol. Since a variable loss of receptor occurred during the

Table 3. *Changes in uterus weight and oestrogen receptor content in relation to the oestrous cycle*

The results are obtained by relating individual measurements in each phase to the average value for the four combined phases within each age group shown in Table 2, and combining these values from the five age groups to give a mean value $\pm$ s.d. Where results from one or more experimental group were not available, the number used is indicated in parentheses.

	Wet wt. of uterus	Receptor		
		Total	Cytosol	Nuclear
Metooestrus/early dioestrus	0.80 ± 0.05	0.68 ± 0.08 (4)	0.73 ± 0.07	0.44 ± 0.14 (4)
Late dioestrus	0.89 ± 0.05	0.94 ± 0.11 (3)	0.90 ± 0.10 (4)	1.08 ± 0.32 (4)
Pro-oestrus	1.25 ± 0.05	1.40 ± 0.07 (4)	1.20 ± 0.11	2.07 ± 0.21 (4)
Oestrus	1.06 ± 0.05	0.96 ± 0.07 (4)	1.17 ± 0.08	0.41 ± 0.06 (4)

Fig. 2. *Relationship of cytosol receptor to uterine wet weight*

Values obtained for the amount of cytosol receptor during different phases of the oestrous cycle and at different ages within the age range 50–81 days were plotted against wet weight of uterus, and subjected to regression analysis. Experimental details are given in the text. $\square$, Metooestrus/early dioestrus; $\bullet$, late dioestrus; $\circ$, pro-oestrus; $\blacksquare$, oestrus.

extensive procedures required for purification of brain nuclei, we used a crude nuclear preparation to minimize the loss; our washing procedure, described in the Experimental section, allowed us to overcome the problems associated with the non-specific low-affinity binding of [3 H]oestradiol. Nuclear assays were first performed with adult rats injected with oestradiol to maximize nuclear content of the receptors. At a concentration of 1–2 nM [3 H]oestradiol, 85–90% of the maximum number of receptor sites were exchanged (Fig. 4a). All subsequent determinations of the nuclear receptor were performed at this concentration. The K_d of the nuclear receptor was 0.53 nM (Fig. 4b). A similar experiment conducted with nuclear preparations from uninjected rats

yielded a K_d of 0.35 nM. These K_d values are similar to those of nuclear and cytosol receptors from target tissues (Buller & O'Malley, 1976), as well as to the hypothalamic cytosol receptor shown previously (Fig. 3). This provided further evidence that in our assay procedures we were measuring the same population of specific oestrogen receptors present either in the cytosol or, after translocation, in the nucleus.

(c) *Distribution of oestrogen receptors during the oestrous cycle.* We then measured the content of cytosol and of nuclear receptors in hypothalamic preparations isolated from the same groups of rats as those used for measurements of uterine receptors. The rats, grouped by age, had been separated into the various phases of the oestrous cycle. In contrast with uterine preparations, we found no age-related variation in the cytosol and nuclear receptor content nor in weight of hypothalamic tissue in the rats in each individual phase. The different measurements were all therefore pooled into groups corresponding to the specific stages of the cycle without regard to age (Table 4).

There was no significant variation in the weight of the block of hypothalamic tissue, dissected out in identical fashion by the method of McEwen & Pfaff (1970).

There was no significant difference in the content of cytosol oestrogen receptor between oestrus and metooestrus/early dioestrus. These two phases gave the highest mean values of cytosol receptor content (Table 4). The content during late dioestrus showed a slight, but not significant, decrease compared with that in oestrus and metooestrus/early dioestrus. In contrast, at pro-oestrus receptor content decreased by 50%, representing a significant difference from the contents at oestrus and at metooestrus/early dioestrus.

The nuclear oestrogen receptor content was, at all phases, lower than the cytosol receptor content. There was no significant difference in nuclear receptor contents between oestrus, metooestrus/early dioestrus and late dioestrus, the values being relatively low. During pro-oestrus there was a 3-fold rise in the mean nuclear content of receptor, which represented

Fig. 3. Binding of $[^3\text{H}]$ oestradiol to the cytosol high-affinity oestrogen receptor in hypothalamic tissue
 (a) Hypothalamic cytosol prepared from 14 rats was incubated with the indicated concentrations of $[^3\text{H}]$ oestradiol in the presence or absence of excess diethylstilboestrol (200-fold) for 18–24 h at 4°C. Samples (0.2 ml) were removed and fractionated on columns of Sephadex LH-20 as described in the Experimental section. Each point is the mean of two measurements; variation <5%. A (○), radioactivity bound in the absence of diethylstilboestrol (high- and low-affinity binding); B (□), radioactivity bound in the presence of diethylstilboestrol (low-affinity binding). A-B (●), high-affinity binding. (b) Scatchard-plot analysis of the high-affinity interaction between $[^3\text{H}]$ oestradiol and cytosol oestrogen receptor. The linear relationship obtained from the plot of the ratio of bound/free versus bound radioactivity was used to calculate the dissociation constant (K_d), by the formula: slope = $-(1/K_d)$. K_d was calculated by regression analysis to be 0.73 nM.

a significant increase compared with the values at oestrus and metoestrus/early dioestrus. This increase in the hypothalamic nuclear oestrogen receptor content, as with the uterus, was associated with the increase in circulating oestrogens at pro-oestrus (Brown-Grant *et al.*, 1970; Yoshinaga *et al.*, 1969).

(d) *Changes in receptor-related activities during the oestrous cycle.* From the previous results, it appeared that the translocation of oestrogen receptor into the nucleus at pro-oestrus was accompanied by a decrease in cytosol content of oestrogen receptor. In an earlier report (Thrower *et al.*, 1976) we suggested that the extent of translocation or nuclear binding of oestrogen receptor may, to some extent, be dependent on the concentration of a cytosol factor. This suggestion was based on observations of the binding of cytosol receptor to oligo(dT)-cellulose in a manner that simulated nuclear binding. In our experiments with hypothalamic preparations, the binding of cytosol receptor to oligo(dT)-cellulose was performed in the

absence and presence of diethylstilboestrol, by using a batchwise analytical method rather than the column-chromatography method previously described, to remove unbound material more effectively (see the Experimental section). This allowed us to determine the amount of the specific oestrogen receptor bound to oligo(dT)-cellulose, since, in accord with our observations on uterine preparations, the hypothalamic oestrogen receptor bound to oligo(dT)-cellulose could be released by high KCl concentrations (Fig. 5).

The capacity of the cytosol receptor to bind to oligo(dT)-cellulose was determined (Table 5) on samples of tissue where both nuclear and cytosol receptor content had been previously estimated (see Table 4). There was no difference in the amount of cytosol receptor binding to oligo(dT)-cellulose when preparations from rats in oestrus, metoestrus/early dioestrus and late dioestrus were compared. However, the value at pro-oestrus was significantly lower

Fig. 4. Measurement of nuclear oestrogen receptor by exchange assay

(a) Each of a group of eight rats, regardless of phase of the oestrous cycle, was injected with $2.5 \mu\text{g}$ of oestradiol and killed 1 h later to obtain maximal values for the nuclear content of oestradiol receptor. Nuclei were prepared as described in the Experimental section. Samples ($0.5 \text{ ml} \equiv$ half brain block) were incubated with the indicated concentrations of $[^3\text{H}]$ oestradiol in the presence (200-fold) or absence of excess diethylstilboestrol at 37°C for 1 h. After washing, radioactivity was extracted into scintillation fluid and monitored. Each point is the mean of two measurements; variation $<5\%$. A ($\circ$), high- and low-affinity binding in the absence of diethylstilboestrol. B ($\square$), low-affinity binding in the presence of diethylstilboestrol. A-B ($\bullet$), high-affinity binding. (b) Scatchard-plot analysis of the high-affinity interaction between $[^3\text{H}]$ oestradiol and nuclear oestrogen receptor (cf. Fig. 3b). The value of K_d was calculated by regression analysis to be 0.53 nM .

Table 4. Wet weight, cytosol and nuclear content of the oestrogen receptor of the hypothalamic region during the oestrous cycle. Animals were segregated according to the phase of the oestrous cycle, and tissue was prepared and weighed as described in the Experimental section. Saturating concentrations of $[^3\text{H}]$ oestradiol (5 nM and 2 nM respectively) were used in the determination of cytosol and nuclear content of the oestrogen receptor by the methods described in the Experimental section. There was no significant variation in tissue weight over the age range studied ($50\text{--}81$ days), therefore receptor content was expressed as mean $\pm$ s.d. in each phase regardless of age. Each determination involved a pooled sample of eight rats in each phase. There were five separate determinations per phase for wet weight and for cytosol receptor content, and four separate determinations per phase for nuclear receptor content. Statistical analysis was by Student's t test: *(d) significantly different from (a) or (b) ($P < 0.01$); **(d) significantly different from (a) or (b) ($P < 0.01$).

	Oestrus (a)	Metoestrus/ early dioestrus (b)	Late dioestrus (c)	Pro-oestrus (d)
Wet wt. (g)	0.35 ± 0.06	0.33 ± 0.06	0.34 ± 0.04	0.32 ± 0.05
Cytosol oestrogen receptor (fmol/brain)	64.3 ± 7.0	72.6 ± 13.6	59.9 ± 15.3	$36.4 \pm 11.4^*$
Nuclear oestrogen receptor (fmol/brain)	2.06 ± 0.66	2.40 ± 0.60	2.73 ± 0.72	$6.50 \pm 1.73^{**}$

than that in the preceding late-dioestrus phase. It is noteworthy that, if these values are added to those of the nuclear receptor content in each individual phase, the combined values appear to be similar throughout the phases, except possibly for a slight decrease at oestrus.

Discussion

Distribution of oestrogen receptors in the uterus

Our observations on cyclic changes in nuclear content of oestrogen receptor, showing maximal con-

tent at pro-oestrus, are in agreement with reports that nuclear content is governed by the concentration of circulating oestrogens (see review by Brenner & West, 1975). The variations in wet weight are also in accord with those observed by other investigators, who report that these variations are paralleled by changes in dry weight (Astwood, 1939) and protein (Clark *et al.*, 1972). In contrast with reports indicating that there were changes in the concentration of cytosol receptor, involving at pro-oestrus either an increase (Feherty *et al.*, 1970) or decrease (Lee & Jacobson, 1971), we find that the concentration of cytosol receptor was maintained constant, with

Fig. 5. Binding of the hypothalamic cytosol oestrogen-receptor complex to oligo(dT)-cellulose

Samples of cytosol (0.5 ml) taken from the same sample as the corresponding samples used for Sephadex LH-20 chromatography were incubated with 150 mg oligo(dT)-cellulose at 4°C for 1 h. Material not bound to the matrix was removed by successive washings with TED buffer before bound material was released with 1.2 M-KCl/TED buffer (start of this elution shown by arrow). ○, Elution profile after incubation with a sample of cytosol from a sample containing [³H]oestradiol alone. ●, Elution profile after incubation with a sample of cytosol from a sample containing [³H]oestradiol and excess diethylstilboestrol. Hatched area: difference in the radioactivity eluted with 1.2 M-KCl after incubation in the absence or presence of diethylstilboestrol, giving an estimate of the amount of high-affinity oestrogen receptor bound to oligo(dT)-cellulose.

respect to wet weight, throughout the cycle. This correspondence between cytosol receptor content and wet weight was also maintained throughout a period of uterine growth. These results suggest that in mature rats the content of cytosol receptor is regulated such that translocation of receptor is accompanied by a process of continuous replenishment or synthesis of the cytosol receptor.

Table 5. Oligo(dT)-cellulose-binding capacity of the cytosol oestrogen-receptor complex during the oestrous cycle. High-affinity oestradiol-receptor binding to oligo(dT)-cellulose was measured as described in the legend to Fig. 5. The values obtained are expressed as the mean $\pm$ S.D. of each phase for four separate estimations. * (d) significantly different from (c) ($P < 0.01$) by Student's *t* test.

	Oestrogen-receptor binding to oligo(dT)-cellulose (fmol/brain)
Oestrus (a)	4.21 ± 1.42
Metoestrus/early dioestrus (b)	5.92 ± 3.44
Late dioestrus (c)	5.39 ± 0.71
Pro-oestrus (d)	$2.59 \pm 1.08^*$

The extent of translocation, measured as the proportion of receptor present in the nucleus, increased from 10 to 34% during the period 50–81 days. The measurements were based on the average of the values at the four different phases of each particular age group (Table 2). This increase matches a 3-fold increase in the concentration ($\mu\text{g}/100\text{ ml}$ of blood) of circulating oestrogens, from 0.34 at 50 days to 0.95 at 70 days (Presl *et al.*, 1969), further demonstrating the close relationship between oestrogen secretion and nuclear content of the receptor.

Our results also show that an average increase of 1 pmol of nuclear receptor was associated with an average wet-weight increase of 80–90 mg, demonstrating the uterotrophic effect of oestrogens. These results, obtained from mature females, are comparable with those of Clark *et al.* (1973), who demonstrated that in immature rats a sustained increase in wet weight of the uterus was directly related to the increase in content of the 'long-term' nuclear receptor, i.e. receptor present 6 h after the administration of oestradiol. An increase of 1 pmol/uterus was also associated with an increase of 80–90 mg wet wt. in these rats. A similar relationship can be deduced from the data of Lan & Katzenellenbogen (1976).

Intracellular relationships of oestrogen receptors in the hypothalamus

There are considerable differences between the behaviour of the oestrogen receptor in the hypothalamus and in the uterus during the cycle. In the hypothalamus there is a considerable depletion of cytosol receptor associated with the translocation of the receptor into the nucleus at pro-oestrus. In contrast with its stimulating effects on other target tissues, oestrogen appears to have an inhibiting influence on macromolecular synthesis in the hypothalamus; thus it has been reported that the synthesis of RNA and of protein in the hypothalamus is decreased as a consequence of oestradiol administration

(McEwen & Pfaff, 1973; McEwen *et al.*, 1974). The inhibitory effect on the macromolecular metabolism may be a contributory factor to the non-replenishment of the receptor at pro-oestrus.

The extent of translocation in the hypothalamus is also extremely low compared with that in the uterus. The nuclear content, at pro-oestrus, of 6 fmol of receptor in the hypothalamus represents a translocation of 9% (calculated by using as values of cytosol receptor the maximal content of 73 fmol/brain at metoestrus/early dioestrus to compensate for the considerable depletion of receptor that occurs at pro-oestrus). This translocation is not age-dependent. In contrast, in the uterus receptor translocation at pro-oestrus increased from 16 to 48% during the same 50–81-day period (Fig. 1).

Clark *et al.* (1973) have demonstrated in immature uteri that the effect of oestrogen is related to the 'long-term' nuclear receptor, i.e. receptor present 6 h after oestrogen administration. The maximal content of this receptor is attained on the administration of physiological doses (0.1 µg) of oestradiol. Excess of oestradiol induces translocation of large amounts of 'short-term' receptors, which then disappear rapidly. This can result in a deficit in cytosol receptor at early time periods, since little replenishment will have occurred (Hsueh *et al.*, 1976; Mester & Baulieu, 1975). Similar long- and short-term receptors are present in the immature rat hypothalamus (Anderson *et al.*, 1973). By analogy, the loss of cytosol receptor from the mature hypothalamus at pro-oestrus, in amounts higher than can be explained solely by nuclear binding (Table 1), may be the result of translocation of excess receptor into the nucleus, which then disappears (presumably metabolized), because the maximal extent of 'long-term' nuclear binding has already been reached at a relatively low value.

Regulation of nuclear binding of hypothalamic oestrogen receptor

We have previously demonstrated that there is a factor in uterine cytosol that mediates the binding of the oestrogen-receptor complex to oligo(dT)-cellulose. In binding the receptor is converted into a 5S 'nuclear' form, and we have postulated that this cytosol factor mediates nuclear binding of the receptor (Thrower *et al.*, 1976). Its absence may explain the lack of nuclear binding of oestrogen in tissues that have normal oestrogen receptors, e.g. in oestrogen-independent mammary tumours (Shyamala, 1972). Conversely, its regulated appearance in hypothalamic tissue could be responsible for the dramatic 6-fold increase in nuclear binding of oestradiol seen in 26–27-day-old animals (Plapinger & McEwen, 1973). Evidence for the existence of a similar factor in uterine tissue required for nuclear binding of the

oestrogen receptor has also been presented by Haselbacher & Eisenfeld (1976).

The limited 'long-term' (i.e. *in vivo*) nuclear binding in the hypothalamus may be regulated by the presence of the comparable cytosol factor, which mediates the binding of the oestrogen receptor to oligo(dT)-cellulose *in vitro*, and which is present in limiting amounts. Thus its concentration is decreased in the cytosol when the receptor is translocated into the nucleus at pro-oestrus (Table 5). The decrease in content of this factor matches the increase in nuclear binding of receptor (when these are respectively considered at the 'resting' metoestrus/early dioestrus phase and at pro-oestrus). Correspondingly, the maximal content of the factor (at metoestrus) is also equivalent to the maximal nuclear binding of receptor (at pro-oestrus). In the uterus of mature rats, the concentration of this factor is maintained in the same manner as for the cytosol receptor, remaining at all times in constant proportion (about 30%) to the receptor (S. Thrower & L. Lim, unpublished work). Because of the continued availability of the factor, as of the receptor, it should be able to mediate high degrees of nuclear binding in the uterus (up to 48% at pro-oestrus in the 81-day-old animals).

In the rat, development and differentiation of the brain is completed by puberty and there is no capacity for further growth in the mature animal. The limited nuclear binding of the oestrogen receptor in the hypothalamus, coupled with the depletion of the cytosol receptor after translocation, may be part of the protective measures that ensure in this brain region that there is only a restricted response to increased oestrogen. This response includes release of tropic factors that influence pituitary release of gonadotropins (Barraclough, 1973) and should be compared with that of the uterus, where, even in the mature female, oestrogens stimulate both differentiation and growth.

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