Estrogen Receptors in the Rat Uterus. Relationship between Cytoplasmic and Nuclear Forms of the Estrogen Binding Protein†

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ABSTRACT: In the presence of 4 M urea, 1 M KCl, and reducing agent, both the cytoplasmic and nuclear forms of the uterine estrogen receptor sediment at 3.6 S, suggesting that both forms contain a common subunit which binds estrogens. In the presence of 0.4 M KCl, mixing experiments with cytosol preparations and nuclear extracts do not produce any change in the sedimentation of either form of the receptor. However, if mixing experiments are performed in dilute buffer solutions without added salt, the nuclear "5S" receptor sediments as an 8S species similar to that observed for the cytoplasmic form under the same conditions.

It has been suggested that the response of the uterus and other target tissues to estrogen stimulation involves a cytoplasmic to nuclear migration of the steroid as an early, key event in the hormone's action (Shyamala and Gorski, 1969; Jensen *et al.*, 1968; Gorski *et al.*, 1968). While this cytoplasmic to nuclear translocation is not clearly understood, it appears that the hormone first forms a complex with a cytoplasmic macromolecule, the estrogen receptor protein(s), followed by migration of this protein–hormone complex to the nucleus. This nuclear form of the hormone–receptor complex can then be extracted from the nuclear fraction *in vitro* with concentrated salt solutions (Shyamala and Gorski, 1969; Jensen *et al.*, 1968). Presumably, this translocation involves a change in the structure of the receptor itself, since the cytoplasmic hormone–receptor complex sediments at approximately 4 S under conditions (0.4 M KCl) which yield a value of about 5 S for the nuclear complex (Jensen *et al.*, 1968; Giannopoulos and Gorski, 1971b).

Reports of the cell-free transformation of the 4S cytoplasmic to 5S nuclear forms of the receptor have appeared (Jensen and DeSombre, 1972). However, the conversion of the nuclear receptor to the cytoplasmic form (Puca *et al.*, 1971) is more controversial. We have reinvestigated the differences in sedimentation properties between the nuclear and cytoplasmic forms of the estrogen receptor under various conditions. Evidence is presented which suggests that the two forms contain a common subunit, as judged by gradient centrifugation in urea–KCl solutions. Furthermore, cytoplasmic components are shown to affect the sedimentation of the nuclear receptor, thus making observed differences between nuclear and cytoplasmic forms difficult to evaluate.

Materials and Methods

Preparation of Nuclear Receptor. Uteri were removed from 21–24-day old immature Holtzman rats and stripped of adhering fat. The uteri were then incubated at 37° for 1 hr in Eagle's HeLa medium (Difco) (1 uterus/ml of medium unless otherwise noted) containing 1×10^{-8} M [^{3}H]estradiol. Incubations were performed under an atmosphere of 95% O_2–5% CO_2 with constant shaking. This treatment leads to the migration of approximately 90% of the cytoplasmic estrogen receptor activity into the nuclear fraction (Giannopoulos and Gorski, 1971a; Williams and Gorski, 1973).

Following the incubation the uteri were washed well with dilute buffer (0.01 M Tris–1.5 mM EDTA, pH 7.4 [TE buffer][1]) at 0°, and homogenized in the same buffer (5 uteri/ml unless otherwise noted) using an all-glass homogenizer. The nuclear-myofibrillar pellet was then obtained by centrifugation at 1000*g* for 10 min. The pellet from five uteri was then washed three–four times with 4-ml aliquots of TE buffer. (Identical results were obtained if the pellet was washed with TE buffer containing 0.15 M KCl.)

The volume of the washed pellet was then estimated, and cold 0.8 M KCl (0.01 M Tris–1.5 mM EDTA–0.8 M KCl, pH 7.4) was added to yield a suspension with a final KCl concentration of 0.4 M. The suspension was thoroughly vortexed (0°) and allowed to sit at 0° for 1 hr with brief, intermittant vortexing (every 10–15 min). After 1 hr the suspension was centrifuged at 200,000*g* for 45 min to yield the final nuclear extract. This procedure led to the extraction of 50–60% of the nuclear estrogen receptor.

Unless otherwise noted, sucrose gradients used for sedimentation studies of the nuclear receptor were prepared in TKE buffer (0.01 M Tris–0.4 M KCl–1.5 mM EDTA, pH 7.4).

Baker reagent grade urea was recrystallized from 95% ethanol. All urea solutions were prepared fresh immediately before use (within 60 min), and were never stored. All urea-containing solutions and gradients also contained the following additions at the indicated concentrations: 0.05 M Tris, 1 M KCl, 50 mM β-mercaptoethanol, 50 mM $NaHSO_3$, and 1.5 mM EDTA, pH 7.4. Fractions from urea gradients were counted in a cocktail of toluene-based scintillation fluid:

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[1] Abbreviations for buffer systems used are: TE, 0.01 M Tris–1.5 mM EDTA, pH 7.4; TKE, 0.01 M Tris–0.4 M KCl–1.5 mM EDTA, pH 7.4.

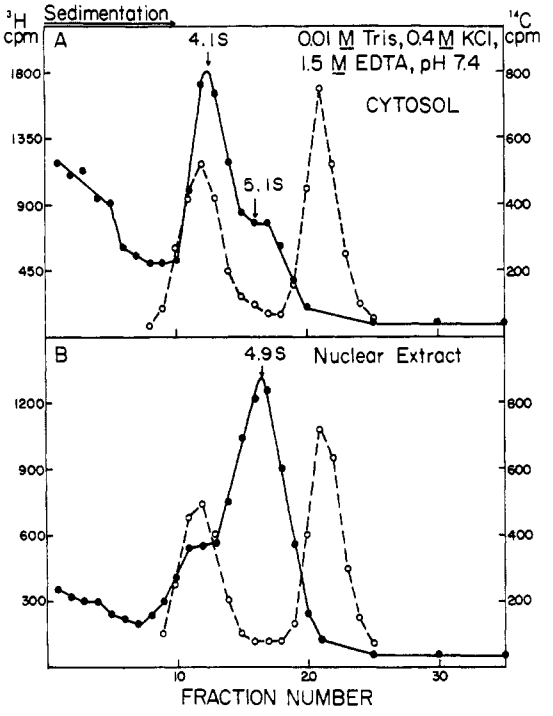

FIGURE 1: Sedimentation of cytoplasmic and nuclear estrogen receptors. Uteri were excised and each uterus was divided to yield two forms, which were placed in different groups. Cytosol (see Methods) was prepared from one group of horns which was washed for 1 hr at 37° in HeLa medium and then homogenized in TE buffer (5 uteri/ml). Cytosol was then adjusted to 0.4 M KCl, and incubated with 10^{-8} M tritiated estradiol for 3 hr (A). The second group of uterine horns was used to prepare the nuclear form of the receptor (B) as described in Methods, after incubation at 37° in the presence of 1×10^{-8} M tritiated estradiol; centrifugation for 14 hr on TKE gradients.

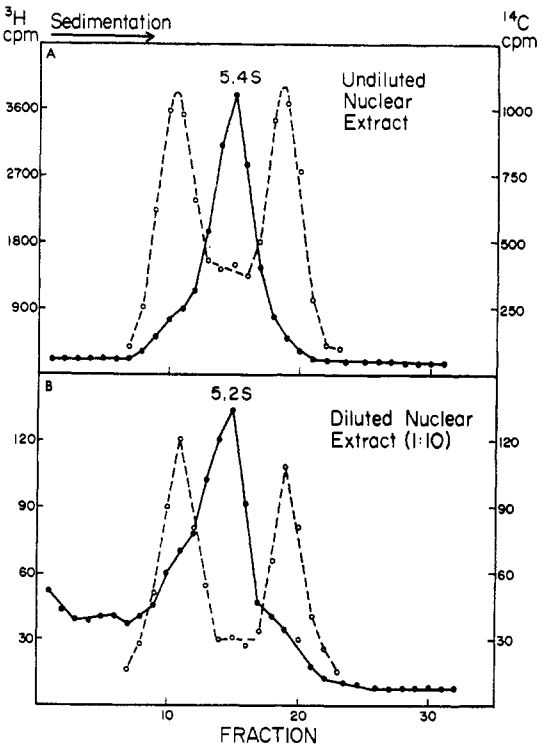

FIGURE 2: Effect of dilution on sedimentation coefficients of nuclear receptor. Whole uteri were incubated with 1×10^{-8} M tritiated estradiol in HeLa medium, and nuclear extract was prepared as described under Methods. Undiluted (A) and diluted aliquots (B) were centrifuged for 14 hr in TKE gradients.

(5 g of 2,5-diphenyloxazole and 0.3 g of 1,4 bis[2-(5-phenyloxazolyl)]benzene/l. of toluene)–Triton X-100–EtOH (14:6:4) with 18–20% efficiency.

Other Reagents and Procedures. All other reagents and procedures have been described in the preceding report (Stancel *et al.*, 1973).

Results

Differences in Sedimentation Properties of Cytoplasmic and Nuclear Receptors. In the preceding paper, we demonstrated that the sedimentation coefficient of the cytoplasmic form of the estrogen receptor was concentration dependent, even in the presence of 0.4 M KCl. We, therefore, carefully reexamined differences between the sedimentation coefficient of cytoplasmic and nuclear forms of the receptor.

As illustrated in Figure 1, cytoplasmic and nuclear forms of the estrogen receptor prepared from the same uteri sediment in different regions of 5–20% sucrose gradients prepared in 0.4 M KCl. The cytoplasmic form sediments at about 4 S with a shoulder at 5 S, and the nuclear form sediments at about 5 S with a small shoulder in the 4 S region. The values show considerable variation between preparations: the cytoplasmic form between 4 and 5.5 S, and the nuclear form between 5 and 6 S. But for any group of uteri the nuclear form always appears to sediment slightly faster than the cytoplasmic.

Effect of Concentration on the Sedimentation Coefficient of the Nuclear Receptor. It was also of interest to determine if the sedimentation coefficient of the 5S nuclear receptor would change with dilution, possibly to a value identical with that of the 4S cytoplasmic receptor. This, however, is not the case. The results illustrated in Figure 2 indicate that the nuclear receptor does not appear to undergo any significant change in sedimentation properties upon dilution.

We have also attempted to alter the sedimentation coefficient of the nuclear receptor by mixing nuclear extracts with cytosol preparations, but no significant effects were observed when the gradients were run in 0.4 M KCl gradients. Likewise, no changes were observed in the sedimentation of the cytoplasmic receptor when cytosol was mixed with nuclear extract. Both of these observations have been previously reported (Jensen *et al.*, 1971).

Effect of 4 M Urea on the Cytoplasmic and Nuclear Receptors. A number of reports support the idea that the nuclear form of the estrogen receptor is derived from the cytoplasmic form by a mechanism involving some type of modification, possibly a conformational change, or the addition of a small molecular weight subunit. Since we could not convert the nuclear form back to the cytoplasmic form by dilution or mixing, we sought other treatments which might bring about such a conversion.

Puca *et al.* (1971) had previously reported that the cytoplasmic receptor was able to retain bound estradiol in the presence of 4 M urea, and we therefore examined the effect of solvents containing 4 M urea on the structure of nuclear and cytoplasmic receptors. The results are seen in Figure 3.

In the presence of 4 M urea and 1 M KCl, both the cytoplasmic (Figure 3B) and nuclear forms (Figure 3A) of the receptor co-sediment with the ovalbumin marker. Note also that in

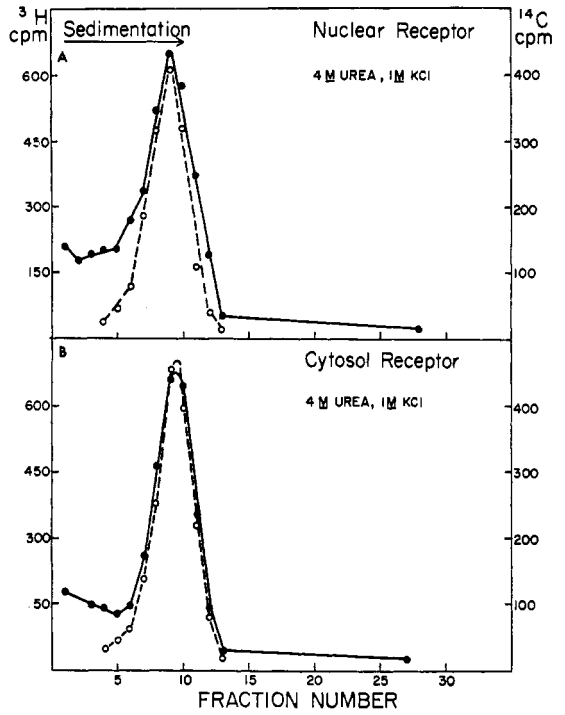

FIGURE 3: Sedimentation of nuclear and cytoplasmic receptors in 4 M urea. Nuclear extract (A) was prepared as described under Methods following incubation of whole uteri at 37° with 1×10^{-8} M tritiated estradiol in HeLa medium. Cytosol (B) was prepared as described under Methods and incubated for 3 hr with 5×10^{-9} M tritiated estradiol. Centrifugation for 23 hr in gradients containing 0.05 M Tris, 4 M urea, 1 M KCl, 50 mM mercaptoethanol, 50 mM $NaHSO_3$, and 1.5 mM EDTA, pH 7.4. Fractions (125 μl) were collected, and 25-μl aliquots were counted as described under Methods.

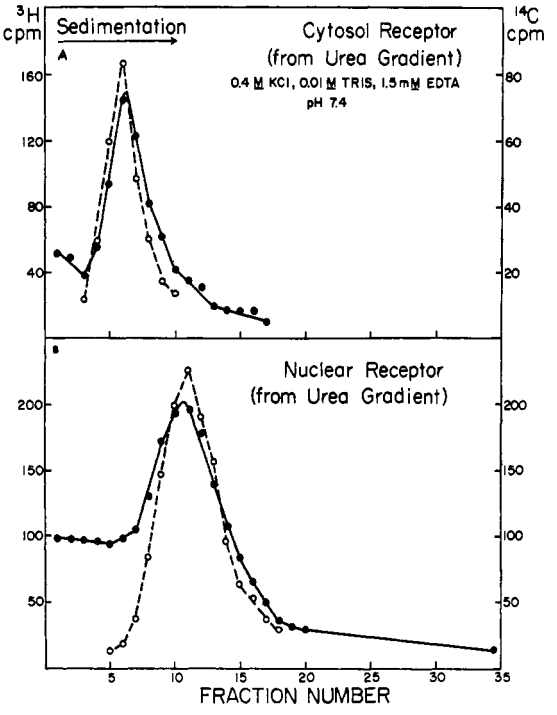

FIGURE 4: Re-run of cytoplasmic and nuclear receptors from urea gradients on TKE gradients. Aliquots (100 μl) from the peaks of Figures 3A and B (fraction no. 9) were diluted with 900 μl of TE buffer. These mixtures (400 μl) were then layered on 7.5–20% sucrose gradients in TKE buffer (4.6-ml gradients), and centrifuged for 17 hr at 50°K in a Beckman SW 50.1 rotor.

the presence of urea, both forms of the receptor sediment as very sharp, symmetrical peaks which resemble the marker peak in shape as well as position in the gradient. This is in contrast to most of the other gradient profiles of different forms of the receptor depicted in this and the preceding paper (Stancel *et al.*, 1973). A 1:1 mixture of cytosol and nuclear extract also co-sediments exactly with the marker.

It is clear from these results that the nuclear and cytoplasmic receptors have been converted to species that are indistinguishable as judged by gradient centrifugation. We cannot precisely determine the sedimentation coefficients of the species present in urea because we do not have precise data on the effect of 4 M urea on the ovalbumin marker. A number of observations suggest, however, that the sedimentation coefficient of the ovalbumin and the receptor is slightly less than 4 S in the urea solutions (see Discussion).

To ensure that the effect of urea on the nuclear receptor represented a structural change in the receptor itself rather than other changes, *e.g.*, differential solvation effects on cytoplasmic and nuclear receptors, the peak fractions from the urea gradients were re-run on gradients containing 0.4 M KCl *without urea*. The results of this experiment are illustrated in Figure 4. Once again, both the cytoplasmic (Figure 4A) and nuclear (Figure 4B) forms of the receptor co-sediment with the ovalbumin marker.

Other experiments also revealed that the nuclear form of the receptor could be converted to a species that co-sediments with ovalbumin (slightly less than 4 S) by a brief exposure (2 hr) to 4 M urea and 1 M KCl, followed by dilution and centrifugation in a gradient containing 0.4 M KCl, but *no urea*.

After these experiments had been performed in 4 M urea and 1 M KCl it was discovered that identical results were obtained if the gradients contained 3 M urea and 0.4 M KCl. At lower urea concentrations, however, the patterns became quite diffuse and multiple peaks appeared in the profile.

Effect of Cytoplasmic Factors on the Nuclear Estrogen Receptor. The results from the urea gradients suggested that both the cytoplasmic and nuclear estrogen receptors contained a common structural unit, which binds estrogens and sediments at about 4 S in urea, or after exposure to urea. The mixing experiments (described in a previous section) performed in 0.4 M KCl, however, did not reveal any effect of cytoplasmic factors on the nuclear receptor as might be expected if the two forms of the receptor contained some identical polypeptide portions. We therefore examined the effect of cytoplasmic proteins on the structure of the nuclear receptor in dilute buffers *without added salt*.

Figure 5A illustrates the profile obtained by centrifuging a preparation of nuclear extract in a TKE gradient and as expected shows a single peak at 5.1 S. As reported by others (Jensen *et al.*, 1971) we have observed that the nuclear receptor aggregates extensively and shows no well-defined peak if run on a TE gradient. If, however, an aliquot of the nuclear receptor used in Figure 5A was first diluted 1:10 with cytosol preparation (incubated with 10^{-8} M cold estradiol) and then placed on a TE gradient, the profile shown in Figure 5B is observed. This profile shows a peak in the 8S region of the low salt gradient, and resembles the profiles routinely observed for the cytoplasmic receptor under these conditions. If the nuclear extract was first diluted with TE buffer alone

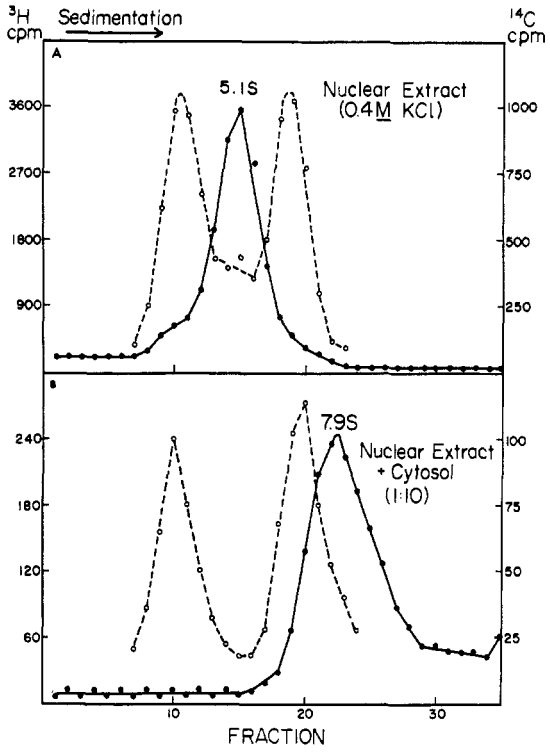

FIGURE 5: Effect of soluble uterine proteins on sedimentation of nuclear receptor. Following incubation of whole uteri with 1×10^{-8} M tritiated estradiol at 37° in HeLa medium, nuclear extract prepared (see Methods) and centrifuged for 14 hr in a TKE gradient (A). Nuclear extract was also diluted 1:10 with a preparation of cytosol (5 uteri/ml in TE buffer) which had been incubated with 1×10^{-6} M cold estradiol, and then centrifuged for 14 hr in a TE gradient (B).

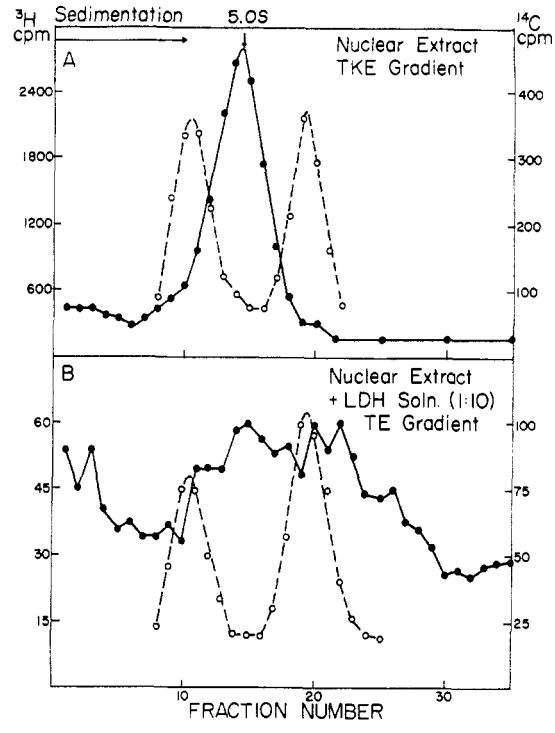

FIGURE 6: Effect of lactic dehydrogenase (LDH) on sedimentation of nuclear receptor. Nuclear extract was prepared (as in Methods) from whole uteri which had been exposed to 1×10^{-8} M tritiated estradiol and an aliquot centrifuged for 14 hr in a TKE gradient (A). Nuclear extract was also diluted 1:10 with a solution of beef heart lactic dehydrogenase, 3 mg/ml in TE buffer, and an aliquot of the mixture centrifuged for 14 hr in a TE gradient.

rather than cytosol, a diffuse pattern without any defined peaks was obtained. Results identical with those shown in Figure 5B are obtained with cytosol preparations containing receptor labeled with cold estradiol, or with cytosol prepared from uteri which do not contain appreciable amounts of cytoplasmic receptor. The later cytosol preparation was obtained from uteri which had been exposed to cold estradiol at 37°, *i.e.*, conditions which cause the nuclear translocation of approximately 90% of the cytoplasmic receptor (Giannopoulos and Gorski, 1971a; Williams and Gorski, 1973). It should be mentioned that in this type of mixing experiment 80–90% of the counts originally bound to the nuclear 5S receptor are routinely recovered in the 8S region of the TE gradients (as in Figure 5B).

In order to ensure that the results from these mixing experiments were not due to a general effect of protein concentration, the following experiment was performed. An aliquot of nuclear extract which contained receptor sedimenting at 5.0 S on a TKE gradient (Figure 6A) was diluted 1:10 with a solution of beef heart lactic dehydrogenase (3 mg/ml) in TE buffer, and then applied to a TE gradient. In this case the counts are spread over the entire gradient with no resolved peaks (Figure 6B). The profile shown in Figure 6B is similar to that observed if aliquots of nuclear extract are run directly on TE gradients, or first diluted with TE buffer and then run on TE gradients.

Discussion

The results obtained from exposing the nuclear and cytoplasmic estrogen receptors to 4 M urea and 1 M KCl suggest that both forms of the receptor share a common unit which binds estrogens. Puca *et al.* (1971) came to the same conclusion based on the observation that DEAE-cellulose treatment of the nuclear receptor (5 S) from calf uterus produced a 4.5S form which was indistinguishable from the cytoplasmic form when examined in high salt. The difference in absolute values between this value of 4.5 S (Puca *et al.*, 1971) and our value of 3.6 S for the receptor from rat uterus in urea may reflect species differences or the ability of urea to prevent small changes due to minor aggregation (compare, for example, Figures 1A and 3B).

Based on the effects of urea on most other proteins, the most likely possibility seems to be that the cytoplasmic to nuclear conversion involves the noncovalent addition of a small subunit to the cytoplasmic form of the receptor. Such a model would also be consistent with the recent observation that the cell-free conversion of the cytoplasmic to nuclear form of the receptor on DNA-cellulose columns is a bimolecular process (Yamamoto, 1972). On the basis of our data alone, however, we cannot rigorously exclude the possibility that the conversion involves a conformational change which is primarily responsible for the change in sedimentation coefficient, since the change in sedimentation coefficient between the "4S" and "5S" forms is small, and since there is so much variability in the measurements (Stancel *et al.*, 1973; Chamness and McGuire, 1972). It seems highly unlikely that the conversion of the cytoplasmic form of the receptor to the nuclear form involves any covalent modification of the receptor, *e.g.*, phosphorylation or acetylation, since such changes would not be reversed by exposure to urea.

The sedimentation coefficient of both the cytoplasmic and nuclear receptors is probably about 3.6 S (the value for ovalbumin) in 4 M urea and 1 M KCl (Figure 3). Even without precise data on the effect of urea on ovalbumin this appears to be the most appropriate value to assign at present, since: (1) concentrations of urea up to 3–4 M do not produce any large changes in the optical rotation (Gordon and Jencks, 1963) or sedimentation properties (Gagen and Holme, 1964), when corrected for viscosity, of ovalbumin; (2) following exposure to 4 M urea, the nuclear receptor sediments at 3.6 S in gradients without urea; (3) if the peak fractions of the nuclear and cytoplasmic receptors are taken off urea gradients and re-run on gradients without urea, values of 3.6 S are obtained (Figure 4); (4) the relative rates of sedimentation of ovalbumin, as measured in sucrose gradients containing 0.4 M urea, are a linear function of the viscosity of urea solutions of the same concentration (unpublished observations). This suggests that in sucrose gradient centrifugation, the major effect of urea (at the concentrations employed in this work) on the sedimentation of ovalbumin is due to increases in viscosity of the working solutions, rather than changes in the ovalbumin molecule.

We cannot be absolutely certain that the 3.8S species observed in urea in this work represents a single polypeptide chain. While this is one likely possibility, a definitive answer to this question will require the isolation of the estrogen receptor in a homogeneous state, since concentrations of urea greater than 4 M destroy binding of estrogens to the receptor (Puca *et al.*, 1971).

In contrast to the cytoplasmic receptor, the sedimentation behavior of the nuclear form of the estrogen receptor is unaltered by dilution. This is true for the "5S" form of the nuclear receptor (Figure 2), obtained by the *in vitro* salt extraction of nuclei. Although not illustrated in this paper, other experiments have revealed that this observation also holds for the "6S" form of the nuclear receptor (Giannopoulos and Gorski, 1971b), obtained by homogenizing whole uteri directly in concentrated salt solutions after prior exposure to estradiol

It is more difficult to interpret the observed sedimentation of the "5S" nuclear receptor as an "8S" species after mixing nuclear extract and cytosol (Figure 5). Superficially, it might appear that the 8S species so formed is the same species as that observed if cytosol is prepared in dilute buffer and sedimented through a TE gradient (see, for example, Figure 1 in the preceding paper; Stancel *et al.* (1973)). This may not be the case, however, since the 8S species formed in the mixing experiment remains as a "5S" nuclear species when examined in TKE gradients, rather than the salt-dissociated "4S" form of the cytoplasmic receptor. These two observations appear contradictory and must be resolved by further studies. Nevertheless, it does appear that the sedimentation of the nuclear receptor is dramatically affected by cytoplasmic factors, and this must be considered when interpreting results based on sedimentation analysis.

A major question that arises from the work in this and the preceding paper (Stancel *et al.*, 1973), as well as that of Williams and Gorski (1973), is whether there are real physical differences between nuclear and cytoplasmic hormone receptors, or merely "apparent" differences. Such apparent differences might be artifacts resulting from interactions between the receptor and other component present in uterine cytosol or nuclear extracts. This question is of obvious importance in determining the mechanism of estrogen action.

In this regard, Williams and Gorski (1973) have recently raised the possibility of a mechanism of estrogen action which is an alternative to previously suggested models (Jensen and DeSombre, 1972). This recently suggested model is as follows (Williams and Gorski, 1973): (1) the estrogen receptor, initially present in the cytoplasm, forms a complex with estrogen; (2) this complex formation somehow leads to a redistribution of the complex, most of the complex undergoing translocation to the nucleus. The nature of the redistribution is such that an equilibrium in the distribution of receptor–hormone complex appears to be established between nuclear and cytoplasmic compartments.

It is thus possible that during disruption and preparation of nuclei by current techniques, the receptor–hormone complex in the nuclear compartment aggregates with other nuclear proteins. The result is the so-called "5S nuclear receptor," rather than the "4S salt-dissociated" form of the cytoplasmic receptor. Further experimentation is needed to prove or disprove the validity of this as well as other proposed models of steroid–hormone action.

Acknowledgment

The authors would like to thank Drs. W. H. Beers, R. E. Leake, and D. L. Williams for helpful suggestions and discussions, and Mrs. T. Spike for assistance in the preparation of manuscripts and figures.

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