A NEW ASSAY METHOD FOR THE DETERMINATION OF OESTROGEN RECEPTORS

By

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ABSTRACT

A new assay method was developed to determine the quantity of oestrogen cytoplasmic receptors which are capable of associating with acceptor sites on chromatin. The quantity of oestrogen receptor complexes associated with chromatin is of paramount importance in the mechanism of action of oestrogen. This method is based on the specific binding properties of oestrogen to the receptor and on the binding of the complex to chromatin. Cytosol was pre-incubated with $[^3]$H]oestradiol-17$\beta$ ($[^3]$HOe$_2$) to form a complex, and further incubated with a constant amount of chromatin. After this the quantity of $[^3]$HOe$_2$ cytosol receptor complexes specifically associated with the chromatin was determined. The binding activity of the chromatin to the $[^3]$HOe$_2$ receptor complex was stable for at least six months in 0.15 M NaCl at -20$^\circ$C. This method enables us to determine the quantity of the biologically active oestrogen receptors more specifically than with other methods.

The association of oestrogen receptor complex with chromatin is known to be important in the mechanism of the action of oestrogen (O'Malley et al. 1969; Mohla et al. 1972). From this, it may be assumed that the quantity of oestrogen cytoplasmic receptor complex which associates with acceptor sites on the chromatin, will determine the response of target tissues to oestrogen.

Various methods have previously been used for determining the concentration of oestrogen binding sites in the cytosol and in the nuclear fraction. These include: charcoal adsorption, Sephadex-chromatography, protamin sulphate precipitation, agar gel electrophoresis, density gradient centrifugation and the like. However, it is difficult with these methods to determine the amount of
the receptor capable of producing the specific biological action of oestrogen (Jungblut et al. 1972; Wagner 1975) since the quantity of oestrogen cytosol receptor complex associated with chromatin was not directly determined.

The purpose of the present investigation is to develop a new assay method for the determination of the quantity of oestrogen cytosol receptors which are capable of associating with chromatin.

**MATERIALS AND METHODS**

1) Steroids

[6,7-3H]Oestradiol-17β (N) (48 Ci/m mole) was obtained from the New England Nuclear Corporation. Non-radioactive steroids obtained from the Tokyo Kase Kogyo Co. (Tokyo, Japan) were used without further purification.

2) Preparation of cytosol

Immature rabbits (1000–1500 g) were killed by decapitation without anaesthesia. All procedures after this stage were performed at 4°C. The uteri were homogenized in a Potter-Elvehjem homogenizer with a teflon pestle with Tris EDTA (Tris 0.05 M HCl, pH 7.4, EDTA 1 mM) buffer (1 ml/0.1 g tissue). The homogenates were then centrifuged at 159,200 × g for 60 min. The supernatants were referred to as cytosol. The protein concentrations were determined by the method of Yoshikawa & Saito (1948) using the Biuret reaction.

3) Preparation of chromatin

Rabbit uteri were minced with scissors and homogenized in Tris EDTA buffer (10 ml/g tissue) in a teflon homogenizer. The homogenate was centrifuged at 800 × g for 10 min. The nuclear pellet was further homogenized in buffer I (0.25 M Sucrose, 0.01 M Tris, 0.002 M CaCl₂, and 0.2% Triton X-100, pH 7.4) and centrifuged at 1500 × g for 10 min by a modification of the procedure of O'Malley et al. (1970). The pellet was re-suspended in 10 vol of buffer I without Triton X-100 and centrifuged at 1500 × g for 10 min. After washing twice, the pellet was re-suspended in 20 vol of the same buffer and then passed through two layers of gauze. The filtrates consisted of pure nuclei as that seen under microscopic examination. The chromatin was extracted according to the method of Spelsberg et al. (1971). The final preparations of chromatin were re-suspended in 0.15 M NaCl (1 ml/g uterine tissue) and stored at −20°C. The DNA content was determined by the procedure of Burton (1956).

4) Assay of [3H]oestradiol-17β ([3H]Oe₂) receptor complex

0.5 ml of cytosol was pre-incubated with 2 × 10⁻⁹ M of [3H]Oe₂ for 60 min at 4°C in triplicate to form a complex. This complex was then incubated with 0.2 ml of the chromatin solution for 60 min at 25°C. After centrifuging at 1500 × g for 10 min, the sedimented chromatin was washed once with 3 ml of Tris EDTA buffer. The washed chromatin was re-suspended in 1 ml of the same buffer and collected on cellulose filter paper disks (Whatman, grade DE81, size 2.3 CMS) under vacuum. Each filter was washed with 8 ml of Tris EDTA buffer and the radioactivity was counted in a mixture of 3 ml of methanol and 10 ml of toluol scintillator (DPO 4 g/l, POPOP 0.1 g/l, in toluene) with a Packard liquid scintillation spectrometer (model 544). A negligible
amount of free $[^3H]Oe_2$ receptor complexes was bound to the cellulose filter. In each experiment, the chromatin was similarly incubated without cytosol and the radioactivity absorbed to chromatin was subtracted as background.

5) Dextran-coated charcoal adsorption assay

One ml of 0.05% dextran grade C (BDH Biochemical Corp.), 0.5% Norite A (American Norite Corp.) suspension prepared in 0.01 M Tris, 0.001 M EDTA buffer, pH 8.0, was added to 0.5 ml of the cytosol or serum pre-incubated with $[^3H]Oe_2$. After incubation at 4°C for 20 min, the mixture was centrifuged at 800 x g for 10 min. The radioactivity of the supernatant was counted. All counts were corrected for the blanks which were similarly incubated without cytosol.

RESULTS

a) Optimum conditions for the incubation

The effect of the incubation temperature on the interaction of the $[^3H]Oe_2$ receptor complex with chromatin was examined. The data shown in Table 1 demonstrate that the pre-incubation at 4°C followed by the incubation with chromatin at 25°C gives the highest binding of this complex to chromatin. After this the time course for the binding reaction at each temperature was

<table>
<thead>
<tr>
<th>Pre-incubation temperature with cytosol</th>
<th>Incubation temperature with chromatin</th>
<th>Bound $[^3H]Oe_2$ on chromatin/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4°C</td>
<td>0.9 ± 0.12 x 10^{-14} mole</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>2.0 ± 0.38</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>1.1 ± 0.32</td>
</tr>
<tr>
<td>4°C</td>
<td>4°C</td>
<td>0.4 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>0.5 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>0.6 ± 0.12</td>
</tr>
<tr>
<td>25°C</td>
<td>4°C</td>
<td>0.3 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td>0.4 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td>0.5 ± 0.13</td>
</tr>
<tr>
<td>37°C</td>
<td>4°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37°C</td>
<td></td>
</tr>
</tbody>
</table>

$[^3H]Oe_2$ (2 x 10^{-9} M) was pre-incubated with uterine cytosol (1.4 mg of protein) of immature rabbits for 60 min at 4°C, 25°C and 37°C. Each pre-labelled cytosol was then incubated with uterine chromatin (64 μg of DNA) of mature rabbits for 60 min at 4°C, 25°C and 37°C. Chromatin was prepared in the standard manner as described in the text. Results are expressed as the mean ± se of three determinations.
examined. During pre-incubation of cytosol at 4°C, equilibrium was achieved in about 60 min (Fig. 1A) and during incubation of chromatin at 25°C, equilibrium was achieved from 60 min to 90 min (Fig. 1B). Hence, pre-incubation of cytosol with [³H]Oe₂ was conducted for 60 min at 4°C, and incubation of chromatin with the [³H]Oe₂ receptor complex was carried out for 60 min at 25°C as a routine procedure.

b) Preparation of optimum chromatin

To test the amount of chromatin, the uterine chromatin of mature ovariectomized rabbits treated with oestradiol-17β-(Oe₂) or of mature rabbits treated with HCG was incubated with same amount of the [³H]Oe₂ receptor complex.
The uterine chromatin of the mature ovariectomized rabbits treated with Oe₂ had almost twice as high binding activity to the [³H]Oe₂ receptor complex as that treated with HCG, although the DNA content in chromatin treated with Oe₂ was smaller than that treated with HCG. The amount of chromatin obtained from the uteri of non-primed rabbits was too small for use. For these reasons, rabbits treated with daily injections of Oe₂ (50 μg in 0.1 ml of sesame oil) subcutaneously for seven days and sacrificed on the fourth day after the last injection, was used routinely for this assay.

**Fig. 2.**

Influence of concentration of [³H]Oe₂ in cytosol on the binding to chromatin.

A) Portions (2.5 mg protein/0.5 ml) of uterine cytosol of immature rabbits were pre-incubated with a range of [³H]Oe₂ (0.25–4.0 × 10⁻⁹ M) for 60 min at 4°C and further incubated with chromatin (64 μg of DNA) for 60 min at 25°C.

B) Optimum amount of chromatin for a [³H]Oe₂ receptor complex. Portions (2.45 mg protein/0.5 ml) of uterine cytosol of immature rabbits were pre-incubated with [³H]Oe₂ (2 × 10⁻⁹ M) for 60 min at 4°C and further incubated with increasing amounts of uterine chromatin for 60 min at 25°C.

Chromatin was prepared in the standard manner as described in the text.
A) Assay using Chromatin

![Graph A]

B) Nuclear Exchange Assay

![Graph B]

Fig. 3.

Comparison of steroid specificity of this assay with the nuclear exchange assay.

A) Portions of rabbit uterine cytosol were pre-incubated with \(^{3}H\)Oe\(_2\) either alone or in the presence of 100-fold of various unlabelled steroids, and further incubated with rabbit uterine chromatin.

B) Rabbits were injected with Oe\(_2\) (0.5 mg/kg) sc and their uteri were removed 60 min later. Portions of the uterine nuclear fraction were incubated with \(^{3}H\)Oe\(_2\) either alone or in the presence of 100-fold of unlabelled steroids.

Oe\(_2\): oestradiol, Oe\(_3\): oestriol, DES: diethylstilboestriol, P: Progesterone, C: cortisol, T: testosterone.

Results are expressed as the mean ± se of three samples.

c) Optimum concentrations of \(^{3}H\)Oe\(_2\), cytosol and chromatin

Fig. 2 A shows the concentration of \(^{3}H\)Oe\(_2\) needed to achieve maximal saturation of receptor sites in the cytosol (2.5 mg protein, 0.5 ml). The equilibrium of reaction was achieved with \(2 \times 10^{-9} \text{ M}\) of \(^{3}H\)Oe\(_2\). Consequently, it was decided that the concentration under 2.5 mg protein/0.5 ml, together with \(2 \times 10^{-9} \text{ M}\) of \(^{3}H\)Oe\(_2\) was adequate for this routine procedure. Then the amount of chromatin needed to saturate a given amount of \(^{3}H\)Oe\(_2\) receptor complex was examined. Fig. 2 B shows that the amount of chromatin, containing over 64 \(\mu\)g of DNA was enough for this assay method under routine conditions. In addition, the range of assay may be widened by using a higher concentration of chromatin solution than the 64 \(\mu\)g of DNA.
d) *Specificity of the chromatin binding of [³H]Oe₂ receptor complex*

Steroid specificity of this assay was determined by competition studies. As seen in Fig. 3 A, the transfer of [³H]Oe₂ to chromatin is remarkably suppressed by the addition of Oe₂, oestriol (Oe₃), diethylstilboestrol (DES), but is not suppressed by progesterone (P) or cortisol (C). Specificity of the binding of [³H]Oe₂ to nuclear oestrogen receptors of rabbit uteri was also examined by [³H]Oe₂ exchange assay according to Anderson et al. (1972). Fig. 3 B shows that the nuclear receptor oestrogen complex is exchanged not only for oestrogen but also for P or C, and there appear to be some non-specific oestrogen binding sites in the nuclei.

e) *Stability of chromatin*

In order to observe the influence of storage of chromatin, different batches of chromatin were incubated with a constant amount of the [³H]Oe₂ receptor complex under the same conditions. As summarized in Table 2, the chromatin can be stored for six months in 0.15 M NaCl at 20°C without significant changes in its binding properties, and moreover the binding activity of these chromatin to [³H]Oe₂ receptor complex is very nearly equal.

f) *Precision*

Intra-assay precision was examined by ten measurements of the same cytosol in the same assay. The coefficient of variation was 10.3 %.

*Table 2.*

Influence of storage on the binding activity of chromatin.

<table>
<thead>
<tr>
<th>Rabbit uterine cytosol</th>
<th>Rabbit uterine chromatin</th>
<th>Bound [³H]Oe₂/µg DNA</th>
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</thead>
<tbody>
<tr>
<td>Cytosol I</td>
<td>Chromatin A</td>
<td>249 ± 42 DPM</td>
</tr>
<tr>
<td></td>
<td>Chromatin B</td>
<td>230 ± 43</td>
</tr>
<tr>
<td>Cytosol II</td>
<td>Chromatin A</td>
<td>498 ± 24</td>
</tr>
<tr>
<td></td>
<td>Chromatin C</td>
<td>494 ± 71</td>
</tr>
<tr>
<td>Cytosol III</td>
<td>Chromatin A</td>
<td>153 ± 6</td>
</tr>
<tr>
<td></td>
<td>Chromatin B</td>
<td>150 ± 26</td>
</tr>
</tbody>
</table>

Portions of uterine cytosol of the different rabbits were pre-incubated with [³H]Oe₂ (2 x 10⁻⁹ M), and then incubated with various chromatins of rabbit uteri. Each mean ± se was calculated from three determinations.
Chromatin A: stored at -20°C for 6 months in 0.15 M NaCl.
Chromatin B: stored at -20°C for 3 months in 0.15 M NaCl.
Chromatin C: fresh.
**DISCUSSION**

The binding of oestrogen to chromatin followed by the stimulation of nuclear RNA synthesis are primary events in early oestrogen action (*Hamilton* 1968), and it is well known that the number of cytoplasmic binding sites reflects the amount of hormone transferred to the nucleus. Subsequently, the quantity of oestrogen cytoplasmic receptors has been measured to determine the response of various tissues to oestrogen. However, it is still uncertain whether all or only part of the cytosol receptor is transferred to the nucleus. Previous studies (*Arnaud et al.* 1971a,b; *DeSombre et al.* 1972) have shown that the 5S Oe$_2$-receptor complex but not the 4S or 8S Oe$_2$-receptor complex stimulates the synthesis of RNA in the uterine nuclei. *Jensen & Desombre* (1973) reported the current opinion namely that the conversion of the 4S receptor to the 5S form, a biologically functional form, is necessary when the oestrogen receptor complex moves into the nucleus. Thus the extent of the response of the target tissue to oestrogen may depend upon the quantity of cytoplasmic Oe$_2$-receptor transformed and bound specifically to chromatin. Moreover the transformation of a specific receptor is a phenomenon dependent on temperature and does not require the presence of nuclei. The essentially similar finding that the binding of the Oe$_2$-receptor complex to chromatin in a cell free system is markedly dependent on the incubation temperature, has been described (*McGuire et al.* 1972).

However, it is difficult to determine directly the quantity of oestrogen receptor specifically associated with chromatin by simple separation methods like the dextran coated charcoal assay. Oe$_2$ binds non-specifically to several serum protein (e.g. albumin, sex steroid binding globulin, corticosteroid binding globulin etc.), and a considerable concentration of these proteins are contained in all target tissue extracts. However, since simple separation with dextran coated charcoal solution does not eliminate all the non-specifically bound $[^3H]$Oe$_2$; a misleading result is sometimes obtained by the dextran coated charcoal assay. Consequently, methods which not only separate steroid receptor complexes from excess unbound steroid but at the same time also characterize the receptor protein, are necessary (*Jungblut et al.* 1972; *Wagner* 1975). When this new method is used, even if serum contamination is present in the cytosol of target tissues, the above-described hazard is virtually eliminated (Fig. 4), and moreover only biologically active oestrogen receptor complexes which can bind directly to acceptor sites are measured.

*Anderson et al.* (1974) have recently reported that the entire quantity of nuclear oestrogen complexes are not bound to nuclear acceptor sites and that only physiologically active oestrogen receptor complexes can be protected from degradation by their association with acceptor sites in the nucleus. Although the exchange assay is an efficient and useful procedure, it may have some
Portions of serum or uterine cytosol of immature rabbits were incubated with [³H]Oe₂ for 60 min at 4°C, then bound [³H]Oe₂ per mg protein was determined by this assay or by the dextran-coated charcoal method as described in Methods.

Results are expressed as the mean ± se of three samples.

limitation at present. Even so, the quantity of oestrogen receptor complexes will be more specifically determined by its association with chromatin rather than a nuclear binding.

In this respect it seems likely that this new assay technique reported here is a more specific and more accurate method for the measurement of Oe₂ receptor than the other methods reported so far.

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REFERENCES


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