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A MICROMETHOD FOR MEASURING THE ACTIVATION OF ESTRADIOL RECEPTORS OF THE RAT UTERUS

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SUMMARY

A method is described to estimate the activation of the estradiol–receptor complexes in the cytosol of the rat uterus. It measures the incorporation of a given amount of [^{3}H]-estradiol complexes into the nuclei. This nuclear uptake is increased after prolonged storage of [^{3}H]-estradiol containing cytosol at 0°C. Experiments have shown that this increase in nuclear uptake is not due to free metabolized estradiol, but that it is proportional to the amount of estradiol receptor complexes, provided they have been stored on ice for the same length of time. It is concluded that the nuclear uptake reflects the degree of activation of the labeled cytosol receptors.

INTRODUCTION

An early event in the interaction of estradiol with its target cell is the binding of the hormone to an extra nuclear receptor protein [1, 2]. This complex is subsequently transformed and activated resulting in a change in its sedimentation constant [3, 4] and an increased affinity for nuclei [5]. The steroid–receptor complex is then translocated into the nucleus [2, 6–8].

A better understanding of these events is essential for further investigations into the mechanism of estradiol-mediated gene expression. The transformation process has been extensively studied using sucrose density gradients to analyze precursors and products [3, 4, 8–11]. Activation of the receptor-steroid complex has been demonstrated by a variety of methods, most of which require much effort and material [5, 6, 10]. In this paper, we describe a micromethod for the study of the activation of the steroid cytosol receptor complex.

Experimental

[6,7^{3}H]-Estradiol 17β, 48 Ci/mmol was obtained from New England Nuclear Company. Only reagent grade chemicals were used. Uteri were obtained from Sprague–Dawley rats (170–200 g) ovariectomized 84 h previously.

After incubation with estrogen–receptor complex, nuclei were separated by vacuum filtration using a device designed in this laboratory. This apparatus permitted simultaneous filtration of 60 samples through Whatman GF/A filters 5 mm dia. Samples were placed in 1 ml chambers above the filters. Wash solution was introduced through 15 ml tubes which were located above the sample chambers. The radioactivity of the isolated nuclei and other samples was measured in a Packard spectrometer, Model 3375, with 40% efficiency for tritium.

All preparations and incubations of the various cell fractions were carried out at 0 to 4°C. Cytosol and nuclei were prepared from minced uterine tissue, homogenized in 20 vol of buffer using a polytron PT 10. Nuclei were prepared from tissues homogenized in 0·5 M sucrose in buffer A (10 mM Tris, 2 mM $MgCl_2$, pH 7·4). The homogenate was filtered through two layers of cheese cloth and centrifuged 10 min at 6000 *g*. The pellet was rehomogenized by hand in the original volume in a solution of 1·75 M sucrose in buffer A and then centrifuged for 20 min at 25,000 *g*. The nuclear pellet was resuspended as before in the original vol. of 1·25 M sucrose in buffer A and stored at −15°C. Cytosol was prepared from tissue homogenized in 10 mM Tris, 1·5 mM EDTA, pH 7·5 (buffer B) by centrifuging the homogenate for 1 h at 105,000 *g*.

The uptake of the estradiol–receptor complex by the nuclei was measured as follows: the cytosol was incubated at ice bath temperature with [^{3}H]-estradiol for varying lengths of time. A sample of the cytosol, 160 μl, was then added to 40 μl of freshly melted nuclei and the mixture was incubated for the time indicated, usually 60 min. After the incubation period, the nuclei were collected by vacuum filtration on filters. The incubation tubes were washed with 1 ml of buffer B, and the filters with 2 ml of buffer B containing 1 μM unlabeled estradiol to displace any loosely bound [^{3}H]-estradiol. The filters were then air-dried and counted in 10 ml of a toluene base scintillation fluid. In all experiments, the background level of

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radioactivity of the filters was determined by incubating samples containing no nuclei. The background radioactivity increased with prolonged storage at 0°C of the cytosol containing [^{3}H]-estradiol.

After counting, the filters were removed from the vials and allowed to dry in the air. The DNA content of the nuclei on the glass filters was determined by the method of Burton[12]. The material from at least four filters was pooled to provide measurable quantities of DNA. For each incubation with nuclei, the amount of estradiol receptor complex in the cytosol was estimated by removing free estradiol with dextran-coated charcoal [13].

The specific nuclear uptake was calculated by subtracting the background radioactivity on the filters without nuclei from the corresponding experimental values. This was then corrected for the amount of DNA on the filter and for the initial number of estradiol receptor complexes in the cytosol of the nuclear uptake assay. This was expressed as c.p.m. in nuclei per μg DNA and 100 c.p.m. in charcoal treated cytosol. When appropriate, [^{3}H]-estradiol was removed from the labeled cytosol by extracting twice with ether. The ether was allowed to evaporate and the residue was dissolved in buffer B.

RESULTS AND DISCUSSION

Uterine cytosol incubated with [^{3}H]-estradiol at ice bath temperature exhibited a linear increase with time in specific nuclear uptake (Fig. 1, curve a). The total amount of [^{3}H]-estradiol–receptor complex in the cytosol, however, remained essentially constant (Fig. 1, curve b). The specific nuclear uptake of [^{3}H]-estradiol extracted from this cytosol at various times during the incubation was small and constant (Fig. 1, curve c). This permits the inference that the measured increase in nuclear uptake is a result of the activation of the [^{3}H]-estradiol-receptor complex.

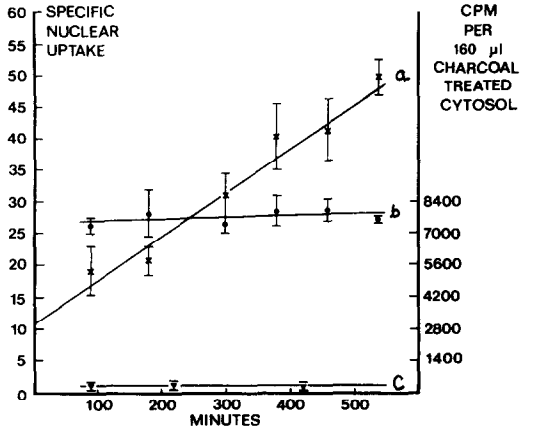

Fig. 1. Activation of the estradiol receptor complex. Cytosol containing 1 nM [^{3}H]-estradiol was incubated over a period of 9 h at ice bath temperature. At the times indicated aliquots were removed for analysis. The nuclear uptake was measured after incubating the cytosol with nuclei for 1 h and the specific nuclear uptake was calculated as c.p.m. in nuclei per μg DNA and 1000 c.p.m. in charcoal treated cytosol. Statistical analysis showed that these points lay on a straight line $N = 18$ $r = 0{\cdot}8482$ $P < 0{\cdot}01$. (Curve *a*). The total amount of [^{3}H]-estradiol–receptor complex remained essentially unchanged over a period of 90 min to 540 min (curve *b*, right hand ordinate). From certain aliquots [^{3}H]-estradiol was extracted and incubated with nuclei for 1 hr. The specific nuclear uptake of free estradiol was essentially zero (curve *c*). All experiments were carred out three times with different cytosol preparations.

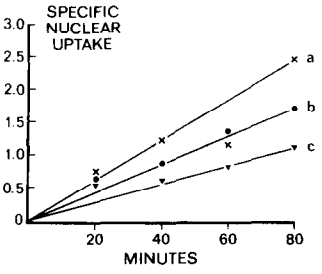

Fig. 2. Nuclear uptake of free [^{3}H]-estradiol. Aliquots of buffer B containing 0·2 (curve *a* ×——×). 1 (curve *b* ●——●) and 5 (curve *c* ▼——▼) nM [^{3}H]-estradiol were incubated with nuclei. At the times indicated samples were removed and the specific nuclear uptake was measured. The results shown are the means of two experiments.

Figure 2 shows that the specific nuclear uptake of free [^{3}H]-estradiol is only 2–4% of the corresponding value for the [^{3}H]-estradiol–receptor complex. The nuclear specific uptake of free estradiol is linear with time for 80 min and decreases as the concentration of free [^{3}H]-estradiol is increased.

The experiments summarized in Table 1 demonstrate that the nuclear uptake is proportional to the amount of [^{3}H]-estradiol complex rather than to the total concentration of [^{3}H]-estradiol in cytosol provided they are incubated at 0°C for the same length of time. The data summarized in Fig. 3 demonstrate that the nuclear uptake is directly proportional to the time of interaction of nuclei and cytosol. The proportionality is independent of the time during which cytosol is incubated with labeled estradiol. The rate of nuclear uptake increases as the time of incubation of cytosol with estradiol is increased. The data summarized in Table 1 and Fig. 3 suggest, as has been found by others [14, 15], that there is an excess of nuclear acceptor sites and that there is a small rate of dissociation of estradiol receptor complexes from the nuclei. These experiments indicate that the specific nuclear uptake reflects the degree of activation of the labeled cytosol. Since receptors occur in at least two different forms [3, 4, 8, 16] and both may have some affinity for nuclei, the specific nuclear uptake may not necessarily be a direct measure of the amount of activated [^{3}H]-estradiol–receptor complexes in the cytosol.

The method described here is a microadaptation of procedures which have been designed to investigate the interaction between the [^{3}H]-estradiol–receptor and chromatin [17, 18]. The use of very small filters

Table 1. Nuclear uptake is proportional to the amount of [^{3}H]-estradiol complex

	Specific nuclear uptake Mean ± SD	Amount of [^{3}H]-estradiol–receptor complexes Mean ± SD	Concentration of [^{3}H]-estradiol Mean ± SD
cytosol 5 nM [^{3}H]-estradiol / cytosol 1 nM [^{3}H]-estradiol	1·128 ± 0·134	1·186 ± 0·098	4·306 ± 0·070
cytosol 5 nM [^{3}H]-estradiol / cytosol $\frac{2}{5}$ nM [^{3}H]-estradiol	5·042 ± 0·810	5·0	5·0
cytosol 1 nM [^{3}H]-estradiol / cytosol $\frac{2}{5}$ nM [^{3}H]-estradiol	4·520 ± 0·911	4·245 ± 0·375	1·161 ± 0·02

Cytosol was divided into two parts and [^{3}H]-estradiol was added to a final concentration of 5 nM and 1 nM respectively. A portion of the former solution was then diluted 1:5 with buffer B and designated cytosol $\frac{2}{5}$ nM [^{3}H]-estradiol. The three cytosols were incubated on ice. Aliquots were removed after 1,2,3 and 4 h and the total concentration of [^{3}H]-estradiol, the amount of estradiol receptor complexes, and the specific nuclear uptake were measured as described under Experimental. All measurements were made in triplicate. Only the nuclear uptake of the diluted cytosol was measured. The two other values were calculated from the cytosol containing 5 nM [^{3}H]-estradiol.

Each time when aliquots had been removed from the cytosols, the means of the measurements were calculated and used to determine the ratios of the specific nuclear uptake, the amount of estradiol receptor complex, and the total concentration of [^{3}H]-estradiol between the cytosols. This gave 4 values for each particular ratio from which the mean ± SD shown in the table was computed.

to recover the nuclei keeps the background low and makes it unnecessary to remove the free [^{3}H]-estradiol before interacting the labeled cytosol with nuclei [18]. It is not necessary to wash the nuclei before placing them on the filters [17, 18].

In this procedure, the nuclei are left on the filters of the filtration apparatus until the entire experiment is concluded. The filters are kept dry by maintaining a vacuum in the lower chamber all the time. There is no decrease in the DNA content of the nuclei that are stored on the filters over a prolonged period of time. This procedure makes it possible to begin all of the experiments at the same time with the same cytosol preparation and to handle a large number of samples easily. This becomes important when the activation process is accelerated by heating or by increasing the ionic strength of the incubation of cytosol with [^{3}H]-estradiol.

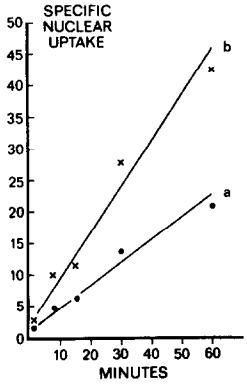

Fig. 3. Nuclear uptake is linear with time. Cytosols containing 1 nM [^{3}H]-estradiol were incubated for 90 and 540 min at ice bath temperatures. They were then allowed to interact with nuclei for the indicated times. The specific nuclear uptake was calculated as c.p.m. in nuclei per μg DNA and 1000 c.p.m. in charcoal treated cytosol. Curve *a* shows the specific nuclear uptake of cytosols incubated 90 min and curve *b* shows the specific nuclear uptake of cytosols incubated 540 min. Statistical analysis showed that these points lay on straight lines with N = 5, r = 0·9874, $P < 0{\cdot}01$ and N = 5, r = 0·9844, $P < 0{\cdot}01$ for the curves *a* and *b* respectively. The figure shows the result of a typical experiment.

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