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Determination of High-Affinity Oestrogen Receptor Sites in Uterine Supernatant Preparations

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An assay method was developed for the determination of high-affinity oestradiol receptors in uterine supernatant preparations. When only high-affinity sites are present in such preparations, or when they predominate, the analysis of the equilibrium between oestradiol and receptor sites based on the Scatchard (1949) plot may be used to determine the dissociation constant of the equilibrium and the molar concentration of the high-affinity sites. When both high-affinity and low-affinity sites are present the Scatchard plot is no longer linear and cannot be used directly to determine high-affinity sites. Determination of the reverse velocity constants of the reaction between high-affinity (k_{-1}) and low-affinity (k_{-2}) receptor sites and [^{3}H]oestradiol has shown that these constants differ by at least one order of magnitude. Advantage has been taken of this difference to introduce an additional step into the assay procedure that eliminates oestradiol bound to low-affinity sites and permits the determination of high-affinity sites in different species and under a variety of physiological conditions.

The observation that immature or ovariectomized rat uteri can selectively retain oestrogens (Jensen & Jacobson, 1962) has led to the recognition of specific oestradiol receptors in the cytoplasm and nuclei of both endometrial and myometrial cells of several species (Jensen *et al.* 1968). The binding of tritiated oestradiol to the cell supernatant fraction of these tissues has been demonstrated *in vivo* and *in vitro* and at least two different types of receptor molecules have been recognized. Receptors characterized by high affinity for oestradiol appear to mediate the action of oestrogens on the uterus and are usually referred to as the specific binding sites. In many preparations non-specific binding sites are present that have considerably lower affinity for oestradiol. Relatively little is known of the binding characteristics of these sites in relation to oestrogens and other steroids.

High-affinity and low-affinity oestradiol receptor molecules can be separated by sucrose-density-gradient centrifugation (e.g. Toft, Shyamala & Gorski, 1967) and the relative amounts can be assessed, but the method lacks precision and is unsuitable for detailed physiological studies. Alternative methods of measuring high-affinity receptor sites by equilibrium dialysis (Baulieu & Raynaud, 1970), or by precipitation (Steggles & King, 1970) of the oestradiol complex have been described.

The aim of the present study was to find a method of eliminating the effect of binding to low-affinity sites and thus to develop a sensitive and reliable method for the determination of high-affinity receptors in uterine supernatant preparations under a wide variety of physiological conditions.

MATERIALS AND METHODS

Materials. [2,4,6,7-^{3}H]Oestradiol (100Ci/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. It was stored in benzene (20ng/ml) at 6–10°C and was checked as a routine for purity by t.l.c. on silica gel in two systems [cyclohexane–ethyl acetate (13:7, v/v) and chloroform–acetone (9:1, v/v)]. No significant deterioration (<5%) was detected after 7 months. Buffer solution contained 1mM-EDTA and 250mM-sucrose in 10mM-tris–HCl buffer, pH8.0 (Korenman, 1968). The dextran–charcoal suspension contained 0.0025% dextran (mol.wt. 60000–90000) and 0.25% Norit A in buffer.

Measurement of radioactivity. β-Scintillation counting was carried out in a Packard model 3375 β-scintillation spectrometer. Aqueous samples (0.5–1.0ml) were transferred to a counting vial containing 10ml of scintillation liquid [0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene] and the contents were agitated for 10s on a Whirlimixer (Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.). The vials were left for 2h in the dark before the radioactivities were counted (efficiency approx. 35%). Equivalent results were obtained with 10ml of an alternative scintillation liquid [20% (w/v) naphthalene, 2% 2,5-diphenyloxazole

and 0.5% 1,4-bis-(5-phenyloxazol-2-yl)benzene in dioxan] (efficiency approx. 25%).

Preparation of uterine supernatants. Albino rats (Wistar strain) were obtained from the Courtauld Institute colony. Virgin pubertal New Zealand White rabbits (approx. 4 months old, weighing 2–2.5kg) were obtained from commercial sources. Human uterine tissue was obtained at biopsy or by curettage from the Department of Obstetrics and Gynaecology, The Middlesex Hospital, London W1P 8AA, U.K. The tissues were stored in 0.9% NaCl at 4°C until processed on the same day.

Rabbits and rats were killed by cervical dislocation and the uteri were dissected out and washed with ice-cold 250mM-sucrose. After being weighed the tissue was sectioned into small pieces and homogenized at 2°C in buffer (10ml) in a Silverson tissue emulsifier (Silverson Machine Ltd., Waterside, Bucks., U.K.) under controlled conditions to ensure complete cell breakage with minimal damage to the binding sites (i.e. three 10s bursts at low speed interspersed with 20s cooling intervals). The homogenates were centrifuged at 4°C and $100000g_{av.}$ for 1h and were stored with added mercaptoethanol (100mM) at −10°C. No loss of binding capacity was observed in a rabbit uterine preparation over 2 months, but the stability of rat and human preparations was not checked for periods greater than 1 week. In the absence of mercaptoethanol and at a concentration of 10mM-mercaptoethanol deterioration occurred within 2 weeks. Rabbit uterine supernatant preparations were diluted tenfold (v/v) with buffer before use. Rat and human supernatants were used without dilution.

RESULTS AND DISCUSSION

Binding equilibrium in the presence of high-affinity receptor sites

$$E + P \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EP \qquad (1)$$

Stability of high-affinity receptors. The uterine supernatant preparation used in this section was obtained from New Zealand White rabbits (2–2.5kg) and contained a high proportion of high-affinity oestradiol-binding sites. Equilibrium between oestradiol (E) and the high-affinity sites (P) of the preparation was established by incubating portions (0.1ml) of the uterine supernatant with [^{3}H]oestradiol (10pg) in buffer solution (0.1ml). The time-course was followed by stopping the reaction at various time-intervals between 0 and 20h. This was achieved by cooling the equilibrium mixture in ice, adding dextran–charcoal suspension (0.5ml) to remove free oestradiol and centrifuging at 4°C and $800g_{av.}$. The radioactivity remaining in solution was that associated with oestradiol bound to high-affinity receptors and this was measured by liquid-scintillation counting. The experiment was repeated at temperatures ranging from 4° to 37°C.

The total amount of [^{3}H]oestradiol present was known and the results were expressed by plotting the percentage of oestradiol bound to high-affinity receptors against time (Fig. 1). It is clear that at 4°C equilibrium is not reached at 3h; at 30°C equilibrium is reached within 0.5h and is maintained, whereas at higher temperatures (37°C) breakdown of the high-affinity receptors occurs. With rat uterine and human endometrial supernatant preparations some decomposition of binding sites did occur at 30°C, but over a period of 30min this did not exceed 5% and did not prevent the establishment of equilibrium for assay purposes. Similar time- and temperature-dependence was observed with uterine supernatant preparations containing both high-affinity and low-affinity receptors when the assay method for high-affinity receptors was used.

Similar experiments carried out at 30°C with buffers of different pH values showed that rabbit receptor sites are stable for at least 0.5h at pH8, but are destroyed in acid (pH6.0) and in alkaline (pH8.9 and 9.8) solution.

Scatchard-plot analysis of the equilibrium between [^{3}H]oestradiol and high-affinity receptor sites: the dissociation constant (K_p) and saturation conditions. The uterine supernatant preparations used contained predominantly high-affinity sites. Equilibrium between oestradiol and the receptor sites was established by incubating the uterine supernatant preparation (0.1ml) at 30°C for 0.5h with increasing amounts of [^{3}H]oestradiol (5–100pg, 0.018–0.367pmol). At the end of this period the mixtures were chilled in ice and dextran–charcoal suspension (0.5ml) was added to remove free oestradiol. After centrifugation, the radioactivity

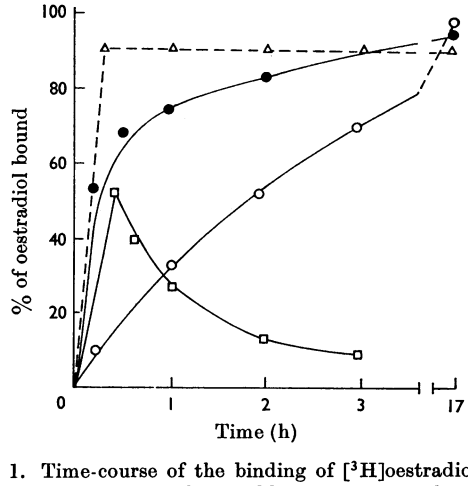

Fig. 1. Time-course of the binding of [^{3}H]oestradiol to high-affinity receptors from rabbit uterine cytoplasm at different temperatures. Details of the assay are given in the text. □, 37°C; △, 30°C; ●, 20°C; ○, 4°C.

in the centrifuge supernatant was measured to determine bound oestradiol and to construct a Scatchard plot of the equilibria. Under these experimental conditions uterine supernatant preparations containing mainly high-affinity receptors consistently gave Scatchard (1949) plots that were linear.

The ratio of bound/free oestradiol is given by a formula

$$\frac{\text{Bound E}}{\text{Free E}} = \frac{e}{e_o - e} = 1/K_p(p_o - p)$$

where e_o is [total oestradiol] (M), e is [bound oestradiol] (M), p_o is [total binding sites] (M) and p is [occupied binding sites] (M). From the straight line obtained by plotting the bound/free oestradiol ratio against the bound oestradiol it is possible to determine both the p_o and K_p values (Scatchard, 1949; Toft *et al.* 1967).

Binding equilibrium in the presence of high-affinity and low-affinity receptor sites

$$\underset{\text{High-affinity sites}}{E + P \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EP} \qquad \underset{\text{Low-affinity sites}}{E + Q \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EQ} \qquad (2)$$

Scatchard-plot analysis of the equilibrium: the dissociation constants K_p and K_q. The uterine supernatant preparations used contained both high-affinity and low-affinity oestradiol receptor sites. Equilibrium between [^{3}H]oestradiol and mixed high-affinity (P) and low-affinity (Q) sites was established, as before, by incubating uterine supernatant preparations (0.1ml) at 30°C for 0.5h with increasing amounts of [^{3}H]oestradiol (0.018–0.357pmol). At the end of this period the bound oestradiol was determined as described above and a Scatchard plot constructed. Under these experimental conditions supernatant preparations from rat uteri and from human endometria gave non-linear Scatchard plots from which it was impossible to obtain reliable values for K_p and p_o directly (*A–B* in Fig. 2).

The distribution of oestradiol in the equilibrium solution containing the high-affinity and low-affinity receptor sites is characterized by a relationship

$$\frac{\text{Bound E}}{\text{Free E}} = \frac{e}{e_o - e} = \frac{p_o}{K_p} + \frac{q_o}{K_q} - \frac{p}{K_p} - \frac{q}{K_q}$$

where q_o is [total receptor sites Q] (M), q is [occupied receptor sites Q] (M) and $e = (p+q)$ is [bound oestradiol] (M). A Scatchard plot of the bound/free oestradiol ratio against the total bound oestradiol concentration is a curve that can be resolved into two linear components on the assumptions that

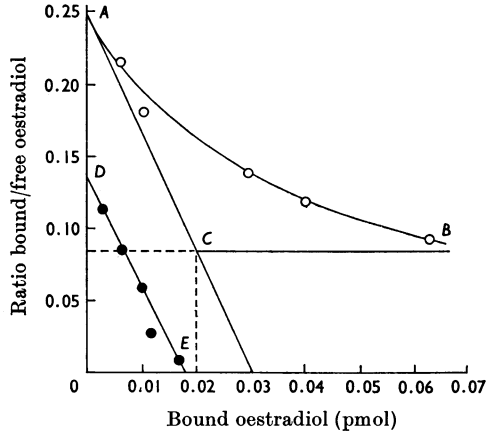

Fig. 2. Effect of incubation with dextran–charcoal on the Scatchard plot: analysis of a 'non-corrected' Scatchard plot (*A–B*) and construction of the 'corrected' Scatchard plot (*D–E*). Curve *A–B* (○), Scatchard plot obtained with a uterine supernatant preparation containing low concentrations of high-affinity oestradiol receptors; curve *D–E* (●), corrected Scatchard plot obtained with the same uterine supernatant preparation subjected to the dextran–charcoal incubation step. The abscissa co-ordinates of the points *C* and *E* represent the concentration of high-affinity receptors as estimated by analysis of the curves *A–B* and *D–E* respectively. Experimental details are given in the text.

$p_o/K_p \gg q_o/K_q$ and $K_p \ll K_q$. The intercept at $e/(e_o - e) = 0$ will then be $p_o + q_o$, the asymptotic slope being $-1/K_q$, and the intercept at $e = 0$ will be $p/K_p + q/K_q$, the asymptotic slope being $-1/K_p$ (cf. Scatchard, Coleman & Shen, 1957). The intersection of the two asymptotes occurs at a point with the abscissa co-ordinate equal to p_o (Fig. 2). In this way it is possible to determine the concentration of the high-affinity receptor sites, as well as the K_p value, if the two asymptotes can be drawn with confidence. As the p_o/q_o ratio decreases, a point is reached when p_o can no longer be measured as the assumption that the high-affinity sites predominate ($p_o/K_p \gg q_o/K_q$) is no longer valid. It is also clear from the experimental curve that the values of K_q and q_o are of such a high order of magnitude that they cannot be determined in this way.

Removal of oestradiol bound to low-affinity receptors by incubation with dextran–charcoal

Reverse reaction velocity constants of high-affinity (k_{-1}) and low-affinity (k_{-2}) receptor sites. [^{3}H]-Oestradiol–receptor complexes were prepared by

incubating a uterine supernatant preparation containing both high-affinity and low-affinity receptors (1ml) with [^{3}H]oestradiol (6000pg) at 30°C for 0.5h. The solution was chilled in ice, dextran–charcoal (2ml) was added and the suspension was centrifuged at 4°C and $800g_{av.}$ for 5min to remove free oestradiol. The chilled centrifuge supernatant contained high-affinity and low-affinity complexes loaded with [^{3}H]oestradiol. The rate of dissociation of these complexes was studied by incubating portions of the solution (0.1ml) with buffer (0.1ml) and dextran–charcoal suspension (0.5ml) at 30°C with constant shaking for various time-intervals between 0 and 120min. The reaction was stopped by cooling in ice and centrifuging and the amount of bound [^{3}H]oestradiol remaining in the supernatant was measured by scintillation counting. It was assumed that oestradiol released by the dissociation of the complexes was immediately removed by charcoal. Over the time-interval studied, t, dissociation of both high-affinity and low-affinity complexes proceeded with decreasing radioactivity in the bound form. The radioactivity remaining in the bound form after time t is given by the equation:

$$a(t) = a_1(0)\cdot\exp(-k_{-1}t)+a_2(0)\cdot\exp(-k_{-2}t)$$

where $a_1(0)$ and $a_2(0)$ are the radioactivities of the high-affinity and low-affinity complexes respectively at zero time. The experimental relationship between $\log a(t)$ and time is shown in Fig. 3. By extrapolation of the linear part B–C of the curve A–B–C, values of $\log[a_1(0)\cdot\exp(-k_{-1}t)]$ were determined and these values were used to determine points on the line D–E representing $\log[a_2(0)\cdot\exp(-k_{-2}t)]$. These straight lines ($B$–$C$ and D–E) represent the decay rates of radioactivity associated with the high-affinity and low-affinity complexes respectively. From the slopes of these lines values for the reverse reaction velocity constants k_{-1} and k_{-2} were calculated (Table 1). Additional values for k_{-1} and k_{-2} in Table 1 were determined by two alternative methods: (1) by incubating the ^{3}H-loaded complexes with excess of non-radioactive oestradiol and measuring the rate of release of radioactivity; (2) by incubating non-radioactive oestradiol-loaded complexes with excess of [^{3}H]-oestradiol and measuring the rate of incorporation of radioactivity into the complexes (J. Méšter & D. M. Robertson, unpublished work). All three methods gave similar values for k_{-1} and k_{-2}.

It is clear from Fig. 2 that the Scatchard plot cannot be used to measure the concentration of high-affinity receptor sites in uterine supernatant preparations that also contain a substantial pro-

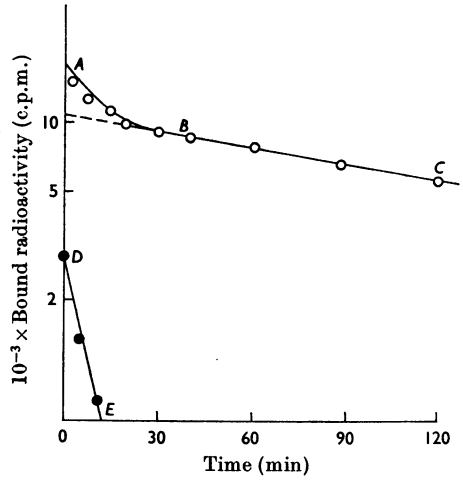

Fig. 3. Incubation of [^{3}H]oestradiol–receptor complexes with dextran–charcoal suspension. Curve A–B–C, semilogarithmic plot of the radioactivity $a(t)$ remaining bound to the rabbit uterine supernatant oestradiol receptors after incubation with dextran–charcoal suspension for time t; curve D–E, semilogarithmic plot of the difference between $a(t)$ and the radioactivity corresponding to the extrapolated linear part of the curve A–B–C. Estimation of the dissociation velocity constants:

$$k_{-1} = 2.303\tan\alpha = 0.74\cdot10^{-4}\,s^{-1}$$

$$k_{-2} = 2.303\tan\beta = 5.0\cdot10^{-3}\,s^{-1}$$

Experimental details are given in the text.

Table 1. *Characteristics of oestradiol-binding protein from uterine tissues of various species*

The values given for rabbit were obtained from pubertal unmated animals; values were obtained throughout the rat oestrous cycle and from human endometrial biopsy and curettage samples obtained throughout the menstrual cycle.

Species	Concn. of high-affinity binding sites (pmol/mg of tissue)	$10^{10}\times$ Dissociation constant K_p (M)	$10^3\times$ Reverse reaction velocity constant (s^{-1})	
			High-affinity k_{-1}	Low-affinity k_{-2}
Rabbit	0.01–0.06	0.8–1.5	0.075	3–5
Rat	0.001–0.008	2–4	0.15	2.2
Human	0.0005–0.002	1–3	0.08	3–5

portion of low-affinity sites. Nevertheless the fact that there is a difference of more than one order of magnitude between the reverse reaction velocity constants of the high-affinity (k_{-1}) and low-affinity (k_{-2}) complexes indicates that a further period of incubation with dextran–charcoal at 30°C for 10min may be used to differentiate between high-affinity and low-affinity sites. Within this time-interval (Fig. 3) almost all of the low-affinity complex has dissociated but less than 6% of the high-affinity receptor sites have been vacated; a correction for the small fraction of high-affinity sites dissociated was applied in routine assays.

Determination of high-affinity oestradiol receptor sites by dextran–charcoal incubation of the equilibrium mixtures

Standard procedure. Equilibrium between oestradiol and receptor sites was established by incubating uterine supernatant (0.1ml) at 30°C for 0.5h with increasing amounts of [^{3}H]oestradiol (0.036–0.367pmol) in buffer (0.1ml). At the end of this period dextran–charcoal suspension (0.5ml) was added and the incubation was continued at 30°C for a further 10min with shaking. The suspension was then chilled in ice, centrifuged at 4°C for 5min at $800g_{av.}$ and the [^{3}H]oestradiol bound to the high-affinity receptors was determined by liquid-scintillation counting. A typical result presented as a Scatchard plot is shown in Fig. 2, where the straight line *D–E*, representing the binding to high-affinity sites only, may be used to determine the dissociation constant, K_p and p_o, the intercept on the abscissa. This line is parallel to the tangent to the curved Scatchard plot and gives similar values for K_p and p_o. [The value of $p/(e_o-e)$, which in theory should be plotted as the ordinate co-ordinate on the Scatchard plot, was in practice approximated to $p/(e_o-p)$. The error introduced by this approximation is reflected only in the estimated value of K_p. The determined value of p_o is unaltered.] This procedure has been applied to supernatant samples prepared from rabbit and rat uteri and from human endometrial curettings. Experimental values are included in Table 1.

Validity of the standard procedure. The validity of the procedure for the determination of high-affinity receptors in the presence of low-affinity binding was established experimentally by measuring high-affinity receptors in the presence of an increasing proportion of low-affinity sites. It has been shown that heat treatment of uterine supernatant preparations for 30min at 60°C inactivates all high-affinity sites but is without effect on the concentration of low-affinity sites. Samples for assay were prepared by mixing heat-inactivated and unheated uterine supernatant preparations in various proportions. The concentration of high-affinity receptors varied from 0.01 to 0.12pmol/0.1ml of sample in the presence of a constant concentration of low-affinity receptors. The standard procedure was applied to the diluted samples and the effect of the dilution on the measured concentration of high-affinity sites is shown graphically in Fig. 4.

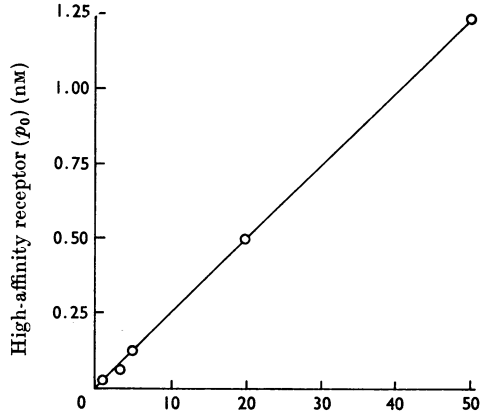

Fig. 4. Measurement of high-affinity receptors, p_0, in samples containing various proportions of unheated (high-affinity and low-affinity sites) and heat-inactivated (low-affinity sites) rabbit uterine supernatant preparations. The concentration of high-affinity receptors was estimated as described in the text.

The standard procedure was sufficiently sensitive to measure the physiological concentrations of high-affinity receptors in supernatant samples prepared from rabbit and rat uteri and from human uterine endometrium. The values ranged from 0.001 to 0.025pmol/mg wet wt. of tissue and the lowest amount that could be distinguished from zero was of the order of 0.001pmol/mg of tissue. Many samples prepared from the uteri of young rabbits gave a linear Scatchard plot and did not justify the use of the dextran–charcoal step.

Low values for p_o in the assay procedure may result from the failure of the dextran–charcoal adsorption to achieve complete separation of the free and bound forms of oestradiol. The amount of dextran–charcoal used (0.5ml) is a compromise, and although free [^{3}H]oestradiol is removed quantitatively a small proportion of the complex is also removed from solution (cf. Murphy, 1967). The percentage of bound [^{3}H]oestradiol remaining in the centrifuge supernatant layer decreased in a linear manner as the amount of dextran–charcoal was varied over the range 0.2–10.0 times that used in the standard procedure, and by extrapolation of the linear relationship it was shown that in the

standard procedure the amount of complex removed was approx. 5%.

The presence of endogenous oestradiol in the uterine supernatant preparations examined could lead to an underestimation of the total high-affinity sites. Under the conditions of the assay dissociation of the high-affinity complex is negligible and the value obtained in the assay represents only the concentration of unoccupied high-affinity sites. That the inaccuracy introduced by endogenous oestradiol is small has been shown by heating uterine supernatant preparations at 60°C for 30min to destroy high-affinity receptor sites and to release the oestradiol so bound. Attempts to determine the liberated oestradiol by competitive binding (Méšter, Robertson & Kellie, 1970) indicate that the amounts released in this way are negligible in comparison with the concentration of unoccupied high-affinity sites.

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