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ESTROGEN RECEPTOR IN CHICKEN OVIDUCT: RECEPTOR DISSOCIATION KINETICS AND TRANSFORMATION

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Summary—Cytosolic and nuclear estrogen receptor forms of chicken oviduct have been studied by (1) measuring hormone dissociation kinetics and by (2) sucrose density gradient analysis on high salt gradients. Estradiol dissociates from the receptor in chicken oviduct cytosol at 22°C following a two-phase exponential process. The fraction of receptor with a fast dissociation rate ($k = 120 \times 10^{-3}\,min^{-1}$) decreases as a function of the pre-incubation at 22°C; after prolonged pre-incubation only the slowly dissociating ($k = 12.3 \times 10^{-3}\,min^{-1}$) form remains. Dissociation of moxestrol, a synthetic estrogen with a higher affinity, from the cytosol receptor at 30°C is similar, showing a transition of a fast dissociating form ($k = 120 \times 10^{-3}\,min^{-1}$) to a slowly dissociating form ($k = 7.6 \times 10^{-3}\,min^{-1}$) as a result of pre-incubation at 30°C. A concomitant temperature-dependent shift of the estrogen receptor from a 4.8 S to a 6.1 S form was observed with moxestrol but not with estradiol as a ligand. Sodium molybdate (20 mM) and NaSCN (400 mM) inhibit the temperature-dependent increase in sedimentation coefficient, but molybdate allows the formation of a receptor form which shows intermediary dissociation kinetics. Estrogen receptor, precipitated with ammonium sulfate (0–35%) shows monophasic dissociation kinetics of estradiol ($k = 39.5 \times 10^{-3}\,min^{-1}$) and for moxestrol ($k = 10.8 \times 10^{-3}\,min^{-1}$), suggesting full receptor activation only with moxestrol as a ligand. Moxestrol–receptor complexes obtained by ammonium sulfate precipitation sediment at 0°C at 4.8 S. Only after subsequent incubation at 30°C a shift from 4.8 S to 5.9 S is observed, suggesting that the formation of the slowly dissociating form of the receptor may precede the formation of a stable transformed receptor complex.

The nuclear estrogen receptor with estradiol as a ligand shows biphasic dissociation kinetics at 22°C ($k = 70 \times 10^{-3}\,min^{-1}$; $k = 14.0 \times 10^{-3}\,min^{-1}$). The ratio of both components (1:1) does not change after preincubation of the nuclear receptor extract at 22°C. Moxestrol dissociates from the nuclear receptor at 30°C monophasically with a slow rate ($k = 6.1 \times 10^{-3}\,min^{-1}$), suggesting that it is extracted as an activated hormone–receptor complex. Our data show that *in vitro* the cytosolic estrogen receptor can undergo a temperature-dependent transition from a low to a high affinity form. The *in vitro* transformation to a form with a higher sedimentation coefficient appears to be dependent on the type of ligand bound to the receptor. The receptor extracted from the nuclei is predominantly present in the higher affinity state.

INTRODUCTION

Administration of estrogens to immature chickens induces growth and cell differentiation of the oviduct and the onset of specific synthesis of egg-white proteins, such as ovalbumin, conalbumin, ovomucoid and lysozyme [1–5]. When estrogen treatment is discontinued the differentiated tubular gland cells lose their ability to synthesize these proteins. These effects are thought to be mediated through the formation of a specific estrogen–receptor complex in the cytoplasm, which is subsequently translocated into the nucleus [6–8]. However recently it has been reported by two groups of investigators that the non-liganded receptor is localized in the nucleus and that the cytoplasmic localisation is based on an experimental artefact [9, 10].

A cytoplasmic as well as a nuclear form of estrogen receptor have been described for chicken oviduct in terms of estrogen specificity and sedimentation characteristics [11–13]. In low ionic strength sucrose gradients the cytoplasmic receptor sediments as an 8 S molecule, which is converted to a 4 S form in 0.15–0.4 M KCl [14–16]. After *in vivo* or *in vitro* exposure of oviduct tissue to estrogens a nuclear receptor with a 5 S sedimentation value can be extracted [15].

In all sex steroid hormone systems studied, the receptors in crude cytosol have been found not to bind to nuclei or DNA unless they undergo some temperature- or salt-induced alteration, called transformation or activation† (for a review see Ref. 17). These phenomena have been studied in detail for the calf uterine estrogen receptor. A sensitive criterion for the transition of receptors from the non-activated

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†In this report "activation" is strictly defined as the reaction which promotes the formation of an estrogen–receptor complex with a higher affinity state, as indicated by the slower dissociation rate of estrogens. "Transformation" refers to changes in the sedimentation coefficient of the estrogen receptor on high salt sucrose gradients.

into the activated state is provided by the hormone dissociation kinetics [18, 19]. With the calf uterine receptor transition of the non-activated (4 S) into the activated state (5 S) is accompanied by a 30-fold reduction of the estrogen dissociation rate [19, 20], indicating the formation of a higher affinity estrogen–receptor complex. This process appears to be of physiological significance, since estradiol dissociates at a similar, slow rate from the nuclear (5 S) receptor after *in vitro* incubation of uterine tissue with estradiol [21], as well as after *in vivo* injection [22]. Little is known, however, about the activation and transformation of the oviduct estrogen receptor. In one report a DNA-stimulated transformation of the receptor from a 4.2 S to a 4.7 S has been described [23] and just recently evidence has been obtained that the estrogen receptor in oviduct nuclei is a dimer composed of similar subunits [24]. In the present study we have studied activation and transformation of the cytosolic estrogen receptor and examined the state of the nuclear estrogen receptor by measuring hormone dissociation kinetics and by sucrose density gradient analysis of sedimentation coefficients on high salt gradients.

EXPERIMENTAL

Animals

Hybrid female chickens (3–4 weeks old) and laying hens of Rhode Island × White Leghorn were used in this study. Immature chickens received subcutaneous implants consisting of silicone tubing (Silastic 602–635 from Dow Corning Corp. Midland, MI; 6 cm long) filled with approx 50 mg of diethylstilboestrol (DES) and closed with a glue (Medical Adhesive Silicone Type A, Dow Corning Corp.). After a period of 2 weeks the implants were removed and 18–20 h later oviduct tissue was collected, cut into small fragments and frozen in liquid nitrogen. The tissue was stored frozen at −80°C.

Materials

[17β-[2,4,6,7-^{3}H]Estradiol (91.5 Ci/mmol) and [11β-methoxy-^{3}H]moxestrol (87.0 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). The radiochemical purity of the estrogens was verified by thin layer chromatography in chloroform–ethyl acetate (3:1) or benzene–ethyl acetate (7:3). Non-radioactive DES was obtained from Sigma Chemical Co. (St Louis, MO). MOPS [3-(*N*-morpholino) propane sulfonic acid] was purchased from Serva (Heidelberg, FRG). Activated charcoal was purchased from Baker Chemicals (Phillipsburg, NJ) and Dextran 250 was obtained from Pharmacia Fine Chemicals AB (Uppsala, Sweden). Ep475 [L-*trans*-epoxysuccinylleucyl-amido-(3-methyl)butane], a powerful inhibitor of cysteine proteases [25, 26] was kindly supplied by Dr Hanada (Taisho Pharmaceutical Co., Tokyo, Japan). All other reagents were analytical grade.

Preparation of cytosol estrogen receptor

Frozen oviduct tissue was homogenized in 3–4 vol cold 40 mM MOPS–1 mM EDTA–11 mM thioglycerol–10% glycerol, pH 7.4 (buffer METG), containing 16 μM Ep-475 (a powerful inhibitor of cysteine protease), by 3–4 ten sec bursts at 50-s intervals using an Ultra-Turrax TP 18N tissue disintegrator (Janke and Kunkel GMBH, Staufen, F.R.G.) at 80% of its maximal speed. The homogenate was centrifuged at 10,000 g_{av} for 10 min, then the supernatant was removed and centrifuged at 164,000 g_{av} for 60 min in a SW 60 rotor (Beckman, Palo Alto, CA). The final supernatant was collected free from floating lipid and is referred to as cytosol. The protein concentration was 9–12 mg per ml.

Preparation of nuclear estrogen receptor

Nuclei from laying hen oviduct were isolated as described by Widnell *et al.*[27]. The nuclear pellet was resuspended in 3 vol of buffer METG containing 16 μM Ep-475. The NaCl concentration was adjusted to 0.5 M by adding a 4 M solution. After 30 min, with intermittent mixing, the viscous suspension was centrifuged at 90,000 g_{av} for 20 min. The supernatant thus obtained was the nuclear oviduct receptor preparation.

Assay of [^{3}H]estrogen binding

Cytosol was equilibrated with 5–6 nM [^{3}H]estrogen for the indicated time periods at 0°C. The non-specific binding of the tritiated ligand was measured by incubating aliquots with [^{3}H]estrogen plus 1 μM non-radioactive DES and was between 15 and 20%. Nuclear extracts were incubated at 0°C for 18 h with 6 nM [^{3}H]estrogen or with 6 nM [^{3}H]estrogen plus 1 μM DES. Non-specific binding of [^{3}H]estrogens was approx 10%. To achieve maximal hormone exchange the extracts were further incubated at 22 or 30°C for an additional period of 30–90 min. The binding of the tritiated ligand was measured after adsorption of free steroid by 0.3% (w/v) charcoal −0.03% (w/v) dextran for 15 min at 0°C and centrifugation at 2000 g_{av} for 5 min. Dissociation of [^{3}H]estrogens from the receptor, measured by exchange of the [^{3}H]estrogen with an excess of non-radioactive DES, was performed as previously described [28]. The data were corrected for inactivation of the receptor and non-specific binding of the tritiated ligands. The detailed method of the assay and data analyses were as described by Weichman and Notides[18, 19].

In some experiments the equilibrated cytosol was mixed with saturated ammonium sulfate solution in buffer METG to a final concentration of 35% saturation. After stirring for 30 min, the precipitate was collected by centrifugation at 10,000 g_{av} for 10 min, rinsed with a little buffer METG and dissolved in

buffer METG (1/3–1/5 of the original volume of the cytosol). After this procedure the protein concentration varied between 2 and 3 mg/ml.

Sucrose density gradient centrifugation

Cytosol and ammonium sulfate precipitates dissolved in buffer METG were treated with charcoal-dextran to remove unbound ligand and were mixed with alkaline phosphatase (6.2 S), and [^{14}C]ovalbumin (3.6 S) as internal sedimentation markers. Aliquots of 0.25 ml were layered onto linear 10–25% (w/v) sucrose density gradients prepared in 40 mM Mops–11 mM thioglycerol–400 mM KCl, pH 7.4. After centrifugation in a SW 60 rotor at 0°C for 17 h at 380,000 g_{av}, the gradients were collected in 40 fractions of 0.1 ml and assayed for markers and radioactivity.

Radioactivity measurements

Cytosol, ammonium sulfate fractions, nuclear extracts or gradient fractions (0.1–0.2 ml) were counted in 5 ml Atomlight (New England Nuclear Corp.) in a Packard Tri-Carb 460 CD liquid scintillation counter (Packard, Downers Grove, IL) with an efficiency of 25–43%.

RESULTS

Extraction of cytosol estrogen receptors from oviduct tissue

In our initial experiments the oviduct tissue from DES withdraw chickens was homogenized in buffer METG containing 0.3 M KCl according to Kon *et al.*[16] and the cytosol fraction was equilibrated with [^{3}H]estradiol at 0°C for 3 h. Under these conditions only the non-occupied form of the estrogen receptor becomes associated with the ligand (not shown). Subsequent incubation at 30°C for 60 min resulted in a 1.8–3.7-fold increase in specifically bound [^{3}H]estradiol, most likely due to exchange of receptor-bound DES by [^{3}H]estradiol (results not shown). In order to examine whether this additional steroid binds to a receptor component, which has leaked from the nuclear compartment in the presence of 0.3 M KCl, we have prepared cytosol in buffers lacking this salt. Indeed, in the absence of KCl a cytosolic receptor preparation was obtained, which contained a similar number of binding sites after incubation at 0°C, but which lacked the temperature-dependent increase in [^{3}H]estradiol binding. To avoid this complication we have used a receptor preparation which could be fully charged with estrogens at 0°C. Therefore buffer METG without KCl was used in the following experiments to extract only the non-occupied cytosol receptor. The average level of specifically bound [^{3}H]estradiol thus obtained was 53.8 ± 11.2 fmol/mg protein (mean ± SD; seven determinations).

Dissociation of [^{3}H]estradiol and [^{3}H]moxestrol from the cytosol receptor

Dissociation of steroids from receptors is most conveniently studied at temperatures that allow detection of both slowly and fast dissociating components [29]. On basis of preliminary experiments [^{3}H]estradiol dissociation kinetics was studied in detail at 22°C. At this temperature receptor inactivation was negligible for 120 min. Dissociation of [^{3}H]estradiol from the estrogen receptor occurred in two exponential phases (Fig. 1); the rate constant of the fast component was $k = 120 \times 10^{-3}$ min^{-1} (Table 1), whereas the slower component dissociated with a rate constant, $k = 12.3 \times 10^{-3}$ min^{-1}. After pre-incubation of the cytosol at 22°C prior to the start of the dissociation, the proportion of the slow component was increased from 58% to 71% after 30 min and to 100% after 60 min. The proportion of this component reflects the fraction of the receptor with the higher affinity for the estrogen. Thus as previously reported in uterus [18, 19] temperature elevation induces the transition of the non-activated form of the oviduct receptor into the activated form.

Because moxestrol dissociated 6–10-fold slower than estradiol from the estrogen receptor, as has been reported for mouse uterus [30] and chicken liver [28],

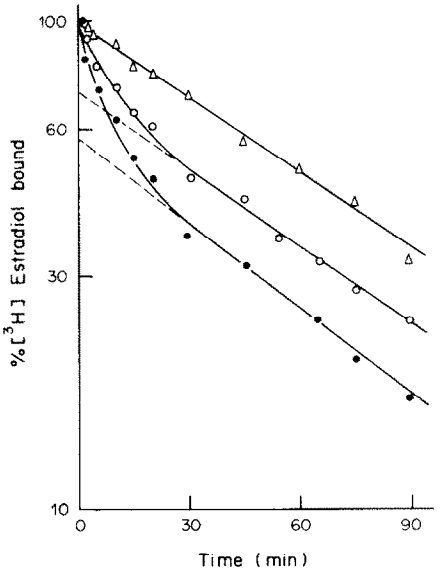

Fig. 1. Dissociation of [^{3}H]estradiol from the chicken oviduct cytosol receptor. Oviduct cytosol was equilibrated with 5 nM [^{3}H]estradiol for 3 h at 0°C, either in the absence or presence of 1 μM DES. Aliquots were incubated at 22°C for 30 min (○) and for 60 min (△) or kept at 0°C (●). The dissociation of [^{3}H]estradiol was subsequently measured at 22°C after the addition of 1 μM DES. Each point is a single determination and was corrected for non-specific binding. Receptor inactivation was nihil during the time period of incubation. The dissociation rate constants were: $k_{-1} = 130 \times 10^{-3}$ min^{-1} (●); $k_{-2} = 13.3 \times 10^{-3}$ min^{-1} (●); $k_{-1} = 100 \times 10^{-3}$ min^{-1} (○); $k_{-2} = 12.1 \times 10^{-3}$ min^{-2} (○) and $k_{-2} = 11.6 \times 10^{-3}$ min^{-1} (△). The estrogen receptor concentration was 0.78 nM.

Table 1. [^{3}H]Estrogen dissociation rate constants and half-times of the cytosol receptor

[^{3}H]Estrogen	Temperature (°C)	k_{-1} ($10^{-3} \times min^{-1}$)	$t_{1/2}$ (min)	k_{-2} ($10^{-3} \times min^{-1}$)	$t_{1/2}$ (min)	n
Estradiol	22	120 ± 20	5.8 ± 0.9	12.3 ± 0.9	56 ± 4	3
Moxestrol	30	120 ± 40	5.8 ± 1.7	7.6 ± 2.1	91 ± 25	4–12

Cytosol prepared in buffer METG was equilibrated with 5 nM [^{3}H]moxestrol or with 5 nM [^{3}H]estradiol at 0°C for 5 h. To measure non-specific binding levels, parallel incubations were carried out in the presence of 5 nM [^{3}H]estrogen plus 1 μM DES. The dissociation of the [^{3}H]estrogens from the receptor was assayed at the indicated temperatures after the addition of 1 μM DES. The data, after correction for receptor inactivation and non-specific binding, are presented as the mean ± SED. The number of experiments is given under n.

Table 2. Effect of different salt treatments on the [^{3}H]estrogen dissociation from the cytosol receptor

Cytosol treatment	[^{3}H]Estrogen	Temperature (°C)	Dissociation rate constant ($10^{-3} \times min^{-1}$)	Half-time (min)	n
MoO_4	Estradiol	22	37.3 ± 6.4	18.6 ± 3.2	6
0–35% $(NH_4)_2SO_4$	Estradiol	22	39.5	17.5	1
MoO_4	Moxestrol	30	19.9 ± 3.4	34.8 ± 6.0	3
0–35% $(NH_4)_2SO_4$	Moxestrol	30	10.8 ± 0.4	64.2 ± 2.6	3
NaSCN	Moxestrol	30	140	5.0	2
KCl	Moxestrol	30	9.8 ± 0.2	70.7 ± 1.4	4

Oviduct cytosol prepared in buffer METG or in buffer METG containing 20 mM molybdate was equilibrated with 6 nM [^{3}H]estradiol or [^{3}H]moxestrol at 0°C for 4 h. Aliquots of cytosol in buffer METG were saturated with ammonium sulfate to 35% and the precipitates were collected at 0°C at 10,000 *g* for 10 min. The precipitates were dissolved in one-third of the original cytosol volume buffer METG. Aliquots of cytosol in buffer METG, containing the temperature-activated receptor obtained after incubation at 30°C for 30 min, were made 0.4 M with respect to NaSCN or KCl, 15 min prior to the start of the dissociation at 30°C. The dissociation of [^{3}H]estrogens was assayed at the indicated temperature after the addition of 1 μM DES. The data, after correction for receptor inactivation and non-specific binding, are expressed as the mean ± SD and the number of experiments is given under n. The receptor concentration in the cytosol was 45–56 fmol/mg protein and after ammonium sulfate precipitation 250–300 fmol/mg.

dissociation of this ligand was carried out at 30°C. At this temperature the [^{3}H]moxestrol–receptor complex was stable for at least 180 min. Dissociation of [^{3}H]moxestrol at 30°C from the estrogen receptor also followed biphasic, first order kinetics (Fig. 2).

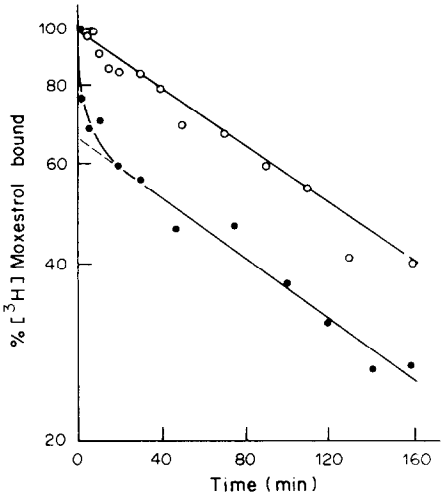

Fig. 2. Dissociation of [^{3}H]moxestrol from the chicken oviduct cytosol receptor at 30°C. Oviduct cytosol was equilibrated with 5 nM [^{3}H]moxestrol for 4 h at 0°C, either in the absence or presence of 1 μM DES. Aliquots were incubated at 30°C for 30 min (○) or kept at 0°C (●). The dissociation was subsequently assayed at 30°C after the addition of 1 μM DES. Each point is a single determination and was corrected for non-specific binding. Receptor inactivation was nihil during the assay. The dissociation rate constants were: $k_{-1} = 110 \times 10^{-3} min^{-1}$ (●), $k_{-2} = 5.9 \times 10^{-3} min^{-1}$ (●) and $k_{-2} = 4.9 \times 10^{-3} min^{-1}$ (○). The receptor concentration was 0.67 nM.

The dissociation rate constants of the fast and slow component at 30°C were: $k = 120 \times 10^{-3} min^{-1}$ and $7.6 \times 10^{-3} min^{-1}$, respectively (Table 1). As a result of pre-incubation of the cytosol for 30 min at 30°C the fraction of the receptor showing the slow rate increased from 65 to 100% (Fig. 2), indicating a temperature-dependent transition of the moxestrol–receptor complex into a higher affinity receptor form. So the receptor complex with estradiol as well as with moxestrol as a ligand shows a temperature-dependent activation.

Effect of salt treatment on the [^{3}H]estrogen dissociation from the cytosol receptor

To further investigate the two different forms of the estrogen receptor in oviduct cytosol we have carried out dissociation studies in the presence of 20 mM molybdate. This compound inhibits the transition into a higher affinity complex of the estrogen receptor in calf uterus [31, 32], and of the glucocorticoid receptor in fibroblasts [33]. In the presence of 20 mM molybdate the majority or all of the oviduct estrogen receptor showed monophasic dissociation kinetics at intermediate rates with [^{3}H]moxestrol as well as with [^{3}H]estradiol (Fig. 3), indicating that one receptor form was predominant. For moxestrol as well as for estradiol the rate constants were higher than measured for the activated cytosol receptor, but lower than those obtained for the non-activated receptor in the absence of molybdate (Table 2), suggesting the presence of an intermediary form of complex. Pre-incubation at 22 or 30°C for 30–60 min did not change the dissociation

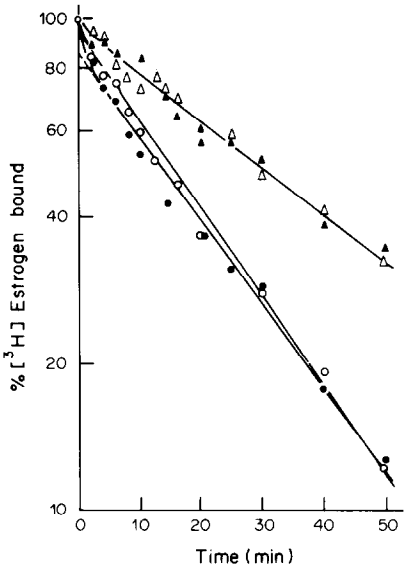

Fig. 3. Effect of sodium molybdate on the dissociation of [^{3}H]estrogens from the chicken oviduct cytosol receptor. Oviduct cytosol prepared in the presence of 20 mM molybdate was equilibrated with 5 nM [^{3}H]estradiol (○, ●) or 5 nM [^{3}H]moxestrol (△, ▲) at 0°C for 5 h. Aliquots were incubated for 30 min at 22°C (○) or at 30°C (△) or kept at 0°C (●, ▲). The dissociation was subsequently assayed at 22°C (○, ●) or at 30°C (△, ▲) after the addition of 1 μM DES. Each point is a single determination and was corrected for non-specific binding. Receptor inactivation was nihil during the assay. The dissociation rate constants were: $k_{-1} = 38.8 \times 10^{-3}\,min^{-1}$ (●), $k_{-1} = 40.2 \times 10^{-3}\,min^{-1}$ (○), $k_{-1} = 21.9 \times 10^{-3}\,min^{-1}$ (△, ▲). The receptor concentration was 0.57 nM.

rates significantly, but resulted in a further increase of the proportion of the estradiol–receptor complex dissociating at this intermediate rate (Fig. 3). A higher concentration of molybdate (50 mM) in buffer METG or preparation of cytosol in buffer TD with 20–50 mM molybdate did not alter the dissociation rates (not shown). In oviduct cytosol molybdate seems to inhibit a full temperature-induced activation, but allows the formation of an intermediary form of estrogen–receptor complex.

A more powerful agent previously shown to inhibit estrogen receptor activation, is the chaotropic salt NaSCN. It reverts the activated calf uterine receptor into a form, which shows the fast dissociation rate, indicative of the non-activated form [19]. In the presence of 0.4 M NaSCN, [^{3}H]moxestrol dissociation from the pre-activated oviduct receptor, followed a single exponential process with a rate constant $k = 140 \times 10^{-3}\,min^{-1}$, identical to that obtained for the non-activated receptor in the cytosol (Table 2). Addition of 0.4 M KCl to the temperature-activated receptor did not affect the dissociation rate of the [^{3}H]moxestrol–receptor complex ($k = 9.8 \times 10^{-3}\,min^{-1}$), indicating that the increase in ionic strength as such is not responsible for the increased dissociation rate (Table 2).

It has been reported by others that addition of salt to the nonactivated oviduct receptor at 0°C to a concentration of 0.4 M and subsequent dilution to 0.1 M, increased the receptor binding capacity for DNA-cellulose approx. 9-fold [34]. We have studied the effect of salt on the receptor dissociation kinetics. In the presence of 0.4 M KCl, added at 0°C 15 min prior to the start of the dissociation at 30°C, the proportion of the slowly dissociating form of the receptor was increased from 58 to 78% (not shown). A similar increase of the fraction of the slowly dissociating form of the receptor to 80% was obtained when cytosol had been incubated at 0°C for extended time periods (16–24 h). Thus an increase in salt concentration and long incubation times do stimulate the formation of the slowly dissociating form of the oviduct receptor, which also binds more effectively to DNA. However as shown above complete activation to the higher affinity state receptor was only obtained after incubation at 22 or 30°C.

The calf uterine estrogen receptor can be activated by ammonium sulfate precipitation, as was shown by its reduced dissociation kinetics at 28°C and this activation was accompanied by a shift in sedimentation coefficient from 4 S to 5 S [19]. For the oviduct estrogen receptor the effect of ammonium sulfate precipitation appeared to depend upon the type of ligand bound. With moxestrol, the hormone dissociation from the receptor at 30°C was monophasically with a rate constant $k = 10.8 \times 10^{-3}\,min^{-1}$ (Table 2), a value very similar to that found for the temperature-activated cytosol receptor. In contrast, the [^{3}H]estradiol–receptor complex dissociated also monophasically, but with a rate constant identical to that obtained for the cytosol receptor in the presence of molybdate ($k = 39.5 \times 10^{-3}\,min^{-1}$) (Table 2). This observation suggests that the same intermediary form of receptor complex is formed in the cytosol in the presence of molybdate, and after ammonium sulfate precipitation.

Sucrose density gradient analysis of the cytosol receptor

The transformation of the calf uterine estrogen receptor from the non-active, cytoplasmic form into the active nuclear form is a consequence of an estradiol-mediated dimerization reaction. Two non-active 4 S receptors, with a molecular mass of approx 75,000, dimerize to form a 5 S nuclear receptor with a molecular mass of 135,000 [35, 36]. The receptor in the oviduct cytosol, whether equilibrated with [^{3}H]moxestrol or with [^{3}H]estradiol sedimented at 0°C in buffer containing 0.4 M KCl predominantly as a 4.6–4.8 S entity. A minor fraction at 6.1 S was also observed (Fig. 4A and Fig. 4B). Preincubation of [^{3}H]moxestrol–receptor complex for 30 min at 30°C, conditions sufficient to bring the receptor into the slowly dissociating form (Fig. 2), resulted in a substantial increase of the fraction of the receptor sedimenting at 6.1 S (Fig. 4A). Heavier aggregates were

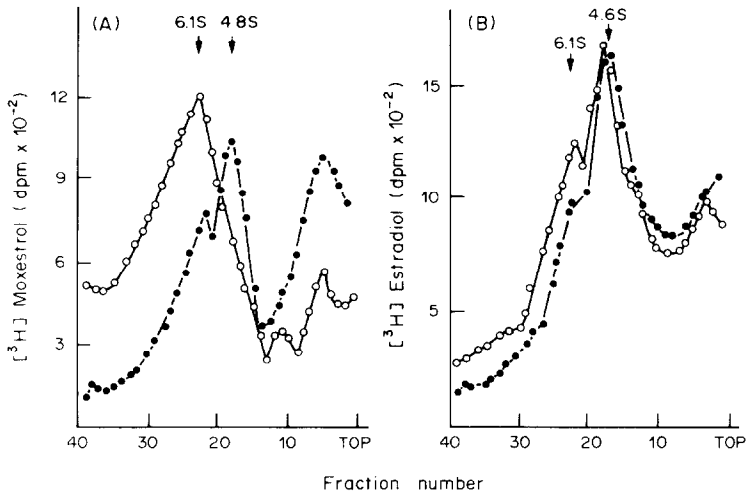

Fig. 4. Sedimentation behaviour of the chicken oviduct cytosol receptor. A. Oviduct cytosol was equilibrated with 5 nM [^{3}H]moxestrol at 0°C for 5 h. One aliquot was incubated at 30°C for 30 min (○), while another was kept at 0°C (●). B. Cytosol was equilibrated with 5 nM [^{3}H]estradiol at 0°C for 5 h. One aliquot was incubated at 22°C for 30 min (○), while another was kept at 0°C (●). After removal of free [^{3}H]estrogens by charcoal–dextran adsorption, 0.25 ml aliquots were layered onto 10–25% (w/v) sucrose gradients containing 0.4 M KCl. Centrifugation was performed at 380,000 *g* for 17 h at 0°C. The data are presented as estrogen specific binding values (differences between incubation without and with excess of DES).

also formed. Thus, the increase in sedimentation value of the complex appears to be associated with the transition of the receptor into the high affinity state and therefore might be related to the temperature-dependent activation. In contrast the fraction of the [^{3}H]estradiol–receptor complex sedimenting at 0°C at 4.6 S did not change as a result of pre-incubation at 22°C for 30 min or 60 min (Fig. 4B). Apparently the transformed estradiol–receptor complex with a higher sedimentation coefficient, after

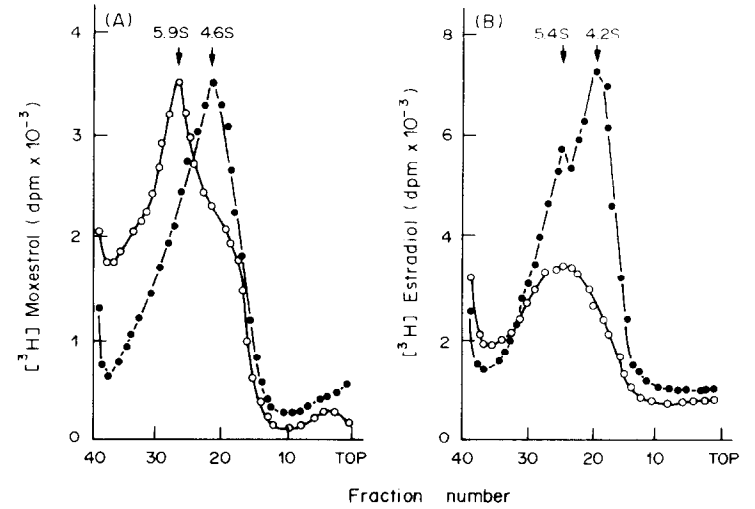

Fig. 5. Effect of ammonium sulfate precipitation and temperature on the sedimentation of the chicken oviduct cytosol receptor. A. Oviduct cytosol equilibrated with 5 nM [^{3}H]moxestrol for 5 h at 0°C was made 35% saturated with respect to ammonium sulfate. After 30 min at 0°C the precipitate was collected by centrifugation at 10,000 g_{av} for 10 min and dissolved in buffer METG (one-fifth of the original cytosol volume). One aliquot was incubated at 30°C for 30 min (○), while the other was kept at 0°C (●). B. Oviduct cytosol equilibrated with [^{3}H]estradiol for 5 h at 0°C was made 35% with respect to ammonium sulfate. After 30 min at 0°C the precipitate was collected by centrifugation at 10,000 g_{av} for 10 min and dissolved in buffer METG (one-fifth of the original cytosol volume). One aliquot was incubated at 22°C for 30 min (○) or kept at 0°C (●). After removal of free [^{3}H]estrogens by charcoal-dextran adsorption, 0.25 ml aliquots were layered onto 10–25% (w/v) sucrose gradients containing 0.4 M KCl. Centrifugation was carried out at 380,000 g_{av} for 17 h at 0°C. The data are presented as estrogen-specific binding values (differences between incubation without and with excess of DES).

its formation at 22°C, is not stable at the assay temperature of 0°C. We have recently reported a similar observation for the estradiol receptor in chicken embryo liver cytosol [28].

With 20 mM molybdate present during the cytosol preparation as well as the sucrose gradient analysis, the [^{3}H]estradiol- and [^{3}H]moxestrol-receptor complexes sedimented predominantly as a sharp peak with a sedimentation value of 4.8 S, irrespective of pre-incubation of the cytosol at 22 or 30°C (not shown). Obviously molybdate effectively inhibited the formation of the 6.1 S form of receptor. In view of its dissociation kinetics, measured in the presence of molybdate, an intermediary form of receptor is formed, which has sedimentation characteristics undistinguishable from the non-activated estrogen receptor. Since it has been reported that molybdate prevents the formation of receptors which interact with DNA or nuclei [37, 38, 39], we assume that the smaller (4.8 S) form of the receptor represents the non-active, non-DNA-binding form of the receptor.

Activation of the calf uterine estrogen receptor achieved by ammonium sulfate precipitation is accompanied by a change in sedimentation coefficient from 4 S to 5 S [19]. This procedure carried out at 0°C was not sufficient to provoke a shift from 4.8 S to 6.1 S when [^{3}H]estradiol or [^{3}H]moxestrol was bound to the oviduct receptor (Fig. 5A and 5B). With both ligands most of the receptor complex sedimented at values between 4.2 S and 4.6 S. Subsequent incubation at 20 or 30°C for 30 min of the receptor precipitated with ammonium sulfate, induced a shift to 5.9 S when [^{3}H]moxestrol (Fig. 5A), but not when [^{3}H]estradiol was the ligand (Fig. 5B). With the latter ligand a considerable loss of receptor-bound hormone was observed. Because 90% of the receptor was recovered with moxestrol as the ligand (Fig. 5A) we conclude that the moxestrol–receptor complex is fairly stable during the incubation at 30°C and the subsequent centrifugation step. Thus the preferential loss of the estradiol–receptor complex sedimenting at 4 S can be totally ascribed to the higher dissociation rates measured for the non-activated and activated forms of the complex. Apparently, the estrogen receptor requires elevated temperatures in order to maintain the transformed state (6.1 S). With moxestrol bound to the receptor the transformed complex is stable on sucroce density gradients at 0°C as well as at 20°C (not shown). With estradiol, the faster dissociation of both forms of the receptor will result in a shorter life-time of the activated estrogen–receptor complex. As the hormone dissociates from the activated state during centrifugation, the receptor may revert to the non-activated state, rebind the hormone, and sediment at 4.6 S.

Dissociation of [^{3}H]estradiol and [^{3}H]moxestrol from the nuclear estrogen receptor

To investigate the state of the estrogen receptor in oviduct nuclei *in vivo*, we have compared the dissociation kinetics of the nuclear receptor extracted from laying hen nuclei with 0.5 M NaCl, with that of the cytosol receptor. Nuclear extracts were incubated with [^{3}H]estradiol or [^{3}H]moxestrol at 0°C for 18 h to obtain a sufficient exchange of the nuclear receptor, which was occupied *in vivo* with endogenous estradiol. These conditions minimize receptor activation during the hormone exchange. Dissociation of [^{3}H]estradiol and [^{3}H]moxestrol from the nuclear receptor was subsequently measured by isotopic dilution at 22 and 30°C respectively. At those temperatures the radiolabeled receptors did not show significant inactivation during 180 min. Dissociaton of [^{3}H]estradiol from the nuclear receptor, when 66% of the total hormone–receptor complex present had been exchanged, followed biphasic kinetics (Fig. 6A). The rate constants of the two components were $k = 70 \times 10^{-3}\ \text{min}^{-1}$ and $k = 14 \times 10^{-3}\ \text{min}^{-1}$ (Table 3). In some experiments the nuclear extract was pre-incubated at 22°C for 30–45 min to obtain a maximal hormone exchange. This treatment changed neither the dissociation kinetics nor the ratio (approx 1) of the two components (Fig. 6B). The rate constants measured for the nuclear receptor were very similar to those obtained for the fast and slowly dissociating receptor forms in the oviduct cytosol. Whether the nuclear receptor forms differ from the fast and slow dissociating forms of the cytosol receptor is still under investigation.

Dissociation of [^{3}H]moxestrol at 30°C from the salt-extracted nuclear receptor was also measured after hormone exchange at 0°C with the tritiated ligand. At an exchange percentage of 34–38% the dissociation process at 30°C occurred as a monopha-

Table 3. [^{3}H]Estrogen dissociation rate constants and half-times of the nuclear estrogen receptor

[^{3}H]Estrogen	Temperature (°C)	k_{-1} ($10^{-3} \times \text{min}^{-1}$)	$t_{1/2}$ (min)	k_{-2} ($10^{-3} \times \text{min}^{-1}$)	$t_{1/2}$ (min)	n
Estradiol	22	70 ± 10	9.9 ± 2.0	14.0 ± 0.2	49.5 ± 0.6	3
Moxestrol	30	—	—	6.1 ± 0.9	114.4 ± 17.8	4

Nuclei isolated from laying hen oviduct were extracted with 3 vol of buffer METG containing 0.5 M KCl. The extract obtained after centrifugation at 90,000 g_{av} was incubated at 0°C for 18 h with 8 nM [^{3}H]estrogen or with 8 nM [^{3}H]estrogen plus 1 μM DES to measure non-specific binding. In some experiments the extract was incubated at 22°C or at 30°C for 30 min prior to the start of the dissociation assay. The dissociation of the [^{3}H]estrogens from the receptor was assayed at the indicated temperature after the addition of 1 μM DES. The data, after correction for receptor inactivation and non-specific binding, are presented as mean ± SD and the number of experiments is given under n. Receptor concentration measured after maximal hormone exchange at 22 or 30°C ranged between 0.72–1.12 pmol/mg of DNA.

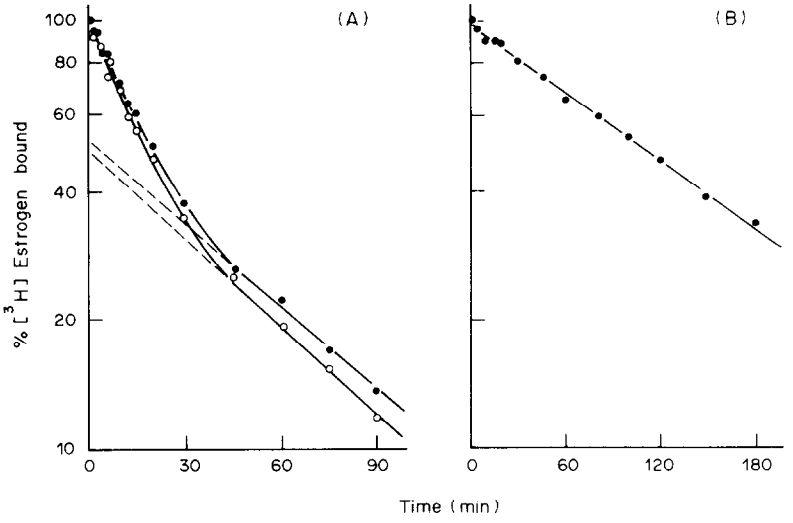

Fig. 6. Dissociation of [^{3}H]estrogens from the salt-extracted nuclear chicken oviduct receptor. A. Nuclear extracts were equilibrated at 0°C for 18 h with 8 nM [^{3}H]estradiol either in the absence or presence of 1 μM DES. One aliquot was incubated at 22°C for 45 min (○), while another was kept at 0°C (●). The dissociation of [^{3}H]estradiol was subsequently assayed at 22°C after the addition of 1 μM DES. B. Nuclear extracts were equilibrated at 0°C for 18 h with 8 nM [^{3}H]moxestrol with or without an excess of 1 μM DES. The dissociation of [^{3}H]moxestrol was assayed at 30°C after the addition of 1 μM DES. Each point is a single determination and was corrected for non-specific binding. Receptor inactivation was negligible during the assay. The dissociation rate constants were: (A) $k_{-1} = 67.1 \times 10^{-3}\,min^{-1}$ (●); $k_{-1} = 60.3 \times 10^{-3}\,min^{-1}$ (○); $k_{-2} = 14.2 \times 10^{-3}\,min^{-1}$ (●) and $k_{-2} = 14.0 \times 10^{-3}$ (○). (B) $k_{-1} = 6.1 \times 10^{-3}\,min^{-1}$ (●). The receptor concentration of the nuclear extract measured after maximal exchange at 30°C with [^{3}H]moxestrol was 0.95 pmol/mg of DNA.

sic process with a rate constant $k = 6.1 \times 10^{-3}\,min^{-1}$ (Fig. 6B) [Table 3]. At a higher degree of hormone exchange (74–85%), obtained after pre-incubation of the extract at 30°C for 30 min, the rate constant was identical. This rate constant is very similar to that of the temperature-activated cytosol receptor, indicating that probably all the receptor had been extracted from the nuclei in its slowly dissociating form. However a small effect of 0.4 M KCl on the receptor activation *in vitro* during the hormone exchange procedure at 0°C cannot be excluded.

DISCUSSION

The estrogen receptor in the cytosol fraction of immature oviduct, obtained from DES-withdrawn chickens, was used to study its hormone dissociation kinetics. Dissociation of moxestrol and estradiol from the estrogen receptor in chicken oviduct cytosol occurs exponentially and shows two components which differ approx 10-fold in dissociation rate constants. The fraction of the receptor with the slow dissociation rate increases as a result of pre-incubation of the hormone–receptor complex at 0°C and higher temperatures, and also after the addition of 0.4 M KCl.

It has been reported that the affinity of the hormone–receptor complex for DNA-cellulose and oviduct nuclei is increased by the same steps as the affinity of the receptor for the hormone [34, 40], suggesting that the slowly dissociating form binds much better to DNA. We have shown that the transformation to a form with a higher sedimentation coefficient proceeds in parallel with the temperature-induced activation, but only when the slower dissociating synthetic estrogen, moxestrol, is used as a ligand. The inability of estradiol to provoke this shift in sedimentation value may be due to the relatively short lifetime at 0°C of the transformed estradiol–receptor complex. Our recent finding that the chicken embryo liver estrogen receptor could reach and maintain the transformed 5 S state at a temperature of 20°C in the presence of an excess estradiol supports this hypothesis [28]. In this respect avian estrogen receptors differ from the estrogen receptor in mammalian tissues.

Sodium molybdate, an effective inhibitor of *in vitro* activation of estrogen receptor and other steroid hormone receptors [31–33, 37] also inhibits oviduct cytosol estrogen receptor activation. Sodium molybdate allows however, the formation of an intermediary form of receptor which sediments as the nontransformed receptor (4.8 S), but which dissociates significantly slower than the activated form of the receptor. With the more potent inhibitor of receptor activation, the chaotropic salt NaSCN, we were able to demonstrate complete inhibition of receptor activation. With moxestrol as a ligand the lifetime of the receptor at 30°C decreased 14- to 18-fold to a value identical to that measured for the

non-activated cytosol receptor in the absence of NaSCN. A similar effect of NaSCN has been reported for the calf uterine receptor [19] and for the estrogen receptor in chicken embryo liver cytosol [28]. In uterine cytosol the fast-dissociating component corresponds to the receptor sedimenting at 4 S, which is the non-activated, non-transformed form and which binds with a 5-fold lower affinity to nuclear acceptor sites than the activated form [21]. Formation of the slowly dissociating form of estrogen receptor in calf uterus can also be achieved at 0°C by ammonium sulfate precipitation (0–35% saturation) and re-dissolving the precipitate in buffer. The formation of the high affinity state of the uterine receptor at 0°C is accompanied by a 4 S–5 S shift in sedimentation coefficient [19]. The effect of ammonium sulfate precipitation on the oviduct receptor was dependent on the type of ligand bound to the receptor. With estradiol, the complex showed dissociating kinetics and a sedimentation coefficient very similar to the intermediary state of estrogen receptor, which is obtained in the presence of sodium molybdate. This suggests that the receptor remained in the non-activated, non-transformed form. With moxestrol, the dissociation rate at 30°C was reduced 11-fold, suggesting complete receptor activation. However, a concomitant shift in sedimentation coefficient to a 6.1 S form, analyzed at 0°C, did not occur. Subsequent incubation at 30°C induced receptor transformation to a 5.9 S form. Our results suggest that oviduct estrogen receptor activation *in vitro*, as defined by a reduction in the hormone dissociation rate of the receptor, can be distinguished from the transition of the receptor from a low to a higher molecular form. Interestingly a recent report on the calf uterine estrogen receptor also described that receptor activation *in vitro*, measured as an increase in nuclear binding, preceded receptor transformation [41].

The nuclear estrogen receptor extracted from laying hen oviduct nuclei with 0.5 M NaCl, showed complex dissociation kinetics. The fast and slowly dissociating forms of the nuclear receptor, measured with estradiol as the ligand, probably represent a non-active and activated state of the receptor. Subsequent temperature elevation however did not convert the low affinity state into the high affinity state, suggesting impairment of the activation process in the nuclear extract. With moxestrol only the slowly dissociating form, representing the activated state of the receptor, was demonstrated. Similar results have been obtained by others when the dissociation of the nuclear receptor was carried out at 0°C [15]. In this study fast and slowly dissociating forms in a ratio of 1:1 were analysed for estradiol, whereas with moxestrol or hydroxytamoxifen, an anti-estrogen, a single, slowly dissociating form was measured. The reason for this discrepancy is unknown. In view of those and the present results, it is unlikely that a fraction of the nuclear receptor becomes damaged during the hormone exchange with estradiol at 0 or 22°C, since (1) incubation at 30°C with moxestrol does not affect the receptor dissociation kinetics indicating receptor stability and (2) dissociation at 0 and 22°C show similar results. It is more likely that the extracted nuclear receptors represent two different forms which were originally present in oviduct nuclei *in vivo*. One may be the activated form and the other a non-active form, which can only be reactivated when it forms a complex with the slow dissociating ligand moxestrol. Recently it has been proposed that *in vivo* the nuclear oviduct receptor with a molecular mass of 118,000 is formed by a dimerization process of two identical subunits with a molecular mass of 58,000 [24]. We suggest that this process is reflected by an increase in sedimentation coefficient and is accompanied by a reduction in the hormone dissociation rate.

For the calf uterus it has been shown that the efficiency of different estrogens to activate the estrogen receptor *in vitro* and to maintain it in its activated, transformed state, correlates with their relative potency *in vivo* [29]. The present results on the activation and transformation of the oviduct receptor by moxestrol and estradiol suggest that moxestrol is a more potent estrogen than estradiol. This is supported by studies on the induction of immature rate uterus growth [42] and induction of specific protein synthesis in chicken oviduct [43].

When the activation and transformation process of the calf uterine and the chicken oviduct estrogen receptor are compared, only minor differences are observed. Both the mammalian and the avian receptor undergo a temperature-dependent activation to a form which has a higher affinity for the steroid. Transformation to a form with a higher sedimentation coefficient differs only in a quantitative way and may reflect differences in equilibrium constant for dimerization. The receptor form which is active under physiological conditions in the nucleus is the slowly dissociating, higher affinity state.

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