Journal of Steroid Biochemistry, 1978, Vol. 9, pp. 1071–1078. Pergamon Press. Printed in Great Britain

A RE-EXAMINATION OF THE INTERACTION OF ESTRADIOL WITH TARGET CELL RECEPTORS

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(*Received* 20 *February* 1978)

SUMMARY

Two intranuclear estradiol receptor complexes can be distinguished by sucrose density gradient ultracentrifugation of the immature rat uterus following either *in vivo* or *in vitro* administration of radiolabelled estradiol. Time course studies demonstrate that the earliest detectable complex sediments as a 4*S* form whereas a 5*S* form accumulates during prolonged exposure to hormone (> 10 min). Pulse chase experiments suggest that the 5*S* complex arises from the lighter 4*S* complex in a precursor-product manner. The properties of both the intranuclear 4*S* and 5*S* forms are shown to be those of authentic estrogen receptors. These observations are not in agreement with the generally held view that receptor 4*S* to 5*S* transformation occurs in the cytoplasm and is a necessary prerequisite to nuclear translocation.

INTRODUCTION

A two-step mechanism of estrogen interaction with target tissue receptors has been proposed by Jensen *et al.*[1, 2] based on a combination of *in vitro* incubations, temperature manipulations and *in vivo* studies using the immature rat uterus. Experimental evidence which supports this concept of estrogen action has received extensive review [3, 4], and in summation holds that hormone freely enters cells, binds to cytoplasmic receptors and then, following a temperature dependent transformation, the hormone receptor complexes move to the nucleus to bind to chromatin acceptor sites. This model of estrogen action has been the subject of a more recent review wherein its less clearly defined aspects were analyzed [5]. In addition, the concept is deficient by virtue of (1) no direct evidence for the *in vivo* cytoplasmic transformation event has been obtained due to the requirements for receptor isolation and (2) the crude methods customarily used to separate nuclear from cytoplasmic compartments preclude precise localization of the native receptors.

We have previously reported the existence of different forms of estrogen binding entities within nuclei of whole cells [6]. Additional studies [7–9] also served to implicate the nucleus as a site for estrogen receptor transformation under physiologic conditions, thereby precluding a cytoplasmic activation step prior to entry into the nucleus. As the transformation (activation) event is generally accepted as fundamental to the mechanism of steroid hormone action, we have continued our investigation of the interaction of estrogen with its receptor as it occurs in whole cells. In the studies to be reported, artifacts of estrogen binding arising from such serum proteins as albumin and alpha-fetoprotein have also been considered. These factors were excluded and allowed the conclusion that the two binding moieties (4*S* and 5*S*) identified in the nuclear compartment are indeed estrogen receptors.

MATERIALS AND METHODS

In vivo *studies.* Experiments were conducted using immature (21–25 days old) and mature (88 days old) rats obtained from Holtzman Co. (Madison, WI.). Animals were injected i.p. with 25μCi ^{3}H-estradiol (^{3}H-E_2, 106 Ci/mM, 64 ng) as a 1% ethanol-saline solution and killed with ether. The excision of uteri and their dissection from extraneous tissues was carried out under cold room conditions (3 ± 1°C).

In vitro *studies.* Uteri obtained as above for use in whole tissue incubations were split along the long axis and rinsed in three changes of Krebs–Ringer–Hanselheit (KRH) buffer (137 mM NaCl, 2.70 mM KCl, 1.50 mM KH_2PO_4, 6.45 mM $Na_2HPO_4 \cdot 7H_2O$, 0.43 mM $CaCl_2 \cdot 2H_2O$, 0.50 mM $MgCl_2 \cdot 6H_2O$ and 5.56 mM glucose·H_2O adjusted to pH 7.4). Incubations were carried out at 4, 10 or 37°C for varying time intervals in KRH with ^{3}H-E_2 at a receptor saturating concentration (5×10^{-9}M). In one experiment,

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whole animal perfusion with saline was carried out prior to the excision of uteri and *in vitro* incubation to reduce possible artifacts arising from steroid binding to serum proteins included in the tissue homogenate.

Preparation of cytosol and nuclear fractions. Following incubation, tissues were successively rinsed and blotted on filter paper in four changes of cold 10 mM Tris–HCl (TE) buffer containing 1.5 mM ethylene–diamine–tetraacetic acid (pH 7.4 at 25°C). All uteri (4/ml TE) were briefly homogenized (5 × 10 s at 30 s intervals) using a Kontes glass homogenizer (Duall 22) immersed in an ice bath (4°C). Homogenates were centrifuged (700 *g* × 10 min) to obtain a crude nuclear fraction which was washed three times in 2 ml volumes of TE; the initial low speed supernatants were centrifuged at 105,000 *g* × 60 min to prepare cytosol fractions. In some experiments, uterine nuclear preparations were also separated from contaminating loose membranous components by passage through a discontinuous sucrose gradient consisting of 0.88 M sucrose (0.2 ml) layered upon 2.2 M sucrose (1.8 ml), (105,000 *g* × 60 min). Bound radioactivity was extracted from nuclei by resuspending the high speed pellet in TEK buffer (TE + 400 mM KCl at pH 8.5) at 4°C for 60 min. The recovery of salt extractable nuclear bound receptor radioactivity ranged from 60 to 70% based upon subsequent ethanol extraction of pellet residual radioactivity.

Sucrose density gradient analyses (SDG). Linear 5–20% sucrose gradients were prepared using a gradient former and allowed to chill prior to use (3 ± 1°C × 60 min). Aliquots (0.3 ml) of high speed cytosol and nuclear extracts were layered on TE sucrose and TEK sucrose gradients respectively, and centrifuged at 40,000–50,000 rev./min (~250,000 *g* × 16–18 h) in a Beckman L2-65B ultracentrifuge at 2–4°C. Fractions (0.1 ml) were collected from the top of gradients by displacement into 15 × 45 mm glass vials using a modified fraction collector and diluted with 3.5 ml scintillation fluid containing toluene, Triton X-100, distilled water and Permafluor (Packard) (2000:1000:210:80).

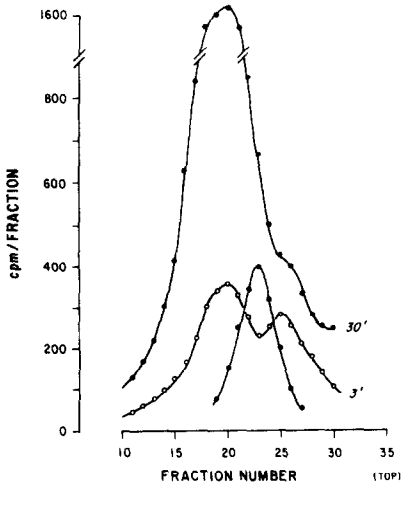

Fig. 1. SDG sedimentation patterns of rat uterine nuclear extracts following 3 (O——O) and 30 (●——●) min exposure *in vivo* to 0.24 nmol ^{3}H-E_2. The internal standard of ^{14}C-human serum albumin is seen to migrate with a uniform peak at fraction number 23.

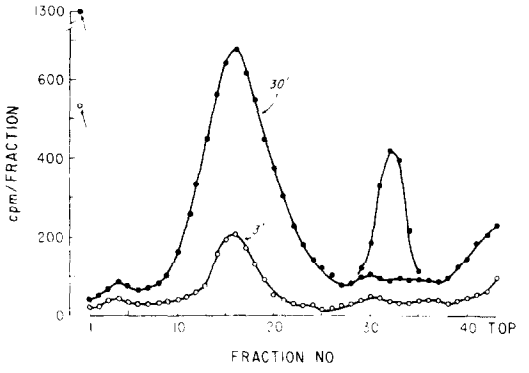

Fig. 2. SDG sedimentation patterns of rat uterine cytosol following 3 (O——O) and 30 (●——●) min exposure *in vivo* to 0.24 nmol ^{3}H-E_2 (^{14}C-HSA at number 32). Binding in the 4*S* region is not apparent.

To precisely characterize the sedimentation profiles of the steroid receptor complexes, an internal standard of ^{14}C acetate-human serum albumin (HSA, 4.6*S*; 2000 c.p.m.) was added to each sample prior to ultracentrifugation as first described by Moriyama and Siiteri[7]. Quantitation of radioactivity was made in a dual channel Packard Tri-Carb Liquid Scintillation spectrometer (efficiency ^{3}H ~ 25%; ^{14}C ~ 60%). Complex sedimentation patterns evident by graphic analysis were further analyzed with a curve resolver (Dupont 310) thereby permitting (1) precise determination of *S* values in relation to the ^{14}C-HSA standard and (2) accurate quantitative analysis of mixtures of nuclear complexes. The total radioactivity associated with each receptor form was then corrected for the efficiency of KCl extraction, procedural losses and counting efficiency and is reported as pmol contained per uterus.

In addition, uterine nuclear extracts obtained following short term whole uteri incubation (5 min × 37°C) and day 21 diluted serum (1:20) were incubated (4°C × 120 min) with 0.05 ml undiluted goat anti-rat α1-fetoprotein serum before analysis by SDG.

RESULTS AND DISCUSSION

In vivo *administration of hormone.* Two discrete salt extractable forms of hormone receptor complex were isolated from nuclei within three minutes of the systemic administration of ^{3}H-E_2 with the more slowly sedimenting 4*S* form accounting for 40% (0.11 pmol/uterus) of the total macromolecular bound radioactivity (Fig. 1). By 30 min, however, the 5*S* form accounted for 85% of the total (0.82 pmol) while the quantity of 4*S* form of hormone receptor complex

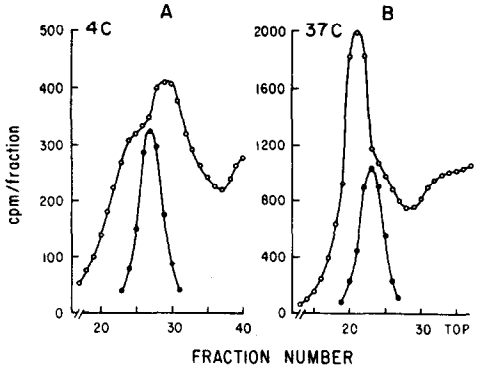

Fig. 3. SDG sedimentation patterns of rat uterine nuclear extracts following *in vitro* whole uterine incubation for 60 min at 4°C (A) and 37°C (B) (^{14}C-HSA migrates at fraction 27 and 23 respectively). Temperature dependence for nuclear accumulation of receptor and/or transformation is suggested. Note the different ordinates and the presence of two binding forms regardless of temperature.

remained approximately the same (0.12 pmol). Cytoplasmic preparations from these studies yielded a mixture of 8*S* and faster sedimenting radiolabelled complexes (Fig. 2). No quantifiable 4*S* receptor form was observed.

In vitro *incubations of whole uteri.* Long term *in vitro* incubation of whole uteri with ^{3}H-E_2 (5×10^{-9}M) at 4°C enhances the apparent level of hormone binding in the cytoplasmic compartment. The translocation of cytoplasmic estrogen receptor complex into the nucleus, however, appears to be temperature dependent since nuclear extracts from uteri obtained at 37°C contain more receptor than those obtained at 4°C (Fig. 3) which is in agreement with previous reports [1, 10]. However, resolution of the nuclear binding peaks obtained following incubation at both 4 and 37°C reveals two components which sediment as 5*S* and 4*S* as was noted following *in vivo* administration of hormone (Fig. 1). The amount of 4*S* estradiol-receptor complex extractable from the nucleus is nearly the same at both 4°C and 37°C (0.10 pmol) whereas a much greater amount of 5*S* complex was found at 37°C (~ 1.0 pmol vs 0.05 pmol).

Intranuclear binding in the 4S *region.* The temperature dependence for both translocation and transformation was further investigated by changing the temperature of the *in vitro* incubation to 10°C. Nuclear extracts from whole uteri incubated at this intermediate temperature and their SDG profiles are shown in Fig. 4. Near maximum nuclear uptake of receptor can be obtained at 10°C. Significantly, the receptor obtained in this manner migrates principally as the 4*S* form. However, brief exposure of such uteri to 37°C results in a shift to the pattern obtained *in vivo* (Fig. 1) in which the 5*S* form is predominant. These results suggest that the translocation process does not necessarily require prior transformation of the receptor to the 5*S* entity as suggested by Jensen and co-workers[1, 2].

Properties of the intranuclear 4S *binding form.* A single nuclear 4*S* binding moiety distinct from the albumin internal standard was obtained (Fig. 5) if uteri were incubated for a very short interval at physiologic temperature (1 min × 37°C). As opposed to albumin, this protein-steroid association is readily destroyed by heating to 56°C and is also resistant to charcoal adsorption. Binding of ^{3}H-E_2 to the 4*S* nuclear form could also be eliminated by addition of excess nonlabelled E_2 or diethylstilbestrol to the incubation medium but not by progesterone or testosterone (results not shown). Furthermore, the pattern of 4*S* and 5*S* complexes obtained following incubation at 37°C for 5 min (equivalent amounts of both binding entities) has been shown to be unaltered by (a) methods which favor outer nuclear membrane dissolution and hence might be expected to remove cyto-

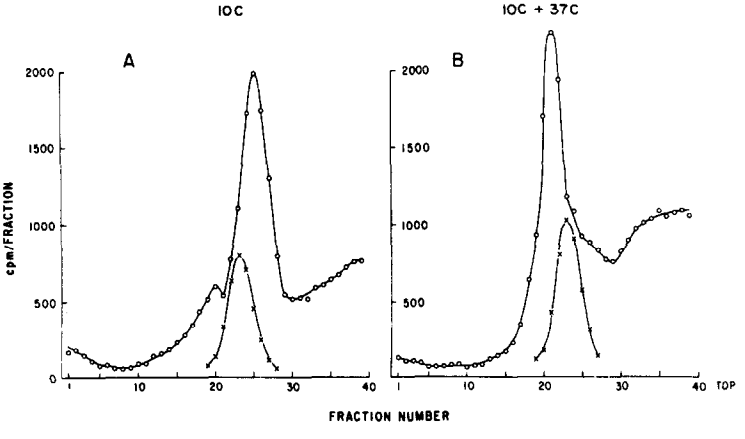

Fig. 4. SDG sedimentation patterns of rat uterine nuclear extracts following *in vitro* whole uterine incubation for 60 min at 10°C (A) followed by 5 min exposure to 37°C (B). ^{14}C-HSA migrates at fraction 23 for both preparations. Note that the near maximal accumulation of nuclear 4*S* receptor at this temperature is readily converted to the more rapidly sedimenting 5*S* state by the brief 37°C treatment.

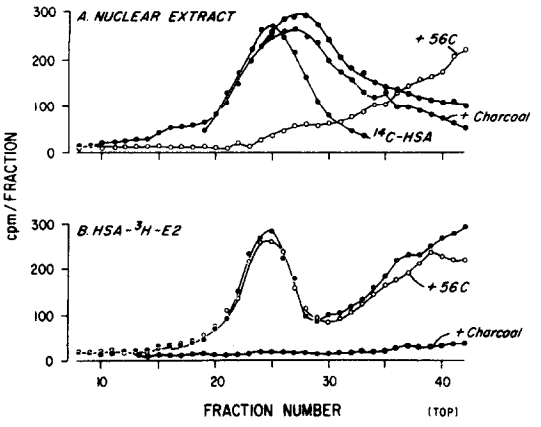

Fig. 5. (A) SDG sedimentation pattern of rat uterine nuclear extract following brief 1 min exposure of whole uteri to 5 nM ^{3}H-E_2 at 37°C. The single (4*S*) binding form (fraction no. 27) is resistant to charcoal adsorption but not elevated temperature. (B) SDG sedimentation pattern of human serum albumin incubated with ^{3}H-E_2, which indicates that albumin binds the steroid, migrates coincident with the ^{14}C-HSA internal standard, and is resistant to an increase in temperature but not charcoal adsorption. ^{14}C-HSA in (A) and HSA-^{3}H-E_2 in (B) both migrate in fraction 25.

plasmic contaminants (digitonin, Triton X-100) or (b) previous sonication of whole nuclei prior to their extraction with KCl [6]. Taken together, these properties of the nuclear 4*S* binding moiety suggest it to be a specific nuclear estrogen receptor and not a nonspecific cytoplasmic contaminant.

In situ whole organ perfusion followed by *in vitro* incubation of uteri (5 min × 37°C) revealed no quantitative or qualitative alteration in the distribution of the nuclear binding forms thereby minimizing possible serum contamination (Fig. 6). Moreover, the more slowly sedimenting (4*S*) binding species has also been demonstrable in nuclear preparations from mature rat uteri (0.11 pmol/uterus) following *in vitro* incubation (5 nM ^{3}H-E_2; 5 min × 37°C). Thus it would appear that short term exposure of uteri to ^{3}H-E_2, regardless of maturity, yields an intranuclear doublet of ER species sedimenting as 4*S* and 5*S*.

Serum and tissue α-fetoprotein (AFP). The extensive tissue washing procedures and the ^{3}H-E_2 incubation levels utilized were associated with an absence of slowly sedimenting (4*S*) cytoplasmic binding moieties as seen by SDG. Nonetheless, AFP ($K_D \sim 1 \times 10^{-8}$) in the cytosol or nuclei of the uterus of the immature rat could seriously impair assessment of ER interactions when using other methods, such as the dextran coated charcoal adsorption assay or gel filtration. AFP migrates as 4.5*S* on SDG [11]; hence immunoadsorption of uterine nuclear extracts and serum from the same animals with anit-AFP serum was carried out to better characterize the binding form(s) (Fig. 7). The results shown indicate that the addition of anti-AFP to serum either diluted in TE or TEK causes the bulk of the bound ^{3}H-E_2 to sediment at the bottom of the gradient but has no effect on the sedimentation patterns of nuclear extracts. Furthermore, AFP is a heat stable serum protein [12] whereas the estrogen 4*S* receptor is destroyed by elevated temperature as demonstrated in Fig. 5. Moreover, AFP-^{3}H-E_2 does not associate with rat liver DNA in contrast to the native estrogen receptor [13]. Taken collectively, these observations allow us to conclude that the nuclear 4*S* form represents estrogen receptor(s) and not estrogen binding contaminants of serum origin.

Time course of intranuclear 4S → 5S *transformation.* Quantitation of the intranuclear forms of estrogen receptor as a function of incubation time at 37°C is shown in Table 1. The ratio of 5*S*/4*S* in these studies increased from 0 to a maximum of 6.41 at 40 min, during which time the 4*S* level was maintained at approximately 0.18 pmol/uterus. During the second hour of incubation, an apparent decrease of the 5*S* form occurred that may represent recycling of receptor or loss of tissue viability resulting in receptor degradation. The level of intranuclear 4*S* complex throughout the two-hour interval at 37°C, however, was comparable to that observed following long-term incubation at 4°C.

Intranuclear transformation 4S → 5S: *Precursor-product relationship.* The time dependent distribution of complexes within the nucleus suggested a possible precursor-product relationship between the 4*S* and 5*S* forms. Supporting evidence for such a relationship was obtained in pulse-chase experiments in which uteri were incubated first with 5 nM ^{3}H-E_2 for 5 min at 37°C to provide equivalent amounts of labeled intranuclear 4*S* and 5*S* receptors. The uteri were then briefly washed and reincubated with either buffer or

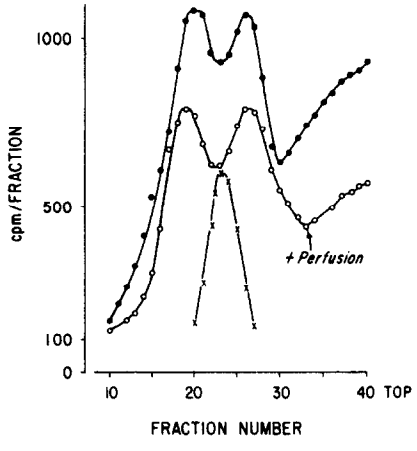

Fig. 6. SDG sedimentation patterns of nuclear extracts following *in vitro* incubation (5 min × 37°C) of whole uteri obtained following *in situ* whole organ perfusion (○——○) are compared to the usual nonperfused treatment (●——●). The qualitative distribution of the two forms remained unaltered. The apparently greater amount of nuclear receptors for the nonperfused preparation reflects a higher baseline level of ^{3}H-E_2 in the extract. ^{14}C-HSA migrates with a peak at fraction 24.

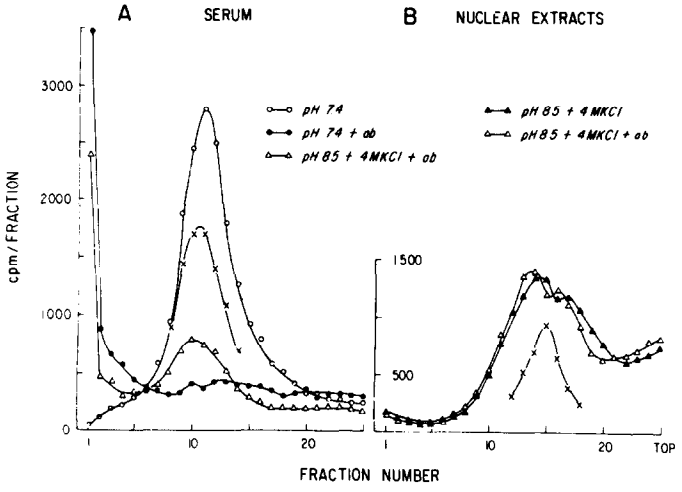

Fig. 7. (A) SDG sedimentation pattern for sera diluted (1:20) with TE buffer ± specific goat anti-rat AFP and TEK buffer. The efficacy of the antibody preparation to complex serum AFP in the different ionic milieus is evident. ^{14}C-HSA (× —— ×) migrates in fractions 10–11. (B) SDG sedimentation pattern for rat uterine nuclear extracts following *in vitro* incubation (5 min × 37°C) selected to yield two binding forms. Both nuclear binding forms are resistant to antibody complex formation. ^{14}C-HSA (× —— ×) migrates in fraction 15. Note that an SW 50 rotor was used and 0.2 ml fractions collected for this experiment thereby contributing to the apparent increase in ^{14}C-HSA sedimentation velocity as well as decreased resolution of the nuclear 4*S*–5*S* separation.

5 nM nonradioactive estradiol. It can be seen (Fig. 8) that incubation with non-labelled hormone resulted in near total disappearance of the nuclear 4*S* component and a marked increase in the amount of 5*S* complex. Radiolabelled uteri (5 min × 37°C) reincubated in buffer alone for up to one hour (not shown), however, gradually lost labeled nuclear receptor but the relative amounts of 4*S* and 5*S* receptor complexes remained constant.

The time related distribution of radiolabelled receptors between cytoplasmic and nuclear compartments in more extensive pulse-chase experiments was also analyzed. As shown in Fig. 9 it is evident that post-incubation of radiolabelled uteri (5 min at 37°C) in the presence of 5 nM unlabelled hormone results in a rapid nuclear accumulation and cytoplasmic depletion of hormone receptor complex. However, when post-incubation is effected in buffer alone the rate of nuclear accumulation of receptor and cytoplasmic receptor depletion is decreased.

Table 1. Distribution of ^{3}H-estradiol-receptors (pmol/uterus) within the nuclear compartment of the immature rat (5 nM; 37°C)

Time (min)	Total	4*S*	5*S*	5*S*/4*S*
1	0.18	0.18	0.00	0.00
3	0.38	0.17	0.21	1.24
5	0.53	0.19	0.34	1.79
10	0.65	0.18	0.47	2.61
20	0.79	0.17	0.63	3.71
40	1.26	0.17	1.09	6.41
60	1.17	0.17	1.00	5.88
120	0.90	0.18	0.62	3.44

GENERAL DISCUSSION

There exists general agreement that the active nuclear estradiol receptor complex sediments as a 5*S* form. However, the presence of a 4*S* estrogen receptor complex in nuclear extracts has been observed by others [14, 15] but its significance generally ignored. The analysis of the kinetics of appearance of nuclear receptors as carried out in these studies demonstrates that the first detectable nuclear radioactivity sediments as a 4*S* complex, and that the amount found

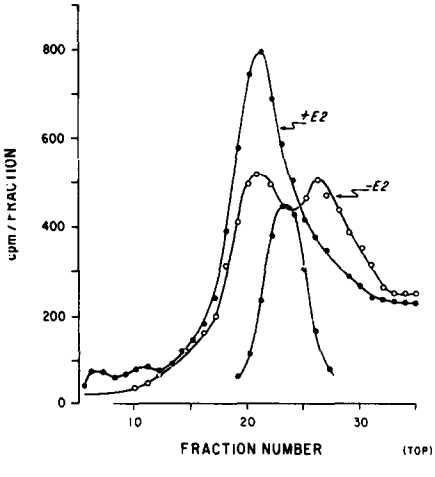

Fig. 8. SDG sedimentation pattern for rat uterine nuclear extracts following *in vitro* incubation (5 min × 37°C). The relative amounts of the two binding forms shown (O——O) when post-incubated in an equimolar environment of non-labelled estradiol (+ E_2) for 15 min change such that the 5*S* exceeds the 4*S* moiety (●——●). ^{14}C-HSA migrates in fraction 23.

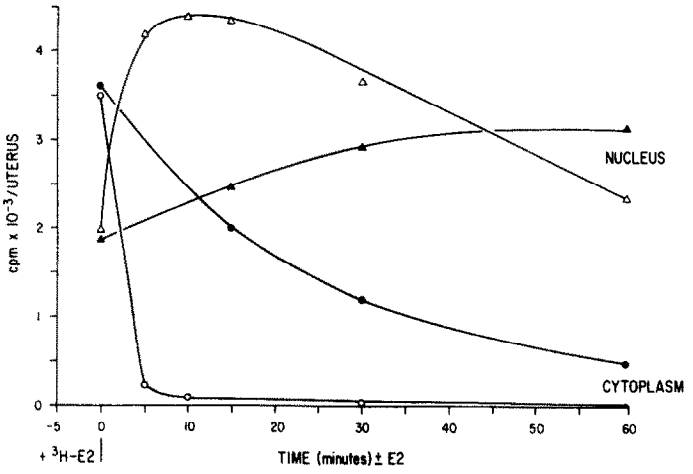

Fig. 9. Immature rat uteri were incubated 5 min at 37°C in the presence of ^3H-E_2 (5 nM). Uteri were then removed, rinsed and transferred to buffer (closed symbols) or buffer containing 5 nM non-labelled E_2 (open symbols) for varying time intervals. The amount of cytoplasmic (circles) and nuclear (triangles) receptors were quantified following SDG. The rate of nuclear accumulation and cytoplasmic diminution of receptor is seen to be influenced by the availability of steroid. This recruitment phenomenon of receptor may be linked to positive co-operative interactions. See text for additional details.

at early times remains constant whereas the amount of 5*S* complex continues to increase until maximal nuclear uptake is achieved (40–60 min). The temporal relationship between nuclear 4*S* and 5*S* receptors suggests that a 4*S* → 5*S* transformation occurs within the nucleus, especially since near maximal total levels of nuclear ER as 4*S* can be achieved at the 10°C intermediate temperature (Fig. 4). Further support for this hypothesis is obtained by the pulse-chase experiment shown in Fig. 8 wherein reincubation of uteri with equimolar nonradioactive estradiol effectively reduced the 4*S* peak while increasing the 5*S* peak accordingly. Indeed, only this type of manipulation consistently yields a single 5*S* nuclear complex.

It could be argued that the 4*S* nuclear component is an artifact arising from contamination of extracts with the serum estradiol binding protein (AFP). However, we have obtained several lines of evidence which appear to negate this possibility; (1) the sedimentation behaviour of the 4*S* nuclear receptor is consistently different from serum AFP, which in our experiments co-sediments with human serum albumin (Fig. 7); (2) the 4*S* nuclear receptor is temperature labile, whereas AFP is heat stable (Fig. 5); (3) *in situ* perfusion of uteri does not reduce the amount of 4*S* nuclear receptor (Fig. 6); (4) prior adsorption of nuclear extracts with AFP antiserum has no effect on the 4*S*–5*S* distribution of receptors (Fig. 7) and (5) the 4*S* nuclear receptor is consistently observed in the adult state. More recent experiments using a sensitive double antibody radioimmunoassay have shown that the amount of AFP in nuclear extracts of immature rats treated with estradiol does not exceed 0.02 pmol/uterus (< 10% of observed 4*S* receptor)*. On the other hand, AFP contamination of cytosol can represent 300–600% of the total 8*S* receptor present if the uteri are not carefully prepared prior to homogenization [16]. This level of cytoplasmic AFP can introduce serious errors in receptor measurements since various levels of binding to AFP will be included in estimates of specifically bound radioactivity under differing receptor assay methods and conditions, i.e. dextran coated charcoal, hydroxylapatite or gel filtration. Furthermore, the commonly used technique employing excess nonlabelled steroid that does not bind to AFP such as diethylstilbestrol to estimate nonspecific binding by saturation analysis can lead to gross errors since the distribution of labelled hormone between receptor, AFP and free hormone in the label-only sample is unknown. The same problem exists when measuring estrogen receptors in human tissues due to the presence of sex steroid binding protein (SBP). The only correct approaches to this problem are to separate the two binding proteins or to block binding to the serum protein by addition of an unlabelled hormone such as dihydrotestosterone which does not bind to the receptor.

Our observations suggest that the nuclear 4*S* component represents a true receptor form and strengthens the hypothesis of nuclear 4*S* → 5*S* transformation. Further support comes from the observations of Yamamoto and Alberts [17] who showed that the 4*S* form of receptor in high salt uterine cytosol was largely transformed to 5*S* by adsorption and elution from DNA cellulose. While the exact molecular mechanisms involved in the 4*S* → 5*S* transition are not clear, rather precise estimates of the molecular weight of these receptor forms has yielded values of

* Linkie, D. and La Barbera, A. In preparation.

76,000 and 132,000, respectively [18]. Since nuclear entry of cytoplasmic proteins with molecular weights much greater than 60,000 is restricted [19, 20], this also argues for receptor transformation occurring after the cytoplasm → nucleus translocation event. However, our observations are also consistent with the possibility that the 4S receptor form is loosely associated with the nucleus in the intact cell rather than in the cytoplasm. As pointed out by Gorski and Gannon[5] the finding of ER in the cytoplasm may be artifactual due to the harsh conditions required for disruption of uteri. It is of interest to recall that in some systems such as chicken liver the estrogen receptor is located primarily in the nucleus [21].

It is pertinent to reexamine the many findings concerning receptor transformation in the cytoplasm prior to translocation. The usual experimental conditions for generating 5S estrogen receptor in uterine cytosol are high salt ($\geq$ 0.4 M KCl) and a temperature elevation to 25–37°C [2–4]. The simultaneous acquisition of the ability of such receptor preparations to then bind isolated nuclei has supported the notion that "transformation" is necessary for "activation" of cytoplasmic receptor to allow nuclear binding. The latter concept has become widely accepted for other steroid hormone receptor interactions such as glucocorticoids and progesterone which do not exhibit the transformation phenomenon [22, 23]. However, such *in vitro* results may not accurately reflect *in vivo* events, especially since the temperature and salt concentration of the cell are relatively constant. Moreover, recent studies by Horowitz and Moore[24] suggest that an exclusion phenomenon is operative in the whole cell, i.e. a greater "free" water concentration in the nucleus than in the cytoplasm tends to drive substances including proteins into the cell nucleus. Thus "activation" may simply represent binding of steroid to receptor associated with cytoplasmic organelles that allows translocation of the complex to the nucleus. The apparent temperature dependency *in vivo* may then simply represent the slow binding kinetics of steroid to cytoplasmic receptor which is necessary for its mobilization. Alternatively, the temperature dependent step *in vivo* may actually be the intranuclear 4S → 5S transformation. As seen in Fig. 3 the amount of nuclear 4S receptor observed after incubation at 4°C is comparable to that seen at physiologic temperature and notably the intermediate 10°C condition portrayed in Fig. 4 results in near total nuclear ER as 4S. This interpretation then implies that the conformational or other change accompanying the formation of the 5S receptor from its precursor(s) or its binding to the nuclear acceptor sites is highly temperature sensitive. This possibility is considered more likely in view of the fact that "activated" receptor preparations can bind nuclei even at 4°C [25]. Thus interactions of the 4S receptor form with another non-identical binding subunit within the nucleus to yield the active 5S receptor prior to binding of the complex to chromatin could account for the present observations. It is important in this regard to note that subtle differences in sedimentation behaviour between the 5S receptor generated from cytosol and that recovered from nuclei by salt extraction have been detected [26].

The formation of the active 5S receptor complex within the nucleus may have important implications concerning the nature of its interactions with the putative chromatin acceptor sites. While we have not addressed this controversial problem directly, the results of the experiment shown in Fig. 9 may bear on this question. If labelled uteri (5 nM ^{3}H-E_2, 5 min × 37°C) are removed and placed in steroid free medium the existing levels of both nuclear forms slowly decrease. In contrast, if the removed uteri are placed in media containing the same concentration of unlabeled E_2 (5 nM) virtually complete translocation of cytoplasmic receptor to the nucleus occurs within 5 min. These results suggest that a recruitment phenomenon, due perhaps to positive cooperativity, is exerted at the level of (1) steroid binding to receptor, (2) transformation of 4S to the 5S receptor form or (3) 5S complex binding to chromatin. Positive cooperative interactions of estradiol with its receptor have been claimed [27], but the bulk of the evidence suggests that binding occurs at non-interacting sites [3–5].

It is tempting to speculate that the intranuclear 4S → 5S transformation and acceptor binding may be linked both temporally and spatially. Many 5S complexes may bind by cooperative interactions sequentially in specific regions of chromatin to alter the superstructure of nucleosomes and thereby expose initiation sites for transcription. While highly speculative, this hypothesis is consistent with (1) the observation of Anderson that a minimal number of estrogen receptor complexes must be retained for 6 h in order to elicit the full sequence of responses to estrogen [28]; (2) the failure of many investigators to demonstrate saturability of acceptor sites *in vitro* [29, 30]; (3) estrogen receptor complex binding for DNA is of low affinity but in large excess of the amount needed to activate a single gene [31, 32] and (4) the fact that receptor preparations tend to aggregate in solution.

Clearly much more work is needed before these mechanisms are fully understood. We would suggest that the early steps in estrogen action proceed as follows: (1) Steroid freely enters the cell and binds to a 4S receptor protein. (2) The resulting complex becomes "free" in the cytoplasm and moves into the nucleus by diffusion. (3) The 4S estrogen complex undergoes transformation to the 5S complex. (4) The 5S complex sequentially binds to chromatin acceptor sites until a critical number is obtained and (5) the resulting changes in DNA superstructure provides access to DNA-dependent RNA polymerases to those initiation sites which permit transcription of new RNA.

Acknowledgements—We are grateful to Dr S. Sell, University of California, San Diego, for supplying the goat anti-

rat-alpha fetoprotein serum used in the immuno-precipitation studies. This work was supported by NIH Grants HD 107 and 10090 and American Cancer Society Grant BC 33.

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