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BINDING CHARACTERISTICS OF ESTRONE, ESTRADIOL AND ESTRIOL TO THE HUMAN MYOMETRIAL ESTROGEN RECEPTOR

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SUMMARY

The human myometrial estrogen receptor in cytosol from pre-menopausal uterine samples has been characterized. At 0° estradiol (K_D 0.38×10^{-10} M) has the highest affinity to the receptor followed by estrone (K_D 0.76×10^{-10} M) and estriol (K_D 1.33×10^{-10} M). The association rate constant is $2.8 \times 10^5\ M^{-1}\ s^{-1}$ for estradiol, $2.1 \times 10^5\ M^{-1}\ s^{-1}$ for estrone and $0.79 \times 10^5\ M^{-1}\ s^{-1}$ for estriol. The dissociation constants and the association rate constants increase with temperature. The calculated thermodynamic parameters indicate a positive change in entropy for the formation of the estrogen receptor complex.

The cytoplasmic estrogen receptor has a sedimentation coefficient of 4 *s* in low salt sucrose gradients. In buffer containing diisopropylfluorophosphate (DFP) to inhibit proteolytic activity the estrogen receptor complex sediments solely as an 8 *s* peak if [^{3}H]-estradiol is added to the buffer prior to homogenization and the tissue sample is used immediately after hysterectomy. Estrogen receptor complexes that sediment at 4 *s* and 8 *s* are found if [^{3}H]-estradiol is omitted from the homogenization buffer and instead added after the cytosol preparation. Most likely a protease is involved the activity of which is not completely inhibited by DFP.

Addition of low concentrations of Cu^{2+} (10 μM) to the cytosol increases the dissociation constant and decreases the estrogen-binding capacity of the receptor. The rate of association is reduced in the presence of 20 μM Cu^{2+}. The estrogen receptor complexes do not show any change in their sedimentation profiles in the presence of Cu^{2+}.

INTRODUCTION

Estrogen-dependent tissues contain estrogen receptors or estrogen-binding proteins of high affinity and specificity, which are intimately involved with the target tissue interaction with the hormone [1–3]. Upon entering the cells of the target tissue, the estrogen forms an estrogen-receptor complex in the cytoplasmic fraction. Apparently a temperature dependent transformation or activation of the complex occurs, which enables its entry to the nucleus [4, 5]. Studies of the estrogen receptor of the uterus of various animals show however a considerable difference in molecular forms and their physiological significance [6–8]. Relatively little is known about the properties of the estrogen receptor in human uterus and the results are inconsistent [8–10]. Furthermore, certain metabolic derivatives, such as estrone and estriol, are not devoid of estrogenic potency and may interfere with hormone action differently than estradiol [11–13]. The observations could indicate a diversity in the action of the high affinity receptor.

The purpose of the investigation in this report was to compare the binding of estrone, estradiol and estriol to the human myometrial estrogen receptor and to measure the rate of the association reactions. The effect of Cu^{2+} ions on the steroid-binding process was tested as it may give one possible explanation to the mechanism of action of the copper intra-uterine device. The molecular forms of the estrogen receptors were characterized by their sedimentation patterns.

EXPERIMENTAL

Materials

[2,4,6,7-^{3}H]-Estrone (98.5 Ci/mmol), [2,4,6,7-^{3}H]-estradiol (100.9 Ci/mmol), [2, 4, 6, 7-^{3}H]-estriol (84.8 Ci/mmol and [^{14}C]-formaldehyde (46.0 mCi/mmol) were obtained from New England Nuclear. They were stored in a benzene-ethanol mixture (9:1) at 0–4°C. Purity was checked by t.l.c. on silica gel using a solvent system consisting of benzene:ethylacetate:acetic acid (60:40:0.5). Diisopropylfluorophosphate (DFP) was purchased from Fluka AG.

Measurement of radioactivity

β-Scintillation counting was carried out in a liquid scintillation counter (LKB Wallac 81,000). Aqueous samples were transferred into counting vials containing 10 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 on a mixer and the vials were left for at least 4 h in the dark before counting.

Preparation of uterine supernatants

Normal uterine samples were obtained from patients immediately after hysterectomy and were considered pre-menopausal. The uterus was cooled on ice immediately after hysterectomy and the myometrial tissue sample (2–5 g) was excised and placed in 40 mM Tris-HCl buffer, pH 7.4 (T-buffer), 4 ml/g tissue. The tissue was 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 homogenate was centrifuged at 100,000 *g* for 1 h. The supernatant was used as the myometrial cytosol. The protein concentration of the cytosol was determined by the method of Lowry[14].

Quantitation of estrogen-binding

Aliquots of myometrial cytosol (0.1 ml, 7–9 mg protein/ml) were incubated for 24 h at 0°C, 80 min at 20°C and 60 min at 30°C with various concentrations of tritiated estrogens (0.05–4.0 nM) plus T-buffer (0.2 ml), with or without a 100-fold excess of estradiol or the same unlabeled estrogen. The ^{3}H-ligand receptor complex was measured by using a dextran-charcoal suspension (0.5% activated charcoal and 0.05% dextran in T-buffer). The dextran-charcoal suspension (0.5 ml) was added, mixed, and incubated for 30 min at 0°C before centrifugation for 10 min at 3000 *g* to remove free estrogen. 0.5 ml aliquots of the supernatant were used for measurement of radioactivity. The calculation of dissociation constants and binding capacity was done graphically from Scatchard plots of the saturation analysis data [15].

Determination of association rate constants

Association was studied at 0°C and 20°C for estrone, estradiol and estriol and at 30°C for estradiol only. Cytosol (2.0 ml) was transferred into a tube containing a radioactive ligand solution (4.0 ml) in T-buffer. At different time intervals an aliquot (0.3 ml) was removed and transferred immediately to a tube containing a 100-fold excess of nonradioactive ligand in 0.1 ml T-buffer at 0°C in order to dilute the unbound radioactive ligand and to stop the formation of the radioactive complex. The dextran-charcoal suspension was used to remove free estrogen. The association rate constants (k_1) were calculated from the linear portions of the second order plots according to Sanborn *et al.*[16]. The reaction is plotted according to the equation $[2.3/([E]_0 - [R]_0)] \log([E]_t/[R]_t) = k_1 t + [2.3/([E]_0 - [R]_0)] \log([E]_0 - [R]_0)$. E refers to [^{3}H]-estrogen concentration, R to receptor binding site concentration as determined from the Scatchard plot, t to time, and the subscripts 0 and t to time 0 and t respectively.

Sucrose gradient centrifugation

[^{3}H]-estrogen to a final concentration of 7.5 nM in 0.5 ml T-buffer was added to 0.5 ml of cytosol (7–9 mg protein/ml) and incubated, unless otherwise noted, for 24 h at 0°C. To assess nonspecific binding parallel samples were treated with a 100-fold excess of non-radioactive estrogen. After incubation, unbound and nonspecific radioactivity was removed during a 1 h treatment with a dextran-charcoal (5% activated charcoal and 0.5% dextran) suspension and 300 μl of the estrogen-charged receptors sedimented through a 2.5–15% sucrose gradient prepared in 40 mM T-buffer. Bovine serum albumin (sed. coeff. 4.6 *s*) was labelled with ^{14}C according to the method described by Rice and Means [17] and used as internal marker. Gradients were centrifuged in a MSE superspeed centrifuge at 40,000 rev/min (175,000 *g*) for 16 h. Fractions of 3 drops (≈ 140 μl) were collected for measurement of radioactivity.

Estrogen metabolism

Aliquots of myometrial cytosol were incubated for 24 h at 0°C and 60 min at 30°C with the highest concentration of tritiated estrogen used in the binding assay. After completed incubation the cytosols were extracted with ether. T.l.c. of the ether extracts, performed as described under Materials, was used to determine metabolic conversion of the estrogens.

RESULTS

The stability of the estrogen-receptor complex

The myometrial cytosol was added to a solution containing estrone, estradiol or estriol in T-buffer at 20°C (Fig. 1). The time course for the formation on the estrogen-receptor complex was followed by stopping the reaction at various time-intervals between 0 and 240 min by transfer of aliquots of the equilibrium mixture to a tube containing a 100-fold excess of nonradioactive ligand in T-buffer at 0°C. A dextran-charcoal suspension wass added to remove free estrogen. The equilibrium is reached within 1 h at

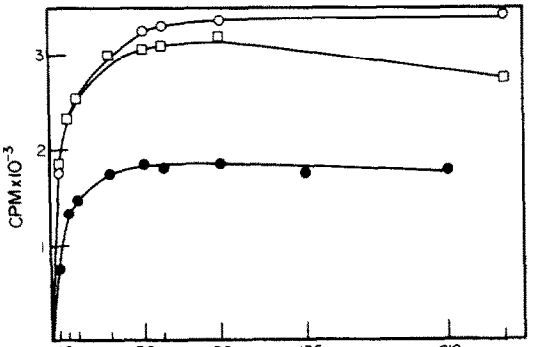

Fig. 1. Time course for the formation of estrogen-receptor complex. Cytosol was incubated at 20°C in the presence of tritiated estrone (□—6.9 nM), estradiol (○—3.4 nM) or estriol (●—8.5 nM) in T-buffer. Aliquots of 300 μl were removed after different intervals of time to a tube containing a 100-fold excess of nonradioactive ligand in T-buffer at 0°C. Free estrogen was removed with a dextran-charcoal suspension. The values have been corrected for nonspecific binding.

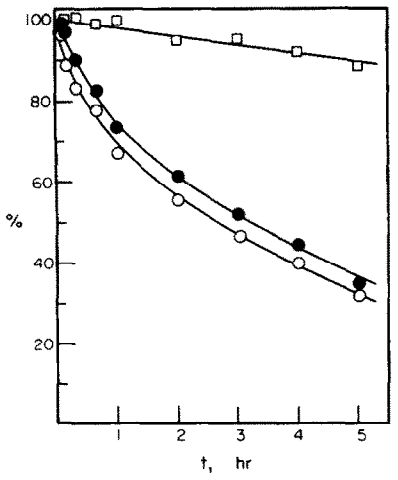

Fig. 2. Stability of the estrogen-receptor complex at 30°C. Cytosol was incubated at 0°C for 24 h in the presence of tritiated estrone (●—6.9 nM), estradiol (□—3.4 nM) or estriol (○—8.5 nM) in T-buffer. The temperature was raised to 30°C and aliquots of 200 μl were removed after various intervals of time and transferred to 0°C. Free estrogen was removed with a dextran-charcoal suspension. The values have been corrected for nonspecific binding.

20°C and maintained. At 30°C the equilibrium for estrone and estriol was not reached because of the lability of the receptor. The time course curves for estrone and estriol (data not shown) showed a maximum after 5–15 min after which the amount of bound steroid decreased fast to the level of unspecific binding. The presence of estradiol seemed to protect the receptor from inactivation at 30°C. This was also indicated when myometrial cytosol was incubated with high concentrations of either estrone, estradiol or estriol for 24 h at 0°C to reach equilibrium after which the temperature was raised to 30°C. At different time intervals an aliquot was removed and transferred immediately to a tube at 0°C. The dextran-charcoal suspension was used to remove free estrogen. The estrogen receptor is quite stable for hours in the presence of estradiol while an inactivation seems to occur with estrone or estriol in the medium (Fig. 2). Hence no binding or association studies with estrone and estriol could be done at 30°C.

Binding studies with radioactive ligands

The myometrial cytosol was incubated with an increasing concentration of ^{3}H-ligand. The dissociation constants at equilibrium, K_D, for estrone, estradiol and estriol were determined according to Scatchard [15] and checked statistically according to the method of least squares. The analysis was done at 0, 10 and 20°C for all the steroids and at 30°C for estradiol only. The influence of Cu^{2+} ions on the affinity of the receptor for estrogens and on the number of binding sites was also tested. Figure 3 shows the results from the binding study at 20°C. The complete data are presented in Table 1. The binding affinity at all temperatures tested was highest for estradiol and lowest for estriol (0°C) or estrone (20°C). An increase in incubation temperature was always followed by an increase in dissociation constant. In the presence of 10 or 20 μM Cu^{2+} ions an obvious decrease in binding affinity and binding capacity was observed for the three steroids.

Association reaction

Association was studied at 0 and 20°C for estrone, estradiol and estriol and at 30°C for estradiol only. The effect of Cu^{2+} ions was also tested. Figure 4 shows the data obtained for 20°C and Table 1 sums up the results for all temperatures. The dissociation rate constants have been calculated from the dissociation constants and the association rate constants.

Sedimentation characteristics

The sucrose gradient analysis was done with estrogen-receptor complexes in cytosol incubated under exactly identical conditions as used for the binding studies. No temperature-dependent *in vitro* receptor-

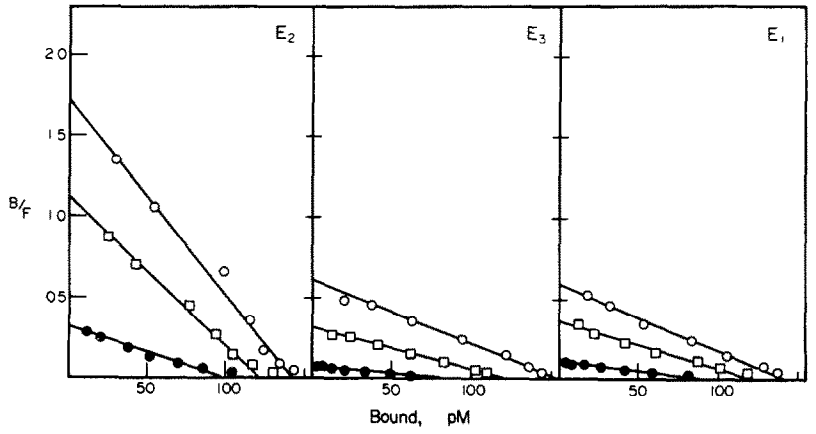

Fig. 3. Scatchard plot of [^{3}H]-estrogen-binding by cytosol at 20°C in the absence and presence of Cu^{2+}-ions. 100 μl aliquots of myometrial cytosol (8.9 mg protein/ml) were incubated for 80 min with various concentrations of tritiated estrone (E_1), estradiol (E_2) and estriol (E_3) with or without a 100-fold excess of unlabeled steroid in 200 μl T-buffer. Dextran-charcoal was used to adsorb free estrogen following equilibrations. (○) no, (□) 10 μM and (●) 20 μM Cu^{2+}.

Table 1. Dissociation constants based on data from the Scatchard plots and association rate constants determined from the initial linear portions of second order plots under different experimental conditions. The dissociation rate constant has been calculated from $K_D = k_{-1}/k_{+1}$

Temp °C	Cu^{2+} μM	Dissociation constant × 10^{10} K_D (M)			Association rate constant × 10^{-5} k_{+1} ($M^{-1}s^{-1}$)			Dissociation rate constant × 10^5 k_{-1} (s^{-1})		
		Estrone	Estradiol	Estriol	Estrone	Estradiol	Estriol	Estrone	Estradiol	Estriol
0	0	0.76	0.38	1.3	2.1	2.8	0.79	1.6	1.1	1.1
	10	2.1	0.53	2.3	2.0	2.7	0.91	4.3	1.4	2.1
	20	4.1	0.96	5.1	1.7	2.8	0.54	7.0	2.7	2.7
10	0	1.4	0.61	1.7						
20	0	2.4	0.81	2.1	10.3	12.3	5.1	24.8	10.0	10.7
	10	3.2	1.1	3.8	11.6	12.4	5.0	37.1	13.4	19.0
	20	8.9	3.1	12.3	6.5	7.8	3.4	57.9	24.2	41.8
30	0		1.4			39.5			54.9	
	10		2.0			35.8			70.2	
	25		7.1			9.5			67.3	
	40		17.0							

transformation was observed, but cytosols incubated at 0 and 30°C showed an estradiol-receptor complex with a sedimentation coefficient of 4 *s* (Fig. 5). A 4 *s* and 8 *s* estradiol-receptor complex appeared only if DFP was added to the buffer prior to homogenization and almost solely a 8 *s* estradiol-receptor complex is obtained if both DFP and estradiol are added to the buffer before homogenization (Fig. 6).

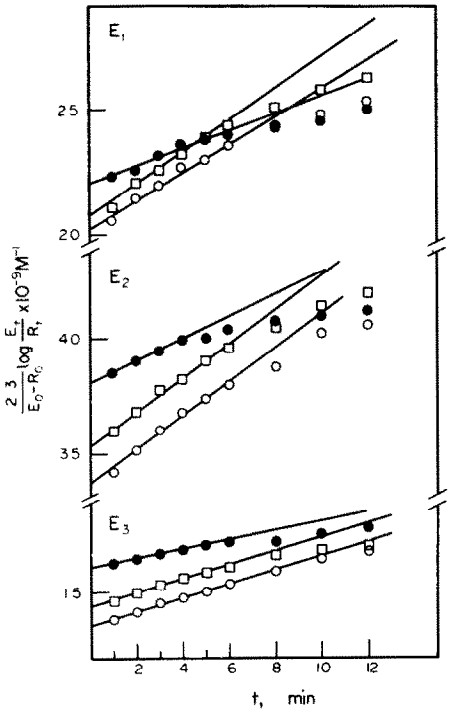

Fig. 4. Association of [^{3}H]-estrogen with cytosol receptor under second order conditions at 20°C in the absence and presence of Cu^{2+}-ions. A 2.0-ml aliquot of cytosol was pipetted into a tube containing tritiated estrone (E_1), estradiol (E_2) or estriol (E_3) in 4.0 ml T-buffer. The final concentration of the steroids was 1.4, 0.6 and 2.1 nM for E_1, E_2 and E_3 respectively. At various intervals of time aliquots of 300 μl were taken and pipetted into a tube containing a 100-fold excess of unlabeled steroid in 100 μl T-buffer at 0°C. Dextran-charcoal was used to adsorb free estrogen. (○) no. (□) 10 μM and (●) 20 μM Cu^{2+}.

The sedimentation profiles of the different estrogen-receptor complexes were analysed without DFP after an incubation at 0°C. The same 4 *s* estrogen-receptor complex was observed for both estrone, estradiol and estriol (Fig. 7). When Cu^{2+} ions were added to the incubation mixture the reduction in binding capacity was verified that was observed in the binding studies. The sedimentation coefficient for the receptor-complex in the presence of Cu^{2+} ions is almost the same, although a slight increase in sedimentation rate is sometimes apparent. In case the cytosol was stored at 0°C for 24 h before use an obvious change in the sedimentation profile of the estrogen-receptor

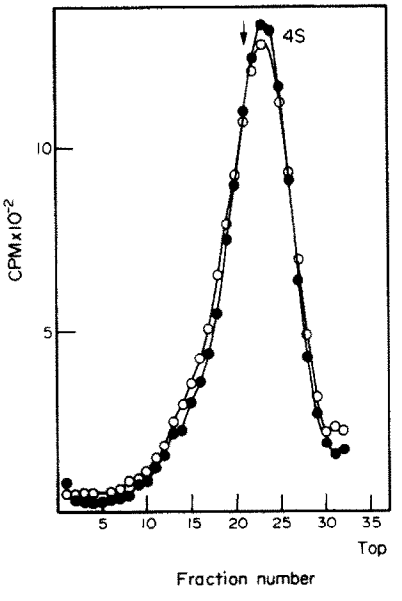

Fig. 5. Sedimentation analysis of the estrogen receptor in human myometrial cytosol after incubation at 0°C (○) and 30°C (●). The tissue was homogenized in T-buffer and the cytosol was equilibrated with 7.5 nM [^{3}H]-estradiol for 4 h at 0°C and 30 min at 30°C. Dextran-charcoal was used to adsorb free estradiol. Aliquots of 300 μl were layered on a 2.5–15% sucrose gradient in T-buffer (4.8 ml). The samples were centrifuged at 175,000 *g* for 16 h at 4°C. Non-specific binding was 7% of the bound radioactivity. (↓)—BSA.

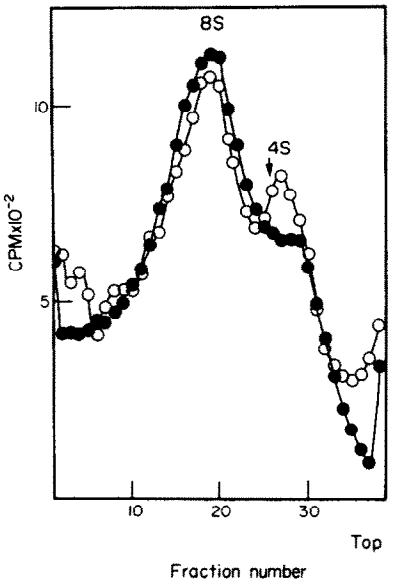

Fig. 6. Sedimentation of the human estrogen receptor isolated with DFP. Human myometrial tissue was homogenized in T-buffer containing 10 mM DFP and 1 mM dithiothreitol (○) or in the same buffer plus 5 nM [^{3}H]-estradiol (●). In the former homogenization [^{3}H]-estradiol was added up to 5 nM after the cytosol preparation and the mixture incubated for 120 min at 0°C. Dextran-charcoal was used to adsorb free estradiol and reduce nonspecific binding. Aliquots of 300 μl were layered on a 2.5–15% sucrose gradient in T-buffer. Samples were centrifuged at 175,000 g for 16 h at 4°C. Nonspecific binding was 3% of the bound radioactivity. (↓)—BSA.

complex is observed in the presence of Cu^{2+} ions. Instead of a 4 s receptor-complex, which represents the main peak in the presence of no Cu^{2+} ions, a 5 s complex appeared with marked shoulders at 4 and 7 s (data not shown).

Estrogen metabolism

The incubation mixture used in the binding studies was checked for metabolic interconversion of the estrogens during the duration of the experiment. According to the results from typical chromatographic distributions of radioactivity after thin-layer chromatography on silica gel no significant metabolization of any of the estrogens occurred. About 96–98% of the steroids were recovered unchanged after the experiment.

DISCUSSION

In this communication the binding of estrone, estradiol and estriol to the high-affinity estrogen receptor in human myometrial cytosol has been analyzed. It has been previously demonstrated that the estrogen receptor in rat uterine cytosol can bind all three estrogens, but with a difference in affinity [11], and that they all can induce the synthesis of an estrogen specific uterine protein [12]. Cytoplasmic binding of estrone in human endometrial tissue has also been observed [9]. In our study the affinity of the estrogens to the receptor at 0°C decreased in the following order, estradiol (K_D 0.38 × 10^{-10} M) > estrone (K_D 0.76 × 10^{-10} M) > estradiol (K_D 1.33 × 10^{-10} M). This is in agreement with previous results which show that estrone competes more strongly than estriol with [^{3}H]estradiol for binding to the myometrial estrogen-binding protein [18]. However at 20°C estriol is bound slightly stronger than estrone. The dissociation constant for estradiol is in the range of reported values [10, 19] although considerably higher values have been obtained [18, 20]. We have

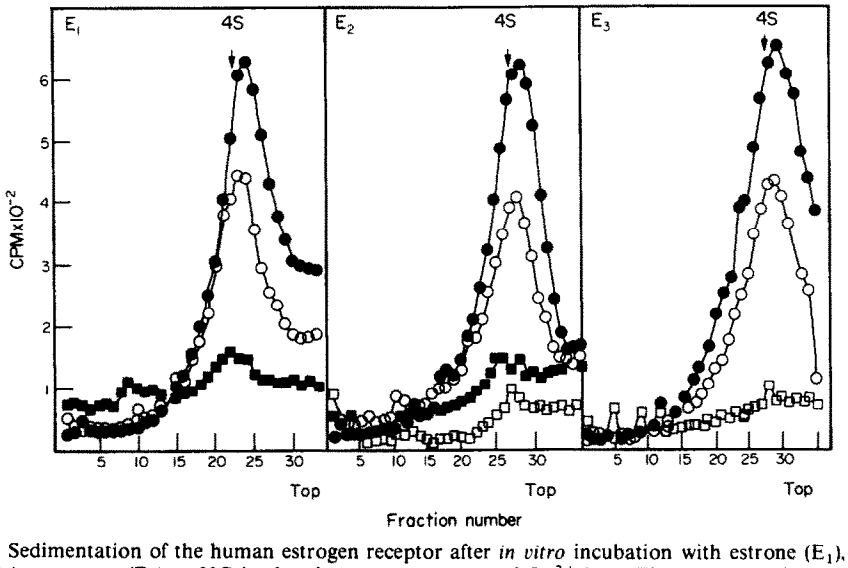

Fig. 7. Sedimentation of the human estrogen receptor after *in vitro* incubation with estrone (E_1), estradiol (E_2) or estrone (E_3) at 0°C in the absence or presence of Cu^{2+}-ions. The tissue was homogenized in T-buffer and the cytosol was equilibrated with 7.5 nM [^{3}H]-estrogen for 24 h at 0°C. Dextran-charcoal was used to adsorb free estrogen. Cytosol equilibrated with 7.5 nM [^{3}H]-estrogen (●), with 7.5 nM [^{3}H]-estrogen plus 33 μM Cu^{2+} (○), with 7.5 nM [^{3}H]-estrogen plus 333 μM Cu^{2+} (■) or with 7.5 nM [^{3}H]-estrogen plus 100-fold excess of unlabeled estrogen (□). Samples were centrifuged at 175,000 g for 16 h at 4°C. (↓)—BSA.

not found previously published values for the binding of estrone and estriol to human myometrial receptor. The rat uterine estrogen-binding protein binds estriol more firmly than estrone and the dissociation constants are more than 10-fold higher compared to our values [11, 12].

The sucrose gradient analysis of the estrogen-receptor complex indicates that the receptor under our conditions sediments at 3.9–4.0 *s* independent of the estrogen used and incubation temperature. No temperature dependent transformation of the estrogen-receptor complex is involved, which implies that the same form of the receptor has been observed during the experiments. Recently Notides *et al.*[8], however, found an increase in sedimentation rate with increasing temperature. For all the estrogens the association rate constant and the dissociation constant increases with temperature. Thermodynamic parameters can be calculated from the data presented in Table 1. The activation energies as calculated from the rate of association at 0 and 20°C are 12.5, 11.8 and 14.8 kcal mol^{-1} for estrone, estradiol and estriol respectively. As the association constant decreases with increasing temperature, ΔH is negative, −9.1, −7.1 and −3.6 kcal mol^{-1} for estrone, estradiol and estriol respectively. The entropy changes, on the other hand are relatively large and positive, 13, 22 and 32 e.u. for estrone, estradiol and estriol respectively, while ΔG 293°C for the estrogens is approximately −13 kcal mol^{-1}. The change in free energy and the activation energy are in good agreement with the reported values for the estradiol receptor complex in rat uterine cytosol [16]. On the contrary we observe a positive entropy change which suggests a conformational change in the protein most likely accompanied by an increase in entropy of the surrounding water molecules. As hydrophobic groups combine, the ordered water layers are liberated into a state of pure water and there is an increase in entropy and a decrease in free energy. The big difference in the entropy change observed for the different estrogens could be used for additional speculations regarding their estrogenic action.

The sedimentation characteristics of the cytoplasmic estrogen receptor from human myometrium have indicated the presence of 7–8 *s* and 4–5 *s* receptor forms [9, 10]. The most extensive analyses has recently been published by Notides *et al.*[8]. According to their results isolation of the estrogen receptor without DFP in the medium yields predominantly a proteolytic fragment of the receptor with a sedimentation coefficient of 3 *s*. In the presence of DFP 8 *s*, 5 *s* and 4 *s* estrogen binding proteins were observed. Uteri from post-menopausal patients were used. Under our conditions, when all the uteri were from pre-menopausal patients, no indication of the occurrence of a proteolytic fragment of the receptor which sediments at 3 *s* has been found. A 4 *s* estrogen-binding protein is observed even in the presence of DFP and no temperature dependent conversion of the 4 *s* estrogen-binding protein to a 5 *s* form was found after incubation at 30°C and centrifugation at low temperature. Notides *et al.*[8] found an increase in the sedimentation coefficient as the temperature at which the centrifugation was carried out increased which is considered as an indication of a rapid 4 *s* ⇄ 5 *s* association–dissociation equilibrium. Our data does not exclude this possibility. We observe a 4 *s* and an 8 *s* estrogen binding protein only when DFP has been added to the buffer before homogenisation. The transformation of the 8 *s* receptor to a 4 *s* receptor depends most likely upon the activity of a protease. It is presumably not completely inhibited in the presence of DFP and has a higher catalytic activity towards the uncomplexed estrogen-binding protein. Notides *et al.* [21] have characterized a human uterine protease by using rat uterine estrogen receptor as a "substrate", but activity (8 *s* → 4.5 *s*) was preferentially observed when estradiol was associated to the receptor.

Addition of low concentrations of Cu^{2+} ions to the myometrial cytosol had a significant effect on the formation of the estrogen-receptor complex. The dissociation constants for both estrone, estradiol and estriol increased with the added Cu^{2+} ion concentrations at all temperatures tested. The effect of Cu^{2+} ions on the association rate constant was negligible at 0°C which indicates that the rate of dissociation increased slightly. At 20°C no effect on the association rate constant was observed in the presence of 10 μM Cu^{2+} but the presence of 20 μM Cu^{2+} reduced the rate constant by 40%. In addition the presence of Cu^{2+} ions has an obvious effect on the estrogen-binding capacity which decreases remarkably with increasing Cu^{2+} ion concentration. The results from the sedimentation analysis in the presence of Cu^{2+} ions confirm the observation. The same sedimentation coefficient (4 *s*) is obtained although the binding of estrogen to the receptor is reduced. Occasionally an increase in the sedimentation coefficient was observed in the presence of Cu^{2+} ions, especially if the cytosol was frozen and stored before the experiment. Obviously the presence of Cu^{2+} ions affects both the affinity and the estrogen binding capacity of the estrogen binding protein. Most likely the functioning of SH groups is impaired as the receptor binding sites are protected by dithiothreitol [22]. Cu^{2+} ions have also been shown to act as competitive inhibitors of the binding of estradiol to the estrogen-receptor protein in rabbit uterine cytosol [23]. The sedimentation pattern showed aggregation, but the analysis was obviously performed only at relatively high Cu^{2+} ion concentrations. An increase in the dissociation constant but with no effect on binding sites has as well been reported for the progesterone-binding protein in human myometrium in the presence of Cu^{2+} ions [24].

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