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Oestrogen Receptors in Mammalian Vas Deferens

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Summary. Cytosolic oestrogen receptors were measured in bovine and rat vas deferens of immature and mature animals. The bindings in the mature animals of both species were similar and specific. Furthermore, our physicochemical studies suggest characteristics identical to those obtained for oestrogen receptors in other target tissues. Our receptor measurements on the calf and the immature rat reveal significantly lower binding capacities than those obtained in the mature animals.

Key words: Vas deferens, Oestrogen receptors.

Introduction

Recently, circumstantial evidence has been presented for oestrogen responsiveness of the vas deferens (VD).

In orchiectomized male beagles, Senge et al. [11] demonstrated 17β-oestradiol-induced hypertrophy or hyperplasia of VD. Evidence for rodent VD as a primary locus of oestrogen action has been reported by Stumpf and Sar [12]. In this autoradiographic study, the epithelial cells of VD were found to be unlabelled, whereas subepithelial connective tissue cells showed nuclear concentration of tritiated oestrogen. Nuclei of smooth muscle cells and some fibroblasts within the muscularis were weakly labelled. The subcellular distribution of the radioactive hormone was assumed to be indicative of the presence of oestrogen receptors.

In an attempt to further substantiate the oestrogen sensitivity of VD, we investigated biochemically the tissue for the presence of oestrogen receptors, key proteins in the mechanism of hormone action and reliable markers of oestrogen responsiveness.

* This paper contains essential parts of cand. med. M. Haupenthal's dissertation (Medical Faculty of the University of Düsseldorf)

Materials and Methods

(6, 7-^{3}H) oestradiol-17β (54 Ci/mmol) was obtained from Amersham Buchler. Radioinert diethylstilboestrol and dexamethasone were purchased from Sigma. All other unlabelled hormones were supplied by Calbiochem. Serva served as a source of Norit A, while Dextran T-500 was obtained from Pharmacia. Agar purum and bovine serum albumin were purchased from Behring. Quickszint® 212 was supplied by Zinsser. All other chemicals were commercial preparations of analytical grade.

Animals

Vasa deferentia (VD) from bulls and calves were collected at the local slaughter house. Rodent VD tissue was obtained from Wistar rats (120–230 g).

Handling of VD

Immediately after resection the vasa were cooled on crushed ice, cleaned from adherent tissue and then immersed in liquid nitrogen. Collected VD were stored at −90 °C for up to one week in the Laboratory.

Cytosol Preparation

Specimens were pulverized for 45 s under liquid nitrogen cooling using a Micro-Dismembrator (Braun-Melsungen). Subsequently, the powder was extracted at 0 °C with three volumes of ice-cold Tris buffer A (10 mmol/l Tris-HCl, 1 mmol/l NaN_3, pH 7.4; for agar gel electrophoresis) or Tris buffer B (10 mmol/l Tris-HCl, 1.5 mmol/l Na_2EDTA, 10 mmol/l monothioglycerol, pH 7.4; for all other assays). The homogenate was centrifuged at 105,000 x g (0 °C) for 45 min and the supernatant (referred to as cytosol) carefully removed with a chilled Pasteur pipette.

Dextran-Coated Charcoal (DCC) Assay

This method was based on Korenman's [6] procedure. Cytosols, prepared in Tris buffer B, were reacted with radiolabelled 17β-oestradiol ($(^3H)E_2$) for 4 h at 0–4 °C, either alone or in the presence of radioinert competitive hormones. Details of individual experimental designs can be seen by reference to the figures and tables. Following

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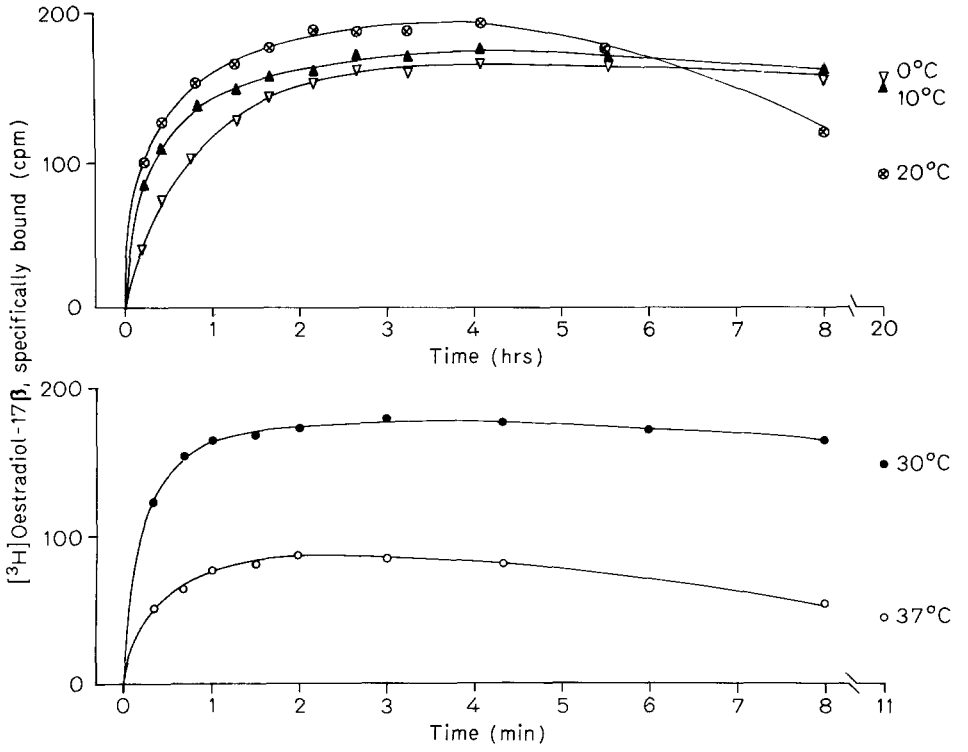

Fig. 1. Time and temperature dependence of the formation of the cytoplasmic (^{3}H) E_2-EB complex. Cytosols were reacted with 10 nmol/l (^{3}H)E_2 ± 1 μmol/l DES. Binding was measured by dextran-coated charcoal (DCC) assay

incubation, the samples (0.1 ml) were combined with 0.25 ml 5 g/l Norit A, 0.5 g/l Dextran T-500 in Tris buffer B. The mixture was incubated at 0–4 °C for 10 min, and then centrifuged at the same temperature for 5 min at 12,000 rev/min in an Eppendorf centrifuge, model 5200. Portions (0.1 ml) of the supernatant fluid were transferred into scintillation vials together with 5 ml of Quickszint® 212 for counting of radioactivity.

Agar Gel Electrophoresis

The electrophoretic procedure was a slight modification [3] of the technique originally described by Wagner [15].

Sucrose Gradient Centrifugation

The assay procedure closely paralleled that originally described by Toft and Gorski [13]. Briefly, aliquots (0.3 ml) of cytosol, prepared in Tris buffer B, were reacted with 10 nmol/l tritiated 17β-oestradiol for 4 h at 0–4 °C, either alone or in the presence of unlabelled diethylstilboestrol (DES) at 1 μmol/l. Portions (0.8 ml) of 5 g/l Norit A, 0.5 g/l Dextran T-500 in Tris buffer B were centrifuged for 2 min at 12,000 rev/min in an Eppendorf centrifuge. The supernatants were discarded. The labelled cytosols were then mixed with the obtained charcoal pellets and processed as described under 'DCC Assay'. Subsequently, the supernatant fluids (0.2 ml portions) were assayed for receptor binding activity by layering over linear 50–200 g/l sucrose gradients in Tris buffer B containing 10% (v/v) glycerol. The gradients were centrifuged in a Beckman L8–80 preparative ultracentrifuge for 2 h at 80,000 rev/min (0 °C), using a VTi-80 (vertical tube) rotor. After centrifugation, 3-drop fractions were collected (Beckman gradient fraction system, LKB drop former) into scintillation vials. Quickszint® 212 (5 ml) was added to each fraction and the radioactivity measured.

Quality Control of Receptor Assays

Quality of receptor assays was routinely controlled using lyophilized reference samples [4].

Protein Determination

Cytosol protein was estimated by Lowry's method [7] using bovine serum albumin as a standard.

Results

Vas deferens (VD) cytosol was investigated for the presence of oestrogen receptors (oestrophilin) by kinetic and equilibrium binding studies, specificity analyses, enzymatic digestion experiments, agar gel electrophoresis, and sucrose gradient centrifugation. Since describing oestrophilin in VD biochemically for the first time, in one of the species investigated (bull) the near complete spectrum of conventional methods for cytoplasmic receptor characterization was used.

After having exemplarily proven the receptor nature of the oestrogen binders (EB) in bull VD, we refrained from determining all these binding parameters for rat VD oestrophilin too, but restricted binding analysis to standard receptor assay procedures.

1. Bull Vas Deferens

1.1. Physicochemical Binding Parameters. The effect of time and temperature on the formation of the cytoplasmic 17β-oestradiol (E_2)-oestrogen binder (EB) complex is shown in Fig. 1. Specific binding of the hormone turned out to be a rapid temperature dependent reaction. At 0 °C maximal binding was obtained in about 3 h and then remained constant for at least 17 h. At 10 °C and 20 °C binding equilibrium was reached earlier. However, at 20 °C stability of the EB

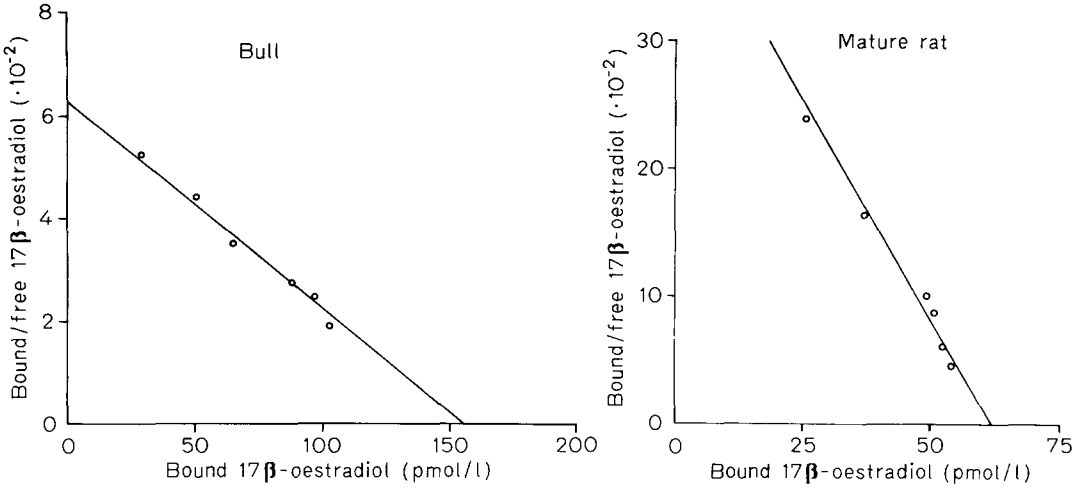

Fig. 2. Representative Scatchard plots of titration data for cytoplasmic EB of bull and mature rat VD. Cytosols, prepared in Tris buffer B, were reacted with ~0.5–5 nmol/l (^{3}H)E_2 ± 1 μmol/l DES at 0–4 °C for 4 h. Specific binding was assessed by DCC procedure

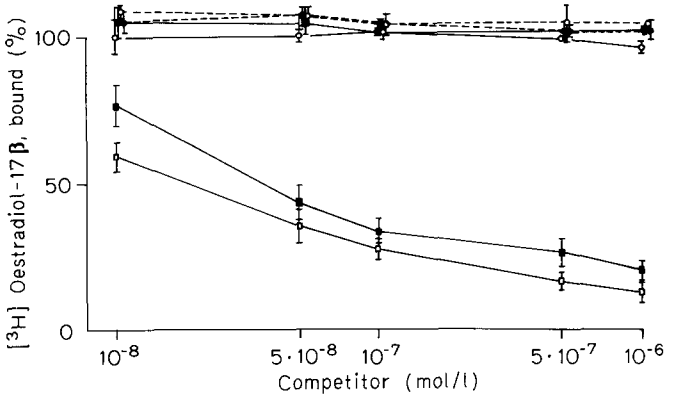

Fig. 3. Ligand specificity of E_2 binding by cytosol from bull VD. Cytosols, prepared in Tris buffer B, were incubated with 8 nmol/l (^{3}H)E_2 for 4 h at 0–4 °C, either alone or in the presence of various unlabelled hormones at 0.01–1 μmol/l (•——•) = aldosterone; •– – –• = dexamethasone. ○——○ = testosterone; ○– – –○ = progesterone; ▪——▪ = DES; □ —□ = E_2). Specific binding was assessed by DCC procedure. (^{3}H)E_2 bound in the absence of any radioinert competing hormone is arbitrarily set to 100%

Table 1. Chemical nature of the E_2 binding entities of bull VD. Cytosols were either heated for 60 min at 45 °C or incubated for 30 min at 0–4 °C with no additions or various enzymes at 1.25 mg/ml. Subsequently, cytosols were labelled with 10 nmol/l (^{3}H)E_2 for 4 h at 0–4 °C, either alone or in the presence of 1 μmol/l DES. Specific binding was assessed by DCC procedure

Treatment	(^{3}H)Oestradiol-17β bound [%]
No treatment	100.0
Hyaluronidase	97.7
RN-ase	96.1
DN-ase	92.3
Pronase	< 1.0
Heat	4.0

complexes significantly decreased as the time of incubation was prolonged. At 30 °C and 37 °C the system exhibited maximal binding within several minutes. Due to an apparent thermolability of EB, binding capacity at 37 °C was found to be drastically reduced as compared to binding at lower temperature.

A Scatchard plot [10] of equilibrium binding data can be seen in Fig. 2 (*left panel*). As indicated by the resulting straight line a single class of binding sites seemed to be present. The apparent dissociation constant (K_d) was found to be 1.9 ± 0.5 x 10^{-9} mol/l ($\bar{x} \pm s_{\bar{x}}$, $n = 5$). The maximal binding capacity (MBC) amounted to 23.7 ± 3.5 fmol/mg cytosol protein.

1.2. Ligand Specificity. The relative affinities of several steroid hormones for the cytoplasmic EB, as measured by competition studies, are summarized in Fig. 3. The ligand specificity for binding to these sites clearly indicated a requirement for oestrogens. The binding of radiolabelled E_2 was inhibited by radioinert E_2 as well as the nonsteroidal compound diethylstilboestrol (DES). All other steroids tested were not competitive.

1.3. Chemical Nature of Cytoplasmic EB. As can be seen in Table 1, EB were sensitive to the proteolytic attack of pronase but turned out to be fairly resistant to the action of hyaluronidase, RN-ase, and DN-ase. Heating of the cytosols for 60 min at 45 °C resulted in dramatic decrease of the ability for specific oestrogen binding.

1.4. Agar Gel Electrophoresis. A representative electrophoretic separation experiment is shown in Fig. 4 (*upper panel*). It can be clearly seen that specific EB migrate to the well-known anodic receptor region of the gel.

1.5. Sucrose Gradient Centrifugation. In the study illustrated in Fig. 5 (*upper left panel*), cytoplasmic (^{3}H) EB were

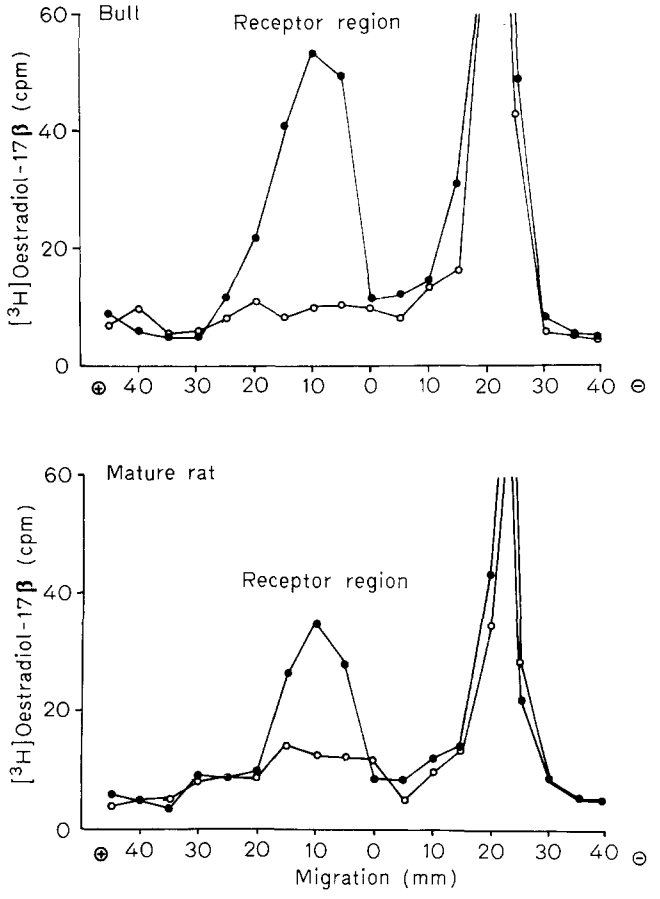

Fig. 4. Electrophoretic separation of cytoplasmic EB from bull and adult rat VD. Cytosols, prepared in Tris buffer A, were reacted with 10 nmol/l (^{3}H)E_2 for 4 h at 0–4 °C, either alone (•——•; total binding) or in the presence of a 100-fold excess of unlabelled DES (o——o; nonspecific binding). Conditions of agar gel electrophoresis: 50 µl portions of labelled cytosols applied to 10 g/l agar gel; 130 mA; 3–4 °C (gel temperature in the receptor region); 5 mm sections counted after in-vial combustion

separated on low ionic strength sucrose gradients. In the resultant pattern, the predominant radioactive complex is a ~9 S binder with a small shoulder in the 4–5 S region of the gradient. The peaks obviously represent saturable steroid binding sites for binding is inhibited by an excess of unlabelled DES.

2. Calf Vas Deferens

In contrast to bull, in calf VD the cytoplasmic concentration of specific oestrogen binding components was found to be low.

2.1. Physicochemical Binding Parameters. By Scatchard's method [10] a single class of specific binding sites was identified (not shown). The K_d amounted to $1.7 \pm 0.4 \times 10^{-9}$ mol/l. MCB was calculated and found to be 5.9 ± 1.2 fmol/mg cytosol protein.

2.2. Sucrose Gradient Centrifugation. The isotopic pattern of (^{3}H) EB from calf VD cytosol is shown in Fig. 5 (*lower left panel*). In contrast to the upper left gradient profile (bull), cytoplasmic EB of calf VD sedimented in the 4 S region only. Moreover, the majority of 4 S EB are nonspecific binders.

3. Rat Vas Deferens

Specific EB could also be demonstrated in rat VD. Similar to EB in bovine VD, the oestrophilic macromolecules in rat VD changed in concentration during sexual maturation of the animals.

3.1. Physicochemical Binding Parameters. As is illustrated in Fig. 2 (*right panel*), for adult rat VD a single category of high affinity EB could be demonstrated in the cystol of mature (MR) as well as immature rats (IR) ($K_{d(MR)}$: $1.6 \pm 0.2 \times 10^{-10}$ mol/l, $MCB_{(MR)}$: 16.3 ± 1 fmol/mg protein; $K_{d(IR)}$: $2.5 \pm 0.9 \times 10^{-10}$ mol/l, $MBC_{(IR)}$: 9.9 ± 1.7 fmol/mg protein ($\bar{x} \pm s_{\bar{x}}$, $n = 4$)).

3.2. Agar Gel Electrophoresis. As can be seen in Fig. 4, the electrophoretic mobility of EB in rat and bull VD cytosol is identical.

3.3. Sucrose Gradient Centrifugation. The experiments illustrated in the upper and lower right panel of Fig. 5 demonstrate that the radiolabelled EB of MR and IR VD cytosol both sediment at about 8–9 S. The additional 4–5 S moiety in the upper right graph of Fig. 5 (MR) represents nearly exclusively nonspecific binding.

Discussion

Our data support the hypothesis that mammalian VD contains oestrogen receptors which seem to be identical with those previously found in well-known oestrogen target organs [5]. EB of VD are thermolabile proteins as are oestrogen receptors. The effect of time and temperature on formation (Fig. 1) and dissociation (unpublished results; this laboratory) of the E_2-EB complex is similar to that reported for oestrogen receptors [1, 2, 9]. The slightly higher binding capacity at 20 °C as compared to 0 °C may be due to exchange of endogenous oestrogens (either directly secreted by adrenal cortex or originating from androgens after peripheral aromatization) with the radioactive tracer. This phenomenon is also expected to occur at higher temperature. However, increasing thermolability obviously counteracts this effect.

The dissociation process was found to be biphasic at all temperatures studied (0–37 °C). Although long-known from the literature, the biphasic dissociation process of (^{3}H) E_2 – oestrogen receptor complexes lacks definite interpretation to date.

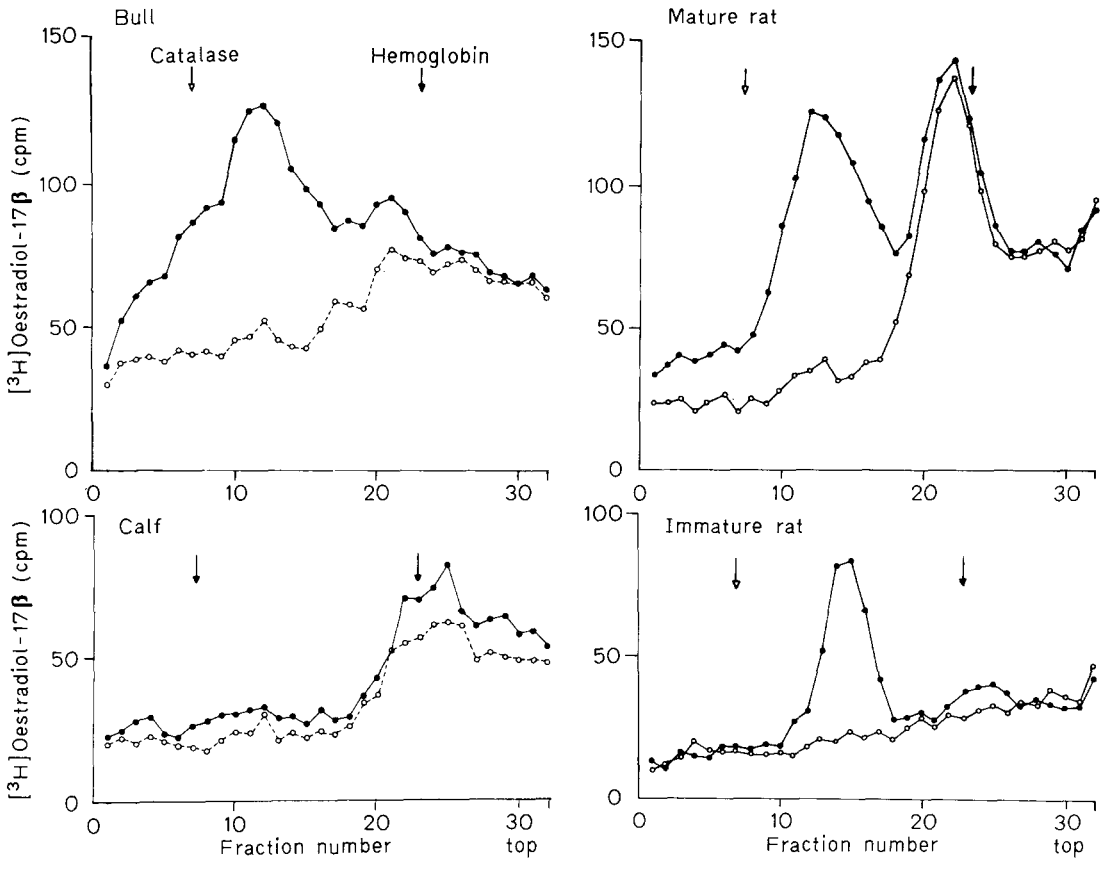

Fig. 5. Isotopic profiles of EB from bovine and rat VD separated by sucrose gradient centrifugation. Aliquots of cytosol, prepared in Tris buffer B, were reacted with 10 nmol/l (^{3}H)E_2 ± 1 μmol/l DES at low temperature. The cytosols were subjected to DCC treatment and then assayed for binding activity by layering over linear 50–200 g/l sucrose gradients in Tris buffer B. The gradients were centrifuged for 2 h at 80,000 rev/min (0 °C) using a VTi-80 rotor (Beckman ultracentrifuge L8-80). ●——● = total binding; ○——○ = nonspecific binding

As cytosols may be contaminated by serum proteins potentially binding oestrogens evidence for nonidentity of the described oestrophilic macromolecules with serum oestrogen binders is briefly discussed. Diethylstilboestrol used for determination of nonspecific binding in this study does not displace E_2 bound to sex hormone binding globulin (SHBG). Therefore, oestradiol eventually attached to SHBG appears as nonspecifically bound hormone in our receptor assay. Consequently, in control experiments with diluted (1:4, v/v) bull and rat serum no specific oestrogen binding could be demonstrated (unpublished results, this laboratory).

Moreover, migration of EB from start (in the middle of the gel) to the anodic region of agar gels definitely excludes interference with SHBG which is known to move to the cathode [15]. Further, E_2-SHBG complexes do not sediment at 8 S as does aggregated oestrophilin in low salt sucrose gradients.

In addition, the ligand specificities of oestrophilin and SHBG are different. The inability of testosterone to compete for 17β-oestradiol binding clearly argues against measuring SHBG. Besides, species like rat do not have SHBG. The high affinity of the oestradiol-binder interaction excludes the involvement of albumin in the binding reaction.

The K_d of the E_2-EB complex from mammalian VD cytosol (~2×10^{-10} to 2×10^{-9} mol/l) is strikingly similar to that of rat mammary gland (~8×10^{-10} mol/l), R 3230 Ac mammary adenocarcinoma (~1.2×10^{-9} mol/l) and endocrine dependent human breast cancer (~9×10^{-10} mol/l) [16, 17].

As has been demonstrated, maturity of the animals is accompanied by an increase in/or occurence of (bull VD) cytoplasmic receptor concentration. This may be related to the rise of blood oestrogen levels during sexual maturation of the animals, because oestrogens have been reported to stimulate their own receptor synthesis [8, 14]. However, a mature dependent change in vas deferens morphology should be considered as a possible explanation, too.

Unpublished data from this laboratory indicate that VD might not be an oestrogen target in all species. For an example, oestrogen-binding studies in beagle VD failed to clearly demonstrate the presence of cytoplasmic oestrogen receptors.