Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 539–544. Pergamon Press. Printed in Great Britain

INFLUENCE OF HEATING AND ESTRADIOL ON THE ACTIVATION AND TRANSFORMATION OF THE ESTRADIOL RECEPTOR OF THE RAT UTERUS

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(*Received* 8 *December* 1975)

SUMMARY

Cytosol from uteri of ovariectomized rats was incubated at different temperatures (0°C, 15°C, 25°C) for various lengths of time before or after tritium labeled estradiol was added. The influence of these treatments on the incorporation of a given amount of estradiol–receptor complex into uterine nuclei and on its sedimentation in sucrose density gradients was studied. Prolonged incubation of the cytosol at 0°C resulted in increased nuclear uptake. Incubating the cytosol at higher temperatures (15°C, 25°C) produced first an increased, then a decreased nuclear uptake. Thus the estradiol-receptor complexes were first activated and then inactivated. Both processes are accelerated with increasing temperature, but the inactivation process has a higher temperature coefficient than the activation process. At higher temperatures (15°C, 25°C) the absolute amount of activation achieved was smaller in the presence than in the absence of estradiol. Prolonged incubation of the cytosol resulted in a 4S to 5S transformation of the estradiol receptor complex, which was enhanced at increased temperature and by the presence of estradiol. At 15°C transformation becomes dominant only after activation has stopped and inactivation has begun. Since activation occurs rapidly it is distinctly different from transformation and inactivation, which occur later.

INTRODUCTION

Radioactive estradiol injected into an immature or ovariectomized female rat rapidly accumulates in the nuclei of target organs [1, 2]. The hormone first becomes associated with a receptor protein in the cytosol before it is concentrated in the nuclei [3–6]. It is generally assumed that the accumulation of estradiol in the nucleus is the result of a translocation of the estrogen–receptor complex from the cytoplasm into the nucleus.

Several lines of evidence suggest that the nuclear and cytosol receptors have the same estradiol binding subunit. Target tissues exposed to radioactive estradiol show a decrease of binding sites in the cytosol and a corresponding increase of binding sites in the nucleus [4, 7, 8]. The estradiol binding subunits of the two receptors cosediment in sucrose density gradients containing 4 M urea and 1 M KCl [9]. Estradiol–receptor complexes are taken up into nuclei from cytosol in cell free experiments [10–13]. However, the two receptors are not identical. In the presence of 0.4 M KCl the nuclear receptor sediments slightly faster in sucrose density gradients than the cytosol receptor [9, 14]. This indicates that the cytosol receptor must be transformed into the nuclear receptor. It is not known whether this transformation involves a change in the conformation of the molecule or an association with a similar or different subunit, but it is thought to occur in the cytoplasm [11, 15, 16]. Another process which must occur to permit the translocation of the receptor into the nucleus is an increase in its affinity to nuclear material. This process, called activation, has been shown to occur in the cytosol *in vitro* [16, 17]. Both of these reactions were thought to be temperature or salt dependent. It was assumed that they were two different aspects of the same underlying chemical reactions. In this paper, we present evidence that activation and transformation are different processes and occur even in the absence of salt at 0°C.

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.

Preparation of cell fractions. All preparations and incubations of the various cell fractions were carried out at 0° to 4°C unless otherwise stated. Cytosol and nuclei were prepared from minced uterine tissue, homogenized in 20 vol. of buffer using a polytron Pt10. 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

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10 min at 6000 *g*. The pellet was rehomogenized by hand in the original vol. 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 up to six weeks before being used in the nuclear uptake assay. Cytosol was prepared from tissue homogenized in 10 mM Tris, 1.5 mM EDTA, pH 7.4 (buffer B) by centrifuging the homogenate for 1 h at 105,000 *g*.

Treatment of cytosol. To measure the influence of estradiol on activation and transformation, the cytosol was divided in two parts and treated as follows. (a) Presence of estradiol: 1 mM estradiol was added to the cytosol and estradiol and receptor were allowed to react for 1 h at 0°C. Then the labeled cytosol was incubated at different temperatures for varying lengths of time. This was followed by a cooling period of 30 min at ice bath temperatures. (b) Absence of estradiol: The cytosol was allowed to stand on ice for 30 min before it was incubated for varying lengths of time at different temperatures. It was then cooled on ice and allowed to react with 1 nM [^{3}H]-estradiol for 1 h.

Nuclear uptake. A sample of the cytosol (160 μl) was added to 40 μl of freshly thawed nuclei and the mixture was incubated for 60 min at 4°C. After the incubation period, the nuclei were collected by vacuum filtration on Whatman GF/A filters 5 mm dia. 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 based scintillation fluid in a Packard spectrometer model 3375 with 40% efficiency for tritium. In all experiments the background radioactivity of the filters was determined by incubating samples containing no nuclei. The background radioactivity increased with prolonged incubation of the cytosol.

After counting, the filters were removed from the vials and allowed to dry in air. The DNA content of the nuclei on the glass filters was determined by the method of Burton[18]. 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 [19]. This estimation was performed at the same time as the nuclear uptake assay. 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 and expressed as c.p.m. in nuclei per μg DNA and 1000 c.p.m. in charcoal treated cytosol. Previous experiments have shown that the nuclear uptake assay specifically measures the translocation of estradiol–receptor complex from the cytosol to the nucleus [13].

Gradients. An aliquot of the receptor-containing solution (200 μl) was layered on top of 5–20% sucrose gradients in buffer B containing 400 mM KCl and centrifuged for 13½ h at 50,000 rev./min in a Beckman SW 50.1 rotor. The tubes were pierced and 36 fractions were collected directly in counting vials. Following the addition of 1 ml NCS and 10 ml of a toluene based scintillation fluid, the radioactivity of each sample was determined.

RESULTS AND DISCUSSION

The activation of the estradiol receptor incubated at different temperatures in the presence or absence of estradiol. The incubation temperature has a marked influence on the time course of the activation process and on the extent of activation achieved. Figure 1 shows the results obtained by incubating the 105,000 *g* supernatant in the presence of 1 nM tritiated estradiol at different temperatures. Maximal activation is obtained more rapidly with increasing temperature but its absolute value decreases. The curves demonstrate that the estradiol receptors are subject to activation and inactivation processes. Neither of these processes is due to a change in the content of the estradiol–receptor complex in the cytosol since the method of calculating the specific nuclear uptake takes this into account. The rates of both activation and inactivation processes are temperature dependent but not to the same extent. [^{3}H]-estradiol containing cytosol incubated on ice shows no apparent inactivation and the specific nuclear uptake increases linearly over the entire incubation period. In contrast, when incubated at 25°C, the specific nuclear uptake increases only for the first 10 min and then begins to

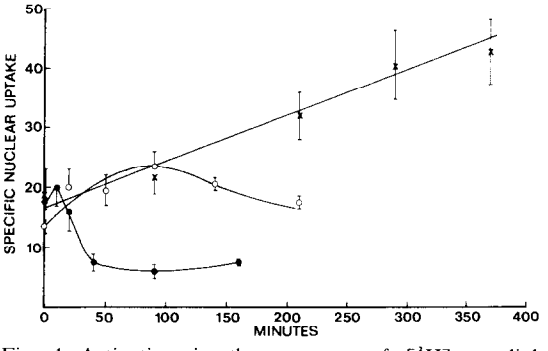

Fig. 1. Activation in the presence of [^{3}H]-estradiol. [^{3}H]-estradiol was added to the cytosol. The labeled hormone and the receptor were allowed to react for 1 h at 0°C. Then portions of the cytosol were incubated at 25°C (●——●), 15°C (○——○) or 0°C (×——×). At the indicated times an aliquot was removed from the incubation mixture, cooled to 0°C for 30 min and interacted with nuclei for 60 min at 0°C. The nuclear uptake was measured and the specific nuclear uptake was calculated as cpm in nuclei per μg DNA and 1000 c.p.m. in charcoal treated cytosol. Each point represents the mean ± standard error of three experiments with different cytosol preparations.

decrease. Figure 1 shows clearly that with increasing temperature inactivation becomes dominant over activation. Therefore, the rate of the inactivation process has a greater temperature coefficient than the rate of the activation process.

The time course of activation of the estradiol receptor incubated at different temperatures in the absence of [^{3}H]-estradiol (Fig. 2) was similar to that in the presence of estradiol (Fig. 1). In both experiments activation and inactivation were temperature dependent in the same general way. No inactivation is evident when the mixture is incubated on ice, whereas inactivation is predominant when the mixture is incubated at 25°C.

The activation curves of cytosol incubated on ice in the presence or absence of [^{3}H]-estradiol are similar. However, when cytosol is incubated at 15°C (Fig. 3) the maximal specific nuclear uptake is reached earlier and is lower when incubated in the presence of [^{3}H]-estradiol than when incubated in the absence of the hormone. Cytosol incubated at 25°C in the presence or absence of [^{3}H]-estradiol exhibits similar, but less pronounced, differences.

The amount of [^{3}H]-estradiol receptors incubated with the nuclei was measured after removing the free [^{3}H]-estradiol by charcoal adsorption (Table 1). The duration of the treatment of the cytosol did not alter the concentration of [^{3}H]-estradiol–receptor complex if the cytosol was incubated either in the presence of labeled hormone or at 0°C. The amount of [^{3}H]-estradiol–receptor complex decreased with the duration of the incubation of cytosol in the absence of labeled hormone (B) at elevated temperatures. This effect was more pronounced at 25°C than at 15°C. As mentioned above, the change in the content of [^{3}H]-estradiol–receptor complex in the cytosol has

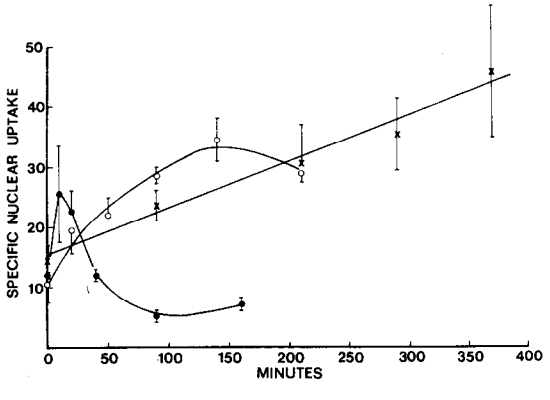

Fig. 2. Activation in the absence of [^{3}H]-estradiol. Freshly prepared cytosol was allowed to stand on ice for 30 min. Then portions of it were incubated at 25°C (●——●), 15°C (○——○) or 0°C (×——×). At the indicated times an aliquot was removed from the incubation mixture, cooled and [^{3}H]-estradiol was added. The receptors were allowed to bind the labeled hormone at 0°C for 1 h before they were interacted with nuclei for another hour. The nuclear uptake was measured and the specific nuclear uptake was calculated. Each point represents the mean ± standard error of three experiments with different cytosol preparations.

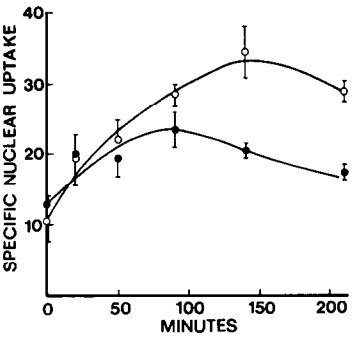

Fig. 3. Activation in the presence and absence of [^{3}H]-estradiol at 15°C. The specific nuclear uptake of cytosol incubated in the presence (●——●) or absence (○——○) of [^{3}H]-estradiol for the indicated time was measured as described under Methods. Each point represents the mean and standard error of three experiments with different cytosol preparations.

no influence on the specific nuclear uptake since the calculation of this number takes this change into account.

The transformation of the estradiol–receptor at different temperatures in the presence and absence of estradiol. The incubation temperature also has a marked influence on the time course and extent of transformation achieved. Figure 4 presents the results obtained by incubating the cytosol in the presence of [^{3}H]-estradiol at 0°C and 15°C. At the start of the incubation an almost symmetrical peak with an S value of 3.8 is visible (BSA = 4.7S). It has a small shoulder in the 5S region. At both temperatures the height of the peak in the 4S region is decreased with prolonged incubation and a peak in the 5S region (5.3S) becomes more prominent. This change of the receptor from 4S to 5S is more impressive when the cytosol is incubated at 15°C than when it is incubated on ice.

Table 1. The radioactivity remaining in 0.16 ml cytosol after charcoal adsorption

0°C		15°C		25°C	
min	c.p.m.	min	c.p.m.	min	c.p.m.
A. Incubation in presence of [^{3}H]-estradiol					
0	7363 ± 188	0	6367 ± 1241	0	6701 ± 227
90	7886 ± 587	20	6948 ± 1241	10	6529 ± 271
210	7418 ± 172	50	6882 ± 1225	20	6625 ± 419
290	8007 ± 358	90	7465 ± 1532	40	6841 ± 77
370	8050 ± 259	140	7714 ± 1203	90	6540 ± 214
		210	7551 ± 1191	160	6011 ± 155
B. Incubation in absence of [^{3}H]-estradiol					
0	7199 ± 665	0	5285 ± 338	0	5621 ± 358
90	7040 ± 182	20	4971 ± 321	10	5282 ± 336
210	6799 ± 274	50	5018 ± 366	20	4521 ± 294
290	6568 ± 212	90	4537 ± 272	40	3338 ± 344
370	6023 ± 510	140	4042 ± 157	90	1953 ± 344
		210	3935 ± 304	160	1192 ± 102

Cytosol was incubated in the presence (A) or absence (B) of [^{3}H]-estradiol at different temperatures. At the indicated times aliquots were removed, the free [^{3}H]-estradiol was adsorbed on charcoal and the amount of [^{3}H]-estradiol–receptor complex was estimated. The values are the means ± standard errors of three experiments with different cytosol preparations.

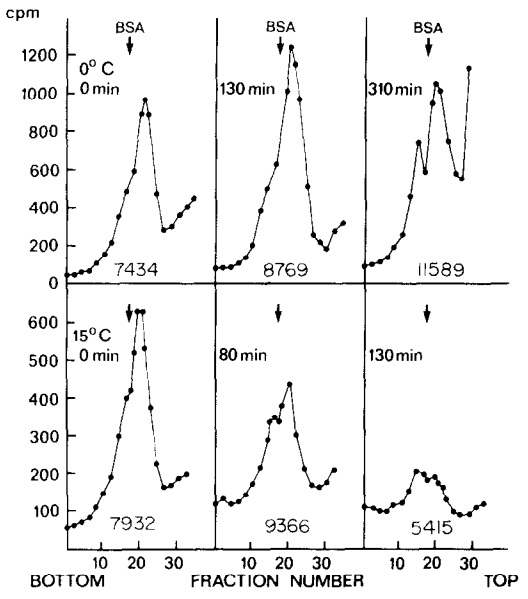

Fig. 4. Transformation in the presence of [^{3}H]-estradiol. [^{3}H]-estradiol was added to the cytosol. The labeled hormone and the receptor were allowed to react for 1 h at 0 C. Then the cytosol was incubated at 15 C or 0 C. At the indicated time an aliquot was removed, cooled to 0°C for 30 min and layered on top of a 5–20% sucrose gradient containing 0.4 M KCl. The gradient was centrifuged in a Beckman SW 50.1 rotor at 50,000 rev./min for 13½ h. The tubes were pierced and 36 fractions were collected directly in counting vials. The radioactivity was determined in a Packard spectrometer model 3375 with 30% and 40% efficiency for the 15 C and 0 C incubation respectively. BSA was used as a marker. The numbers given under each peak are estimates of the amount of [^{3}H]-estradiol–receptor complex added to the gradients. Shown are the means of two measurements. For details see under Experimental.

Incubating the cytosol in the absence of estradiol at 0 C and 15 C (Fig. 5) gave results similar to those obtained in the presence of [^{3}H]-estradiol (Fig. 4) but the extent of transformation achieved is smaller at 15 C.

Figures 4, 5, and 6 show that the area under the gradient profile decreased with prolonged incubation of the cytosol. Two phenomena could account for this observation. First, the adsorption of the [^{3}H]-estradiol–receptor complex to the wall of the nitrocellulose tube might increase just as the adsorption to the glass fiber filter increases (see Experimental Section for details). Second, the amount of aggregated material which sediments to the bottom of the centrifuge tube might increase. The latter phenomenon was more prominent when the cytosol was incubated at 15°C than when it was incubated at 0°C. This might account for the greater decrease in the area under the gradient profile when the cytosol was incubated at 15 C than when it was incubated at 0°C. The low profile seen in the lower panel of Fig. 4 at 130 min incubation was due to the small amount of [^{3}H]-estradiol–receptor complex added to the gradient. The amount of radioactive material detected at the top of the gradient did not increase with the length of incubation, suggesting that no excessive dissociation of the [^{3}H]-estradiol–receptor complex occurred. This suggestion is further supported by the data shown in Table 1.

These results do not resolve the question of whether estradiol must be present for the transformation, but they show that the rate of this process is enhanced in the presence of estradiol at 15°C.

Comparison of activation and transformation. Figure 6 compares the activation and transformation of the receptor in the presence of estradiol at 15°C. Activation occurs during the first 80 min of incubation. During this time, little transformation can be demonstrated. During the second half of the incubation the reverse is true. The receptor is not further activated but it is transformed. Incubation in the absence of estradiol at 15°C (not shown) revealed a similar relationship between activation and transformation. From these results, we conclude that activation and transformation of the estrogen receptors are two different processes and that activation occurs earlier than transformation during the incubation.

The amount of labeled material sedimenting to the bottom of the tube increased with prolonged incubation of the cytosol at 15°C and removed an increasing amount of material from the 4 to 5S region of the gradient. It may be speculated that this aggregation phenomenon may have played a role in the inactivation of the [^{3}H]-estradiol–receptor complex observed

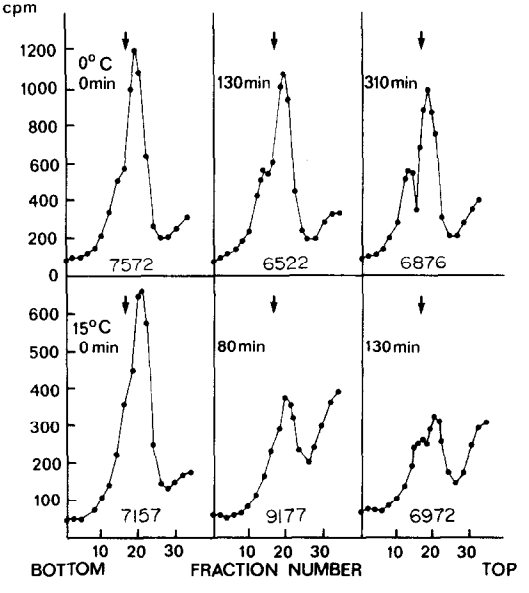

Fig. 5. Transformation in absence of [^{3}H]-estradiol. Freshly prepared cytosol was allowed to stand on ice for 30 min. Then it was incubated at 15°C and 0°C. At the indicated time an aliquot was removed, cooled and [^{3}H]-estradiol was added. The receptors were allowed to bind the labeled hormone at 0°C for 1 h before they were layered on a 5–20% sucrose gradient containing 0.4 M KCl. The gradient was then treated as described under Fig. 4. The numbers given under each peak are estimates of the amount of [^{3}H]-estradiol–receptor complex added to the gradients. Shown are the means of two measurements. For details see under Experimental.

after longtime incubation of the cytosol at higher temperatures.

These experiments demonstrate the occurrence of three different processes, activation, inactivation and transformation, in the uterine cytosol when stored over an extended period of time at different temperatures. Activation and transformation have been assumed to be two different aspects of the same process [16]. The present results suggest that inactivation and transformation occur subsequent to activation which occurs earlier and is a separate process.

The experiments showed that the amount of estradiol present is not rate limiting in the activation process and it may not be necessary at all.

Chatkoff *et al.*[20] showed that estradiol is neither necessary for binding of receptors to chromatin nor does it significantly influence the equilibrium parameters which describe the extent of binding. Rochfort *et al.*[21] and Ruh *et al.*[22] showed that certain androgens are able to translocate the free estradiol receptor from the cytoplasm to the nucleus. The experiments described in this paper provide some indication that the receptor is not activated in the intact ovariectomized animal and becomes activated only after the sacrifice of the animal. In Figure 7, the activation curve obtained by incubating the cytosol on

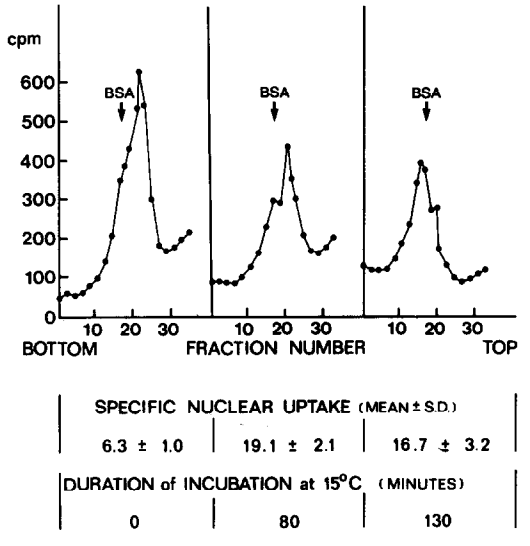

Fig. 6. Comparison between transformation and activation in presence of [^{3}H]-estradiol at 15°C. Estradiol was allowed to bind to the receptors for 1 h at ice bath temperature, before they were incubated at 15°C. At the indicated time an aliquot was taken out and cooled to 0°C for 30 min. Then the specific nuclear uptake and the amount of transformation was estimated as described under Methods. The amounts of [^{3}H]-estradiol-receptor complex added to the assays was estimated by charcoal adsorption as described in the Methods section and is expressed as means of two determinations. Aliquots containing 10776, 9540 and 9516 c.p.m. were added to the gradients and aliquots containing 8621, 7632 and 7613 c.p.m. were added to the nuclear uptake assay. These aliquots were taken from cytosol incubated at 15°C for 0, 80 and 130 min respectively. The figure presents the results of a typical experiment.

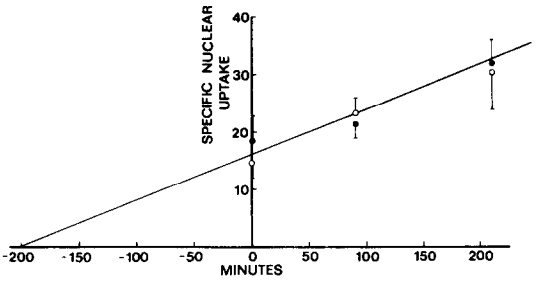

Fig. 7. Extrapolation of the activation curve at 0°C. The activation curves of the cytosol incubated at 0°C in the presence (●) or absence (○) of [^{3}H]-estradiol as described in the legend of Figs. 1 and 2 have been plotted and extrapolated to their intercept with the abscissa.

ice was extrapolated back to its intercept with the horizontal axis. This is about the time of the start of the experiment. These results from the literature and our backward extrapolation taken together would tend to indicate that the receptor has to undergo an activation before it can interact with nuclear material and that the presence of estradiol is not necessary for this process. *In vivo* the receptor may be restricted to some parts of the cell where it is prevented from undergoing spontaneous activation. The microsomal fraction also contains estradiol receptors [23]. One possible role of the estradiol may therefore be to free the receptor so that it can undergo activation. The inactivation observed is not a loss of estradiol-receptor complexes since in calculating the specific nuclear uptake, the values are normalized to a constant number of estradiol receptor complexes. The rate of inactivation is very much enhanced by elevating the temperature. The findings that transformation is much more obvious at higher temperatures and in the presence of the hormone are in accordance with several reports [11, 15]. The 5.3S peak becomes dominant only after activation has stopped and inactivation has started (Fig. 6). The experiments described in this paper show that *in vitro* activation and transformation are two different processes.

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