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ESTROGEN RECEPTOR FORMS IN HUMAN MYOMETRIAL CYTOSOL

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SUMMARY

The estrogen receptor forms in human myometrial cytosol have been characterized in a low salt medium (without KCl) in the absence and presence of the protease inhibitor, diisopropylfluorophosphate (DFP). The sedimentation coefficients of the receptors were 9.0 ± 0.1 S, 5.4 ± 0.16 S and 4.3 ± 0.17 S. The amount of the receptor forms present was dependent on the time of the DFP addition. Obviously a 9 S → 4.3 S receptor transformation was catalyzed by the protease in the cytosol while the 5.4 S receptor form seemed to be unaffected.

The estradiol binding capacity of the receptors in cytosol without DFP was increased compared to cytosol prepared in the presence of DFP. The protease catalyzed transformation which resulted in a reduction in the amount of estradiol bound to the 9 S receptor form was accompanied by a two-fold increase in the amount bound to the 4.3 S receptor. No change in the dissociation constant of estradiol receptor complex was observed by the dextran-charcoal technique. Either the proteolytic 9 S → 4.3 S transformation has to uncover hidden estradiol binding sites or then part of the estradiol bound to the 9 S receptor dissociates rapidly so that it cannot be detected under the conditions used.

INTRODUCTION

The models for the action of steroid hormones comprise entry into the cell, binding to the cytoplasmic receptor and translocation of the complex into the nucleus. Subsequent steps lead to interaction with the genetic apparatus and stimulation of the DNA-dependent RNA polymerase activity. Recent review articles are available on the subject [1, 2].

Different forms of steroid hormone receptors are characterized by their sedimentation coefficients. The observed forms are very much dependent on the experimental conditions [1–3]. Estrogen receptors require an activation or transformation from a 4 S to a 5 S state before translocation to the nucleus occurs [4, 5]. The transformation seems to be a temperature and estradiol dependent dimerization reaction [5, 6].

Relatively few reports can be found that describe the estrogen receptor forms in human uterus and the obtained results are variable [5, 7–9]. Sedimentation coefficients in the range of 3 S to 9 S have been reported.

In a previous communication on the binding characteristics of estrogens to the human myometrial estrogen receptor we reported on the receptor forms that were observed in a low salt medium [10]. Only a 4 S receptor form was present if the protease inhibitor diisopropylfluorophosphate (DFP) was omitted. The presence of DFP was a necessity for the occurrence of a 8 S receptor form. We have continued our investigation of the protease catalyzed receptor transformation in human myometrial cytosol. Furthermore, we have observed that protease affects the amount of estradiol bound to the receptors.

EXPERIMENTAL

Materials. [2,4,6,7-^{3}H]-Estradiol (100.9 Ci/mmol) was obtained from New England Nuclear, and stored in a benzene–ethanol mixture at 0–4°C. Diisopropylfluorophosphate (DFP) was purchased from Fluka AG.

Measurement of radioactivity. Aqueous samples from sucrose gradients and from dextran-charcoal assays were transferred into counting vials containing 5 ml of scintillation liquid [0.6% (w/v) 2.5-diphenyloxazole (PPO) and 0.08% (w/v) 1.4-di(4-methyl-5-phenyloxazolyl)-benzene (POPOP) in toluene]. The content was agitated for a few seconds and left at least 4 h in the dark. The β-scintillation counting was carried out in a liquid scintillation counter (LKB Wallac 81000).

Preparation of uterine supernatants. Normal uteri was obtained from patients (35–50 years) immediately after hysterectomy and were considered pre-menopausal. The uteri were cooled on crushed ice and all subsequent procedures were carried out in 0–4°C. The myometrial tissue was cut into small pieces and thoroughly washed with physiological NaCl-solution. 2–5 g samples were placed in 40 mM Tris–HCl–1 mM dithiothreitol buffer pH 7.4 (TD-buffer), 2 ml/g tissue and homogenized with an Ultra-Turrax TP 18/10 shaft 10 N (4 × 10 s). The homogenization was always done within 1 h of hysterectomy. The homogenates

were centrifuged for 15 min at 48,000 *g* in a Sorvall Superspeed RC 2-B (SS-34) centrifuge. The DFP was added either before homogenization, to the homogenate or at different times after centrifugation as a 5.4 mM solution in TD-buffer to a final concentration of 2 mM. The final centrifugation was carried out in a MSE Superspeed centrifuge for 1 h at 100,000 *g*. The protein content of the cytosols, as measured by the method of Lowry *et al.*[11], were 10–14 mg/ml. DFP had no effect on the protein content.

Quantitation of estradiol binding. Saturation analysis was carried out by essentially the same procedure as used earlier [10]. Aliquots of cytosol 0.1 ml were incubated overnight at 0°C with various concentrations of tritiated estradiol (0.16–5.0 nM) in 0.1 ml TD-buffer with or without a 100-fold excess of unlabeled estradiol. Bound and free estradiol was separated by adding 0.5 ml of a dextran-charcoal suspension (1% activated charcoal and 0.1% dextran in TD-buffer) to the incubation mixture. After 30 min at 0°C and subsequent centrifugation 0.5 ml of the supernatant was used for measurement of the radioactivity. The calculation of dissociation constants and binding capacities was done according to the method of Scatchard[12], after substraction of non-specific binding.

Sucrose gradient centrifugation. Fractions of the prepared cytosols 1 ml were equilibrated with 5 nM tritiated estradiol with or without a 400-fold excess of non-radioactive hormone for 4 h at 0°C. In order to separate free and bound hormone and to reduce non-specifically bound estradiol 100 μl of a concentrated dextran-charcoal slurry (10% activated charcoal and 2.5% dextran in TD-buffer) was added to the incubation mixture and occasionally agitated at 0°C for 1 h. After centrifugation 200 μl of the supernatant (2–2.8 mg protein) was layered on 5–20% sucrose gradients in TD-buffer together with 30 μl of an amido-black 10 B stained BSA solution [13]. Occasionally 7.5–17.5% sucrose gradients were used. Centrifugation was carried out for 14 h at 2°C in a MSE 50 ultracentrifuge (6 × 4.2 ml Ti) at 47,000 rev./min (g_{max} 305,000). Fractions of 3 drops (~80 μl) were collected for measurement of radioactivity.

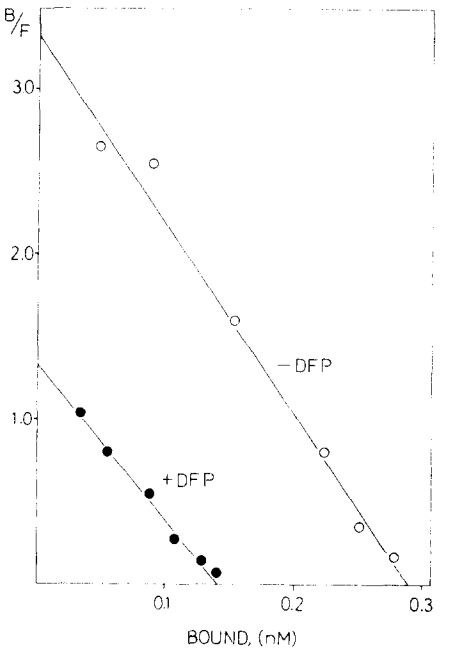

Fig. 1. Scatchard-plot of [^{3}H]-estradiol binding by estrogen receptors in human myometrial cytosol prepared without (○) or with (●) 2 mM DFP. 100 μl aliquots of myometrial cytosol were incubated overnight with various concentrations of [^{3}H]-estradiol (0.16–5 nM) with or without a 100-fold excess of unlabeled estradiol in 100 μl TD-buffer. Dextran-charcoal was used to adsorb free estradiol following equilibrations.

RESULTS

Binding studies.

The presence and absence of DFP in the homogenization buffer affects the receptor forms present in the myometrial cytosol [5, 10]. Determinations of the binding affinity of estradiol to the receptor forms and the number of binding sites were made under the conditions mentioned. Myometrial tissue samples from the same uterus were homogenized in TD-buffer with and without 2 mM DFP. The cytosols obtained after centrifugation were used for saturation and binding affinity analysis. The data for the binding of [^{3}H]-estradiol to the cytosol receptors are presented as Scatchard plots (Fig. 1). The dissociation constant for the estradiol receptor complex in both cytosols was of the same order of magnitude, 0.066 nM without and 0.059 nM with DFP in the cytosol. Estradiol concentrations up to 15 nM has been tried with no change in the dissociation constant. Surprisingly a difference in the number of binding sites was obtained as the binding capacity of the DFP cytosol was reduced by about 50%. When additional experiments were performed the binding capacity of the DFP cytosols were always between 50 and 90% as compared to cytosols prepared without DFP. Occasionally the dissociation constant of the estradiol receptor complex in the DFP cytosol was somewhat lower than the constant for the complex in cytosol without DFP (data not shown).

Obviously the receptor forms present in cytosols prepared with and without DFP in the medium have different binding capacities for estradiol measured by the charcoal-dextran technique. The observation was tested in experiments where DFP was added either before the tissue sample was homogenized, after homogenization, after centrifugation to the cytosol or then it was omitted. Typical binding curves for estradiol receptors in the four cytosols are shown in Fig. 2. An evident shift occurred in the binding capacity depending on the time of DFP addition while the dissociation constant can be calculated to be almost unaffected. If the binding capacity of the receptor in cytosol from which DFP was omitted is marked 100% the corresponding values for the cytosol obtained

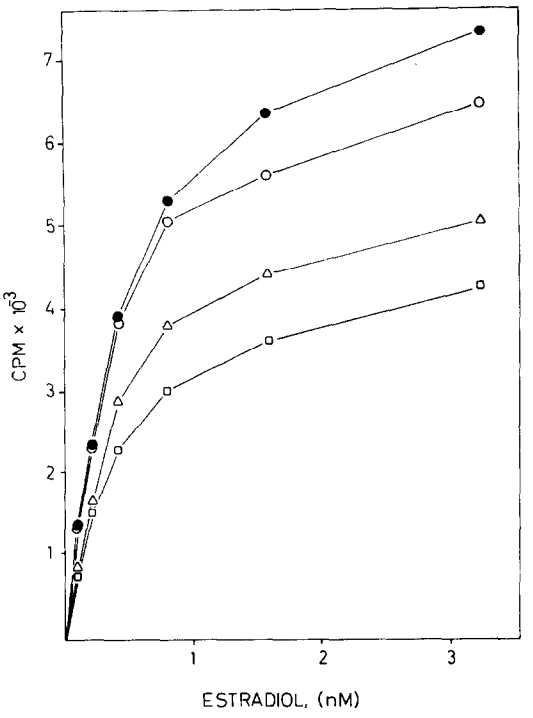

Fig. 2. Saturation binding analysis for the measurement of estrogen receptor concentration in cytosol prepared from human myometrial tissue when DFP was added before homogenization (□), after homogenization (△), after centrifugation (○) or then it was omitted (●). Conditions are the same as those described for Fig. 1.

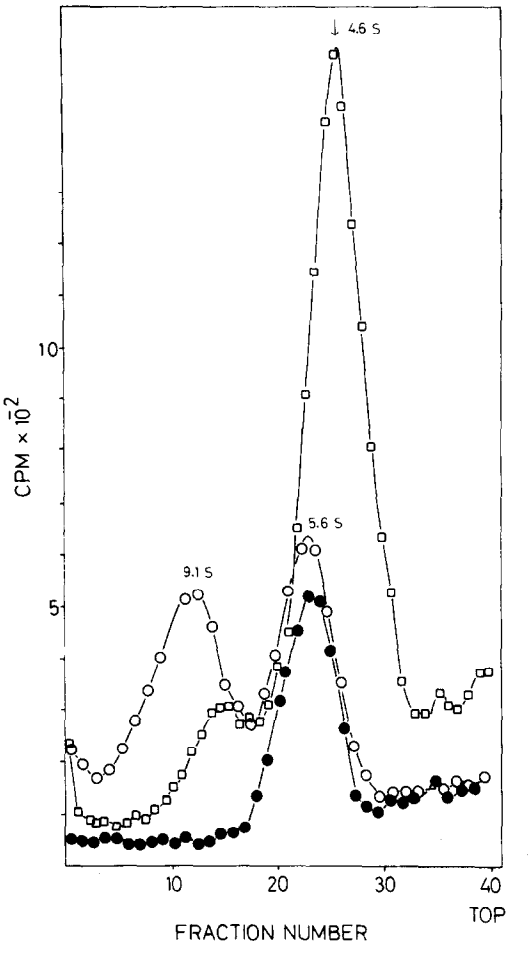

Fig. 3. Sedimentation of the estrogen receptors in cytosol from human myometrial tissue prepared without (□) or with (○) 2 mM DFP. The tissue was homogenized in TD-buffer (with or without DFP) and the cytosol was equilibrated with 5 nM [^{3}H]-estradiol for 4 h at 0°C. A 400-fold excess of unlabeled estradiol was used for estimation of non-specific binding (●). Dextran-charcoal was used to adsorb free estradiol. Aliquots of 200 µl were layered on a 5–20% sucrose gradient in TD-buffer (3.0 ml). The samples were centrifuged at 305,000 *g* for 14 h at 2°C. BSA (↓).

when DFP was added before homogenization, after homogenization and after centrifugation are 54, 68 and 85%, respectively.

Characterization of receptor forms by sucrose gradient centrifugation

We had previously observed only a 4 S form of the estrogen receptor in cytosol without DFP and an additional 8 S form in the DFP cytosol [10]. As the receptor forms in the two cytosols evidently have different binding capacities for estradiol this ought to be confirmed by sucrose density gradients. In Fig. 3 the sedimentation properties of the receptor forms in the two cytosols are shown after incubation with [^{3}H]-estradiol for 5 h at 0°C and removal of free steroid with a dextran-charcoal suspension. The peaks of bound radioactivity sediment at 9.1 S and 5.6 S in the DFP cytosol and at 4.6 S in the cytosol without DFP. When the bound radioactivity was corrected for unspecific binding 5700 c.p.m. was obtained for the DFP cytosol compared to 10,200 c.p.m. for the cytosol without DFP. Several separate runs gave on the average 9.0 ± 0.1 S, 5.4 ± 0.16 S and 4.3 ± 0.17 S for the three peaks.

The non-specific binding in receptor preparations has usually a sedimentation coefficient around 4.5–5.0 S and consists mainly of serum albumin [8]. The receptor form with a sedimentation coefficient of 9 S should thus be seen in DFP cytosol when free [^{3}H]-estradiol is not removed by the dextran-charcoal treatment. The results in Fig. 4A show that this is actually the case. A peak of bound radioactivity was obtained at 8.9 S and the size of the peak decreased if DFP was added later or omitted. The bound radioactivity at 5.2 S that mostly represents non-specific binding decreases considerably after the dextran-charcoal treatment (Fig. 4B) but it did not affect the amount of radioactivity bound to the 8.9 S peak (after correction for non-specific binding). The cytosol without DFP showed after removal of free steroid a peak at 8.7 S. The difference in the amount of bound radioactivity in the 9 S peak of the DFP cytosol and the cytosol without DFP is about 2100 c.p.m. The non-specific binding has been taken into consideration. When non-specific binding had been substracted from the peaks at 4.7 S (cytosol

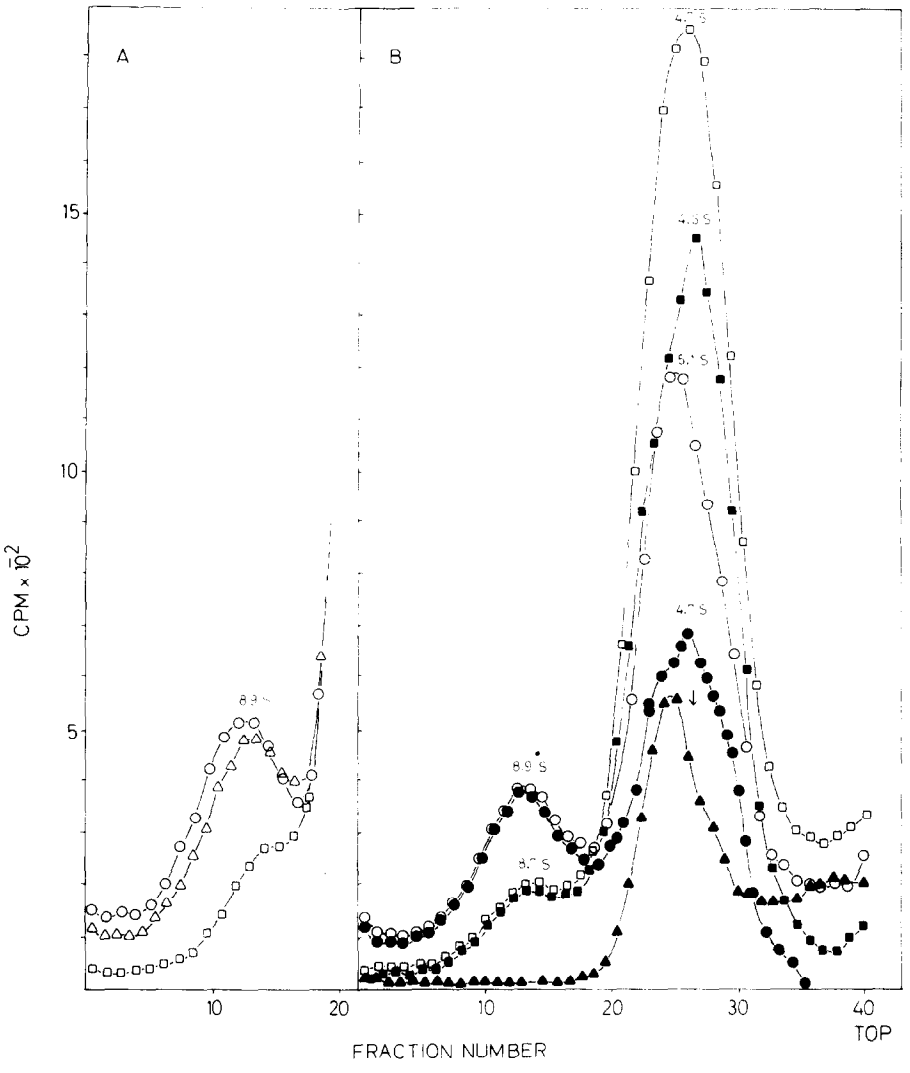

Fig. 4A. Sedimentation of the estrogen receptors in cytosol from human myometrial tissue prepared without DFP (□), with 2 mM DFP (○) or by adding DFP 20 min after homogenization (△). Conditions are the same as those described for Fig. 3 except that free [^{3}H]-estradiol was not removed before centrifugation.

Fig. 4B. Sedimentation of the estrogen receptors in the previous cytosol after removal of free estradiol by dextran-charcoal treatment. Cytosol prepared without DFP (□), cytosol prepared by adding DFP 20 min after homogenization (○), non-specific binding (△), radioactivity bound to the cytosol without DFP when corrected for non-specific binding (■) and radioactivity bound to the cytosol to which DFP was added 20 min after homogenization when corrected for non-specific binding (●). BSA (↓).

without DFP) and 5.1 S (DFP cytosol) the difference in the amount of bound radioactivity in these peaks was about 4200 c.p.m. Hence the decrease in the binding capacity that occurred in the 9 S receptor form in the absence of DFP resulted in an exactly two-fold increase in the binding capacity of the receptor with a lower sedimentation coefficient. After the substraction of non-specific binding from the amount of bound radioactivity in the DFP cytosol a peak was obtained at 4.7 S with an obvious shoulder at about 5.5 S. The corresponding peak for the cytosol without DFP has an unsymmetrical peak at 4.6 S. Evidently the DFP cytosol contains considerable amounts of the 5.4 S receptor form and it seems to be present also in the cytosol without DFP.

As it has not been established that the protease catalyzed transformation of the 9 S receptor form to the 4.3 S form occurs through an intermediate 5.4 S form which has increased binding capacity, attempts were made to determine the amount of radioactivity bound to the 5.4 S form. The result presented in Fig. 5A was obtained when the cytosol was prepared either in the presence of DFP, added 2 h after homogenization or then it was omitted. When the results were corrected for non-specific binding (Fig. 5B) an almost equivalent amount of radioactivity was bound

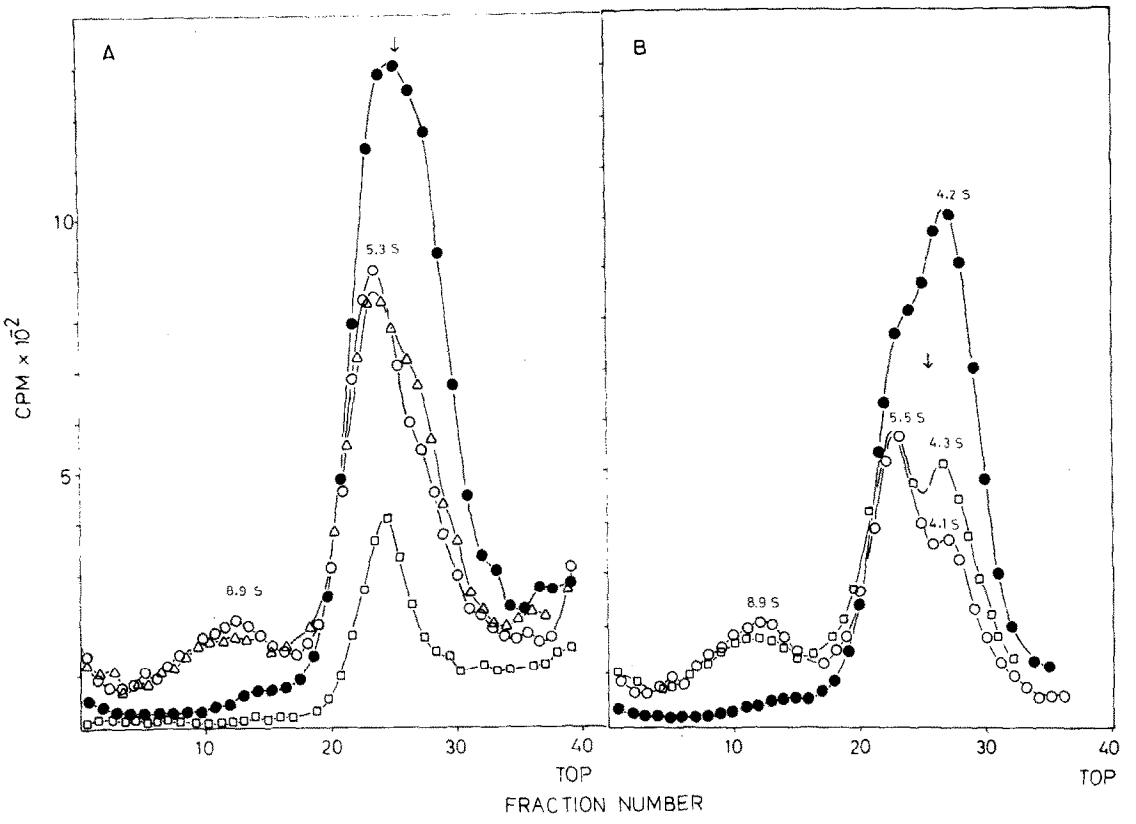

Fig. 5A. Sedimentation of the estrogen receptors in cytosol from human myometrial tissue prepared without DFP (●), with 2 mM DFP before homogenization (○) or by adding DFP 2 h after homogenization (△). Non-specific binding (□). BSA (↓). Conditions are the same as those described for Fig. 3 except that a 7.5–17.5% sucrose gradient was used.

Fig. 5B. The result of Fig. 5A after substraction of non-specific binding. The amount of radioactivity bound to cytosol without DFP (●), cytosol with DFP (○) and cytosol to which DFP was added 2 h after homogenization (□).

to the 5.5 S peaks of the DFP cytosol and the cytosol to which DFP had been added after 2 h while the amount of radioactivity in the 4.3 peak increased due to the proteolytic transformation of the 9 S form. The DFP cytosol also showed a peak for the 4.3 S receptor form. Most likely the 5.4 S receptor form should not be considered as an intermediate in the transformation. The shoulder in the 4.2 S peak for the cytosol without DFP indicates the presence of the 5.4 S form. Analogous experiments, when the incubation for the cytosol without DFP and in the presence of estradiol were extended to 24 h, still showed a 5.4 S receptor form. This amount became difficult to evaluate because of the radioactivity bound to the 4.3 S peak.

DISCUSSION

The presented experimental data on estrogen receptors in human myometrial cytosol confirm the observations on the presence of a protease which is inhibited by DFP and affects the receptor forms [5, 8, 10, 14]. Both a 9 S and a 5.4 S receptor form was observed when the cytosol was prepared in a medium containing DFP while a 4.3 S receptor form predominated when DFP was omitted. Most likely the 9 S receptor form was proteolytically transformed to the receptor form that sediments at 4.3 S, as the amount of the 5.4 S receptor seemed to be independent of the time of DFP addition. A close examination of the previously published data reveals slightly unsymmetrical peaks that obviously conceal the 5.4 S receptor form which was not resolved under the conditions used for centrifugation [10].

In a low salt medium Notides *et al.*[5] obtained sedimentation coefficients of 7.9 S, 4.9 S and 3.7 S for a partially purified estrogen receptor solution in the presence of DFP and only a 3 S proteolytic fragment was present when DFP was omitted. The sedimentation coefficients for the estrogen receptor forms that we obtained could be considered consistent with these if a slight displacement is permitted. Our analysis, however, gave no indication of the presence of a 3 S proteolytic fragment, but the estrogen receptor obtained without protease inhibitor sedimented at 4.3 S.

The most unexpected finding in the present experimental data was the evidence for a decreased binding capacity for estradiol without any change in the dissociation constant for the estradiol receptor complex in the presence of DFP. Actually Notides *et al.*[5] already found an indication of the decreased capacity. According to the results the 5.3 S receptor form is

very unlikely to affect the binding capacity for estradiol. Obviously the 4.3 S estrogen receptor form binds twice as much estradiol compared to the 9 S form as measured by the dextran-charcoal technique. It is conceivable that the protease catalyzed 9 S → 4.3 S receptor transformation exposes binding sites for estradiol. Another possible explanation is that twice the measured amount of estradiol may actually be bound to the 9 S receptor but half of it is bound in a fast dissociable form with lower affinity which cannot be measured under the non-equilibrium condition of the dextran-charcoal technique. Consequently, either the 9 S estrogen receptor contains binding sites with different affinities for estradiol or the proteolytic transformation of 9 S → 4.3 S uncovers hidden sites for the binding of estradiol.

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