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ESTRADIOL AND PROGESTERONE RECEPTORS IN DOG PROSTATE CYTOSOL

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SUMMARY

When intact or castrated dog prostate cytosol is incubated with tritiated estradiol, high levels of saturable binding can be observed in the 3–4S region of sucrose gradients. Occasionally a shoulder is also seen in the 8S region. The estradiol binding is specific for natural and synthetic estrogens. It is of high affinity ($K_A = 2 \times 10^9\ M^{-1}$). The binding component also has a relatively high affinity for 5α-androstane-3β,17β-diol, a major metabolite of 5α-dihydrotestosterone in dog prostate cytosol. Estradiol and 5α-androstane-3β,17β-diol can displace each other in competition experiments and they both yield identical concentration of sites by Scatchard analysis. The concentration of estradiol binding sites is quite variable in prostates from 24 h castrated dogs and no apparent correlation can be found between the concentration of sites and prostate wt. By contrast in the prostates from intact dogs there is a highly significant inverse relationship between prostate wt. and concentration of estradiol binding sites.

The presence of progesterone receptors in dog prostate can also be demonstrated by sucrose density gradient analysis using the synthetic steroids R5020 (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione) and R1881 (17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one). The binding component sediments both in the 8S and 4S regions of sucrose gradients. It is present in low amounts under basal conditions. Five days after a single injection of estradiol valerate, the binding is increased about 10-fold. It is almost completely abolished by the addition of progesterone and various unlabeled synthetic progestins but only partly decreased by 5α-dihydrotestosterone. Relatively low and constant levels of progesterone receptors are observed in normal and hyperplastic prostates from adult dogs (30 ± 1 [S.E.M.] fmol/mg prot.)

INTRODUCTION

In the previous paper [1], we have shown that besides the androgen receptor, another binding protein is present in dog prostate cytosol. This protein binds 5α-androstane-3β,17β-diol and is saturated by low amounts of unlabeled diethylstilbestrol. This behaviour was suggestive of an estrogen receptor and this study was undertaken to ascertain the existence of such a receptor. Similar binding proteins have been described for rat [2], guinea-pig [3], human [4, 5] and baboon [6] prostates. This hypothetic estrogen receptor might have important implications in dog prostate since the combined administration of 5α-androstane-3α,17β-diol and estradiol has been shown to induce hypertrophy [7]. In view of the high levels of estradiol receptors in dog prostate revealed by these studies and of the increased estrogenic stimulation in hyperplastic prostates as evidenced by tissular levels of estradiol [8], we have undertaken also to look for the presence of progesterone receptor which appears to be a marker of estradiol action in many target tissues. Progestin binding components have been reported previously by various groups of workers [9–12] in the human prostate in which benign hyperplasia is a common feature of old age.

MATERIALS AND METHODS

Animals. Immature and mature dogs were obtained from Le Centre de Biomédecine, Laval University. They were either castrated 24 h before removing the prostate or in other cases they were used intact.

Steroids. [6,7-^{3}H]-Estradiol (44 Ci/mmol) was purchased from New England Nuclear Corp. and [6,7-^{3}H]-5α-androstane-3β,17β-diol (43 Ci/mmol) from Amersham/Searle Corp. [6,7-^{3}H]-R5020 (S.A. 56.5 Ci/mmol), [6,7-^{3}H]-R1881 (S.A. 55.5 Ci/mmol) and the corresponding unlabeled hormones were generous gifts from Dr. Jean-Pierre Raynaud, Centre de Recherches Roussel-Uclaf, Romainville, France. Medroxyprogesterone acetate was kindly supplied by UpJohn Company, D-norgestrel by Wyeth Company and cyproterone acetate by Schering A.G., Berlin.

Terminology: E_2, estradiol = 1,3,5(10)-estratrien-3,17β-diol; estrone = 3-hydroxy-1,3,5(10)-estratrien-17-one; 3β,17β-diol = 5α-androstane-3β,17β-diol; 3α,17β-diol = 5α-androstane-3α,17β-diol; DES = diethylstilbestrol; DHT = 17β-hydroxy-5α-androstan-3-one; R5020 = 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; R1881 = methyltrienolone = 17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one; D-norgestrel = 13-ethyl-17-ethinyl-17β-hydroxy-4-gonen-3-one; medroxyprogesterone acetate = 17α-acetoxy-6α-methylprogesterone; 19-nortestosterone = 17β-hydroxy-4-estren-3-one; cyproterone acetate = 6-chloro-17α-acetoxy-1,22-methylene-4,6-pregnadiene-3,20-dione.

19-Nortestosterone was purchased from Steraloids, Pawling, N.Y. and estradiol valerate (Delestrogen) in sesame oil from Squibb. Other radioinert steroids were bought from Sigma and Calbiochem.

RESULTS

Sucrose density gradient profiles of dog prostate cytosol incubated with tritiated estradiol shows a high level of binding in the 3–4S region (Fig. 1). This binding is almost completely abolished by low amounts of unlabeled estradiol or diethylstilbestrol but not by 5α-dihydrotestosterone or progesterone. The addition of unlabeled 5α-androstane-3β,17β-diol also results in significant reduction of estradiol binding. Occasionally with other dog prostates, a distinct shoulder could be observed in the 8S region of gradients (results not shown).

This binding of estradiol is of high affinity. The association constant (K_A) at 0°C was determined by Scatchard analysis in dog prostates with the charcoal assay and found to be 1.9 ± 0.2 (S.E.M.) $\times 10^9\ M^{-1}$ in castrated dogs ($n = 18$) and 2.1 ± 0.2 (S.E.M.) $\times 10^9\ M^{-1}$ in intact dogs ($n = 18$).

The binding specificity was studied by incubating a fixed concentration of [^{3}H]-estradiol and increasing concentrations of various unlabeled steroids (Table 1).

Table 1. Ligand specificity of [^{3}H]-estradiol in dog prostate cytosol

Steroid	Competition activity
Estradiol	100
Estrone	29
Diethylstilbestrol	24
Androst-5-ene-3β,17β-diol	23
5α-Androstane-3β,17β-diol	16
5α-Androstane-3α,17β-diol	1
5α-Dihydrotestosterone	<0.1
Cortisol	<0.1
Progesterone	<0.1
5α-Androstane-3α,17α-diol	<0.1

The order of competitor activity for [^{3}H]-estradiol binding was estradiol > estrone > diethylstilbestrol = androst-5-ene-3β,17β-diol > 5α-androstane-3β,17β-diol ≫ 5α-androstane-3α, 17β-diol > 5α-dihydrotestosterone. Progesterone, testosterone, cortisol and 5α-androstane-3α,17α-diol showed no significant competition at the highest concentration used.

In view of the relatively high competition activity of 5α-androstane-3β,17β-diol and taking into consideration that this steroid is a major metabolite of DHT [1, 13], we did further studies of the binding of this compound in prostate cytosol. Similarly to

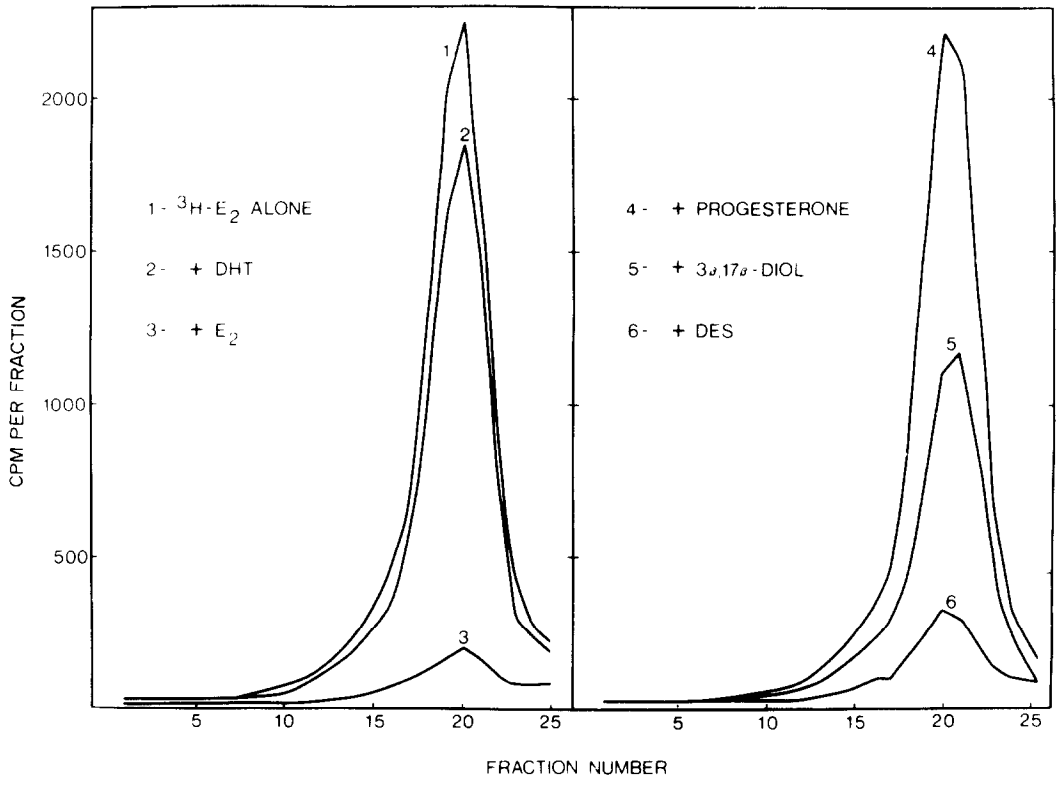

Fig. 1. Sucrose density gradient profiles of the binding of 4.5 nM [^{3}H]-estradiol alone or in the presence of various unlabeled competitors at a 69 nM concentration to dog prostate cytosol from an intact animal. The amount of protein applied on each gradient was 4.2 mg. Fraction 25 is at the top of the gradient. Bovine serum albumin in identical experimental conditions sediments in fraction 18. Unbound steroids were removed by charcoal treatment before centrifugation.

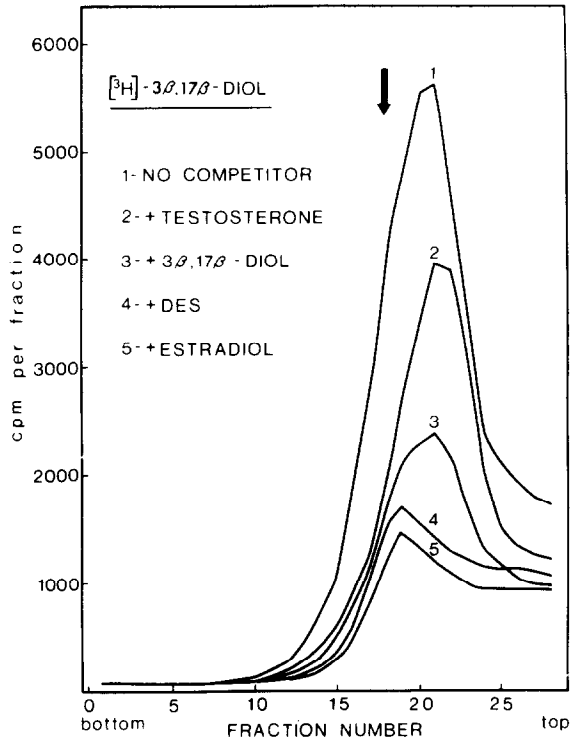

Fig. 2. Sucrose density gradient profiles of the binding of 18.4 nM [^{3}H]-5α-androstane-3β,17β-diol alone or in the presence of various unlabeled competitors at a 35 nM concentration to dog prostate cytosol. The amount of protein applied on each gradient was 10.3 mg. The arrow indicates the sedimentation position of bovine serum albumin.

estradiol, 5α-androstane-3β,17β-diol binding is abolished preferentially by estradiol and diethylstilbestrol and much less by testosterone (Fig. 2). Furthermore estradiol and 5α-androstane-3β,17β-diol yield an identical number of binding sites by Scatchard analysis (Fig. 3).

Similarly to our previous study on androgen receptors in dog prostate [1], we also determined the concentration of estradiol binding sites in relation to prostate wt. (Fig. 4). No significant correlation could be found between the concentration of estradiol binding sites and the prostate wt. of castrated dog and large variations were observed for individual animals with a mean binding site of 178 ± 15 fmol/mg prot. In intact dogs, on the contrary, there is a significant inverse relationship between estradiol binding and prostate wt. The lower amount of estradiol receptor sites in hypertrophic prostates cannot be explained by receptor occupancy by endogenous estrogens since "heat exchanged" cytosols (23°C up to 5 h) showed only 10 to 15% increase in binding over 0°C incubated cytosols (results not shown).

In a preliminary experiment with four different untreated dog prostates ranging from 6 to 43 g in wt, the binding of the synthetic progestin, [^{3}H]-5020, to the cytosol was also analyzed by sucrose density gradient centrifugation (results not shown). A small peak of radioactivity (max at 150 to 400 c.p.m.) could be observed in all cases in the 8S region of the gradients. In one of these experiments unlabeled steroid competitors were also included. It was observed that the 8S binding peak was abolished by unlabeled progesterone and R5020 but not by 5α-dihydrotestosterone or estradiol.

Since there appeared to be only small amounts of progesterone receptors under these conditions, in the next experiment, one dog received a single intramuscular injection of 50 mg of estradiol valerate in order to see if progesterone receptors could be induced by this treatment. Five days after the injection, the animal was killed. Prostate cytosol was incubated with [^{3}H]-R5020, [^{3}H]-R1881 and [^{3}H]-DHT and applied on sucrose gradients (Fig. 5). High levels of binding could be observed in the 8S and 4S regions of the gradients with [^{3}H]-R5020 and it was almost completely abolished by the addition of unlabeled progesterone or D-norgestrel. In this prostate, there was also high levels of androgen receptors as could be observed with [^{3}H]-DHT. When [^{3}H]-R1881 was used as binding probe, the binding seen was roughly equivalent to the sum of the progesterone (R5020) and androgen (DHT) receptors since R1881 has a high affinity for both receptors [9, 14]. The level of

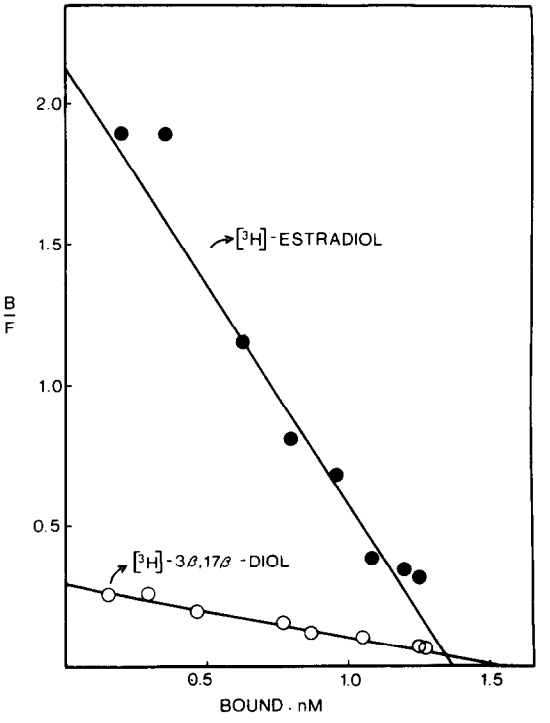

Fig. 3. Scatchard analysis of the binding of [^{3}H]-estradiol and [^{3}H]-5α-androstane-3β,17β-diol at 0°C to aliquots of the same prostate cytosol. The amount of protein was 25 mg per ml. In this experiment, the concentration of [^{3}H]-estradiol varied from 0.3 to 5.2 nM and the concentration of [^{3}H]-5α-androstane-3β,17β-diol from 0.8 to 21.8 nM. The association constant (K_A) was found to be $1.5 \times 10^9\ M^{-1}$ for estradiol and $0.2 \times 10^9\ M^{-1}$ for 5α-androstane-3β,17β-diol.

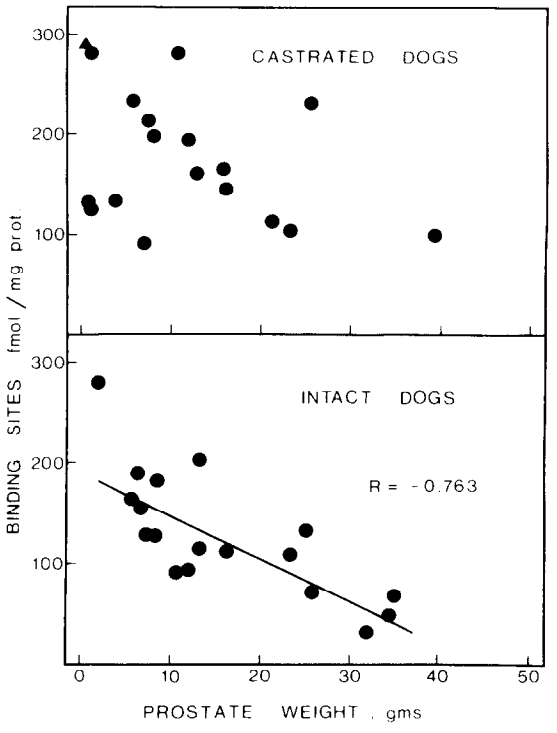

Fig. 4. The relation between prostate wt. and estrogen receptor content in intact and 24 h castrated dogs. All individual determinations were obtained by Scatchard plot. The triangle represents the result for a pool of 3 prostates from immature dogs. The correlation between prostate wt. and concentration of binding sites was determined by computer analysis. The correlation was found to be statistically significant ($P < 0.01$) in intact animals.

Table 2. Ligand specificity of [^{3}H]-R5020 binding in prostate cytosol of an estrogen treated dog

Steroid	Competition activity
R5020	100
R1881	145
D-Norgestrel	138
Progesterone	128
Medroxyprogesterone acetate	110
Cyproterone acetate	28
19-Nortestosterone	4
Estradiol	0.7
5α-Dihydrotestosterone	0.6
Cortisol	<0.1
5α-Androstane-3α,17α-diol	<0.1

progesterone receptors in this treated dog was increased about 10 fold over untreated dogs. Estradiol injection thus provided a convenient procedure to obtain receptor rich tissues in order to study the receptor characteristics.

The receptor specificity was further studied in the prostate of another estradiol treated dog (Fig. 6). The binding of [^{3}H]-R5020 was decreased to very low levels by the addition of unlabeled R1881 and R5020. It was not influenced by cortisol and only partly by DHT. Complete competition curves were also performed with the charcoal assay and the competition activity at 50% binding was calculated (Table 2). The results obtained confirm the sucrose gradient experiments showing that the binding is highly specific for progesterone and synthetic progestins but not for estradiol, DHT and cortisol.

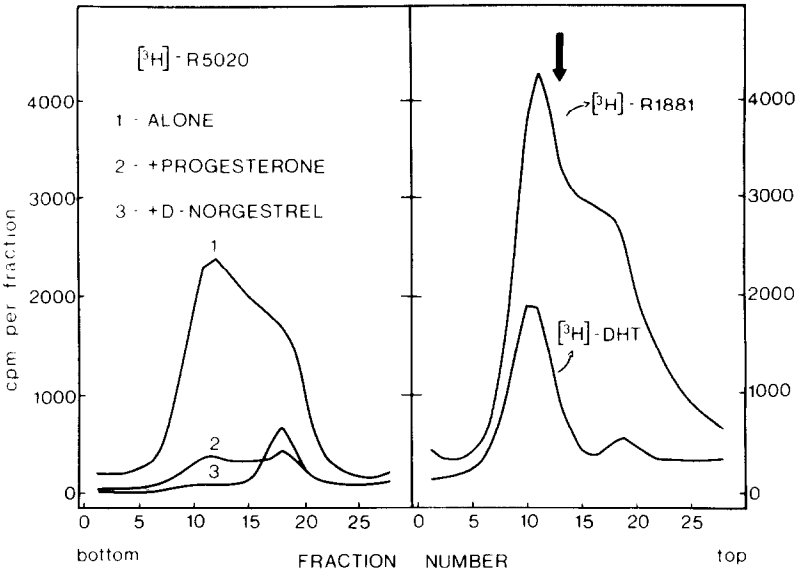

Fig. 5. Sucrose density gradient profiles of the binding of 10.7 nM [^{3}H]-R5020 alone or in the presence of 6.3×10^{-7} M unlabeled progesterone or 6.3×10^{-7} M unlabeled D-norgestrel, of 14.7 nM [^{3}H]-DHT and of 4.0 nM [^{3}H]-R1881 in the prostate cytosol of a dog having received a single intramuscular injection of estradiol valerate 5 days earlier. The arrow indicates the position of bovine gamma globulin.

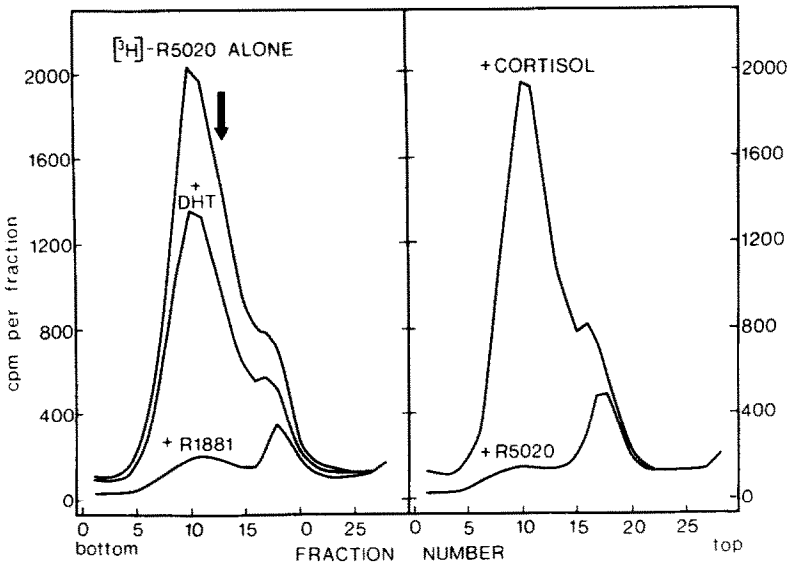

Fig. 6. Effect of various unlabeled steroids at a concentration of 6×10^{-7} M on the binding of [^{3}H]-R5020 in dog prostate cytosol. Other experimental conditions are identical to those described under Fig. 1.

The next series of experiments were aimed at finding appropriate experimental conditions to determine total concentration of progesterone binding sites in dog prostate cytosol. It was observed that [^{3}H]-R5020 was of limited use for that purpose particularly in prostates containing low amounts of receptors since this steroid showed considerable non specific binding. By contrast, non specific binding was low with [^{3}H]-R1881. However since this steroid also binds to androgen receptors, unlabeled DHT (1.7×10^{-7} M) was also included in the incubation in order to abolish specific binding to the androgen receptor. This concentration of DHT has been shown to have no effect on progesterone receptor in competition experiments. Under these conditions in estrogen treated dog, [^{3}H]-R1881 (Fig. 7 panel D) gave a concentration of binding sites identical to [^{3}H]-R5020 (Fig. 7 panel C) by Scatchard analysis. In absence of added unlabeled DHT, [^{3}H]-R1881 gave a curvilinear Scatchard plot (Fig. 7 panel B) resulting from the binding of this steroid to androgen and progesterone receptors. In this experiment, the level of androgen receptors as determined directly with [^{3}H]-DHT (Fig. 7 panel A) is slightly higher than the value that can be calculated from the sum of androgen and progesterone receptors in B minus progesterone receptors in C or D. This could result from an imprecision of the Scatchard plot shown in panel B. The association constants (K_A) at 0°C of [^{3}H]-R5020 and [^{3}H]-R1881 for the progesterone receptor were respectively 1.1×10^8 M^{-1} and 1.8×10^8 M^{-1}. The experimental conditions described in Fig. 7 panel D were chosen for the determination of progesterone receptor levels in further experiments.

Relatively low and constant levels of progesterone receptors were observed in normal and hyperplastic prostates from adult dogs (Fig. 8). The prostate from immature dogs generally showed binding levels similar to adult dog prostates although two dogs had higher levels of binding. The mean level of binding (excluding high values) was 30 ± 1 (S.E.M.) fmol per mg prot. and the association constant (K_A) was 0.9 ± 0.1 (S.E.M.) $\times 10^8$ M^{-1}.

DISCUSSION

Our study shows that high levels of estradiol binding proteins are present in dog prostate cytosol. Such binding cannot be demonstrated in plasma (results not shown). The prostate component shows many of the characteristics of steroid hormone receptors. It is of high affinity and it is highly specific for natural and synthetic estrogens. It is thus clearly different from the androgen receptor described in the previous paper [1]. The estradiol binding component has a sedimentation coefficient of 3 to 4S on sucrose gradients. The absence of binding in the 8S region of gradients is at variance with most other estrogen target tissues of the genital tract of the female rat. In the baboon prostate [6] and in some human neoplastic breast tissue [15], estrogen receptors have also been shown to sediment exclusively in the 4S region. In the genital tract of the rhesus monkey, estrogen treatment changed the sedimentation coefficient from 4S to 8S when compared with the castrate [16]. In the case of the dog prostate cytosol, only the 4S form was observed in intact and castrated animals. However in estrogen treated dogs, a small shoulder could

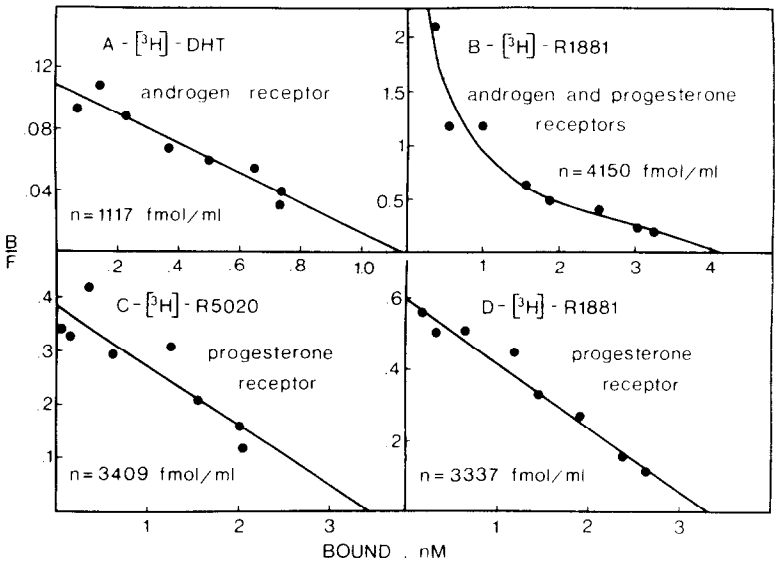

Fig. 7. Determination of androgen and progesterone receptors by Scatchard analysis in the prostate cytosol of an estradiol treated dog. A. The cytosol was incubated with [^{3}H]-DHT alone (total binding) or in the presence of 4.3×10^{-6} M unlabeled DHT (non specific binding). All the tubes also contained 3.7×10^{-8} M unlabeled diethylstilbestrol in order to abolish the binding of DHT metabolites to estrogen receptor [1, 2]. B. The cytosol was incubated with increasing concentrations of [^{3}H]-R1881 alone (total binding) or in the presence of 4.3×10^{-6} M unlabeled R1881 (non specific binding). C. The cytosol was incubated with increasing concentrations of [^{3}H]-R5020 alone (total binding) or in the presence of 3.8×10^{-6} M unlabeled R5020 (non specific binding). D. The cytosol was incubated with [^{3}H]-R1881 alone or in the presence of 3.8×10^{-6} M unlabeled R5020. All tubes in experiment D also contained 1.7×10^{-7} M unlabeled DHT in order to abolish the binding of [^{3}H]-R1881 to the androgen receptor. In all cases (A–D), the protein concentration in the cytosol was 10.5 mg per ml. In A, C and D, the regression lines were determined by computer analysis.

be observed in the 8S region (results not shown). Clearly then, the 4S sedimentation coefficient observed in dog prostate cytosol does not exclude the possibility that estradiol binding is due to a "receptor". This possibility is further strengthened by the observation that estradiol treatment induces progesterone receptors in dog prostate cytosol. Progesterone receptor appears to be a marker of estradiol action in several estrogen target tissues [17–19] and this action is mediated by the estrogen receptor [20].

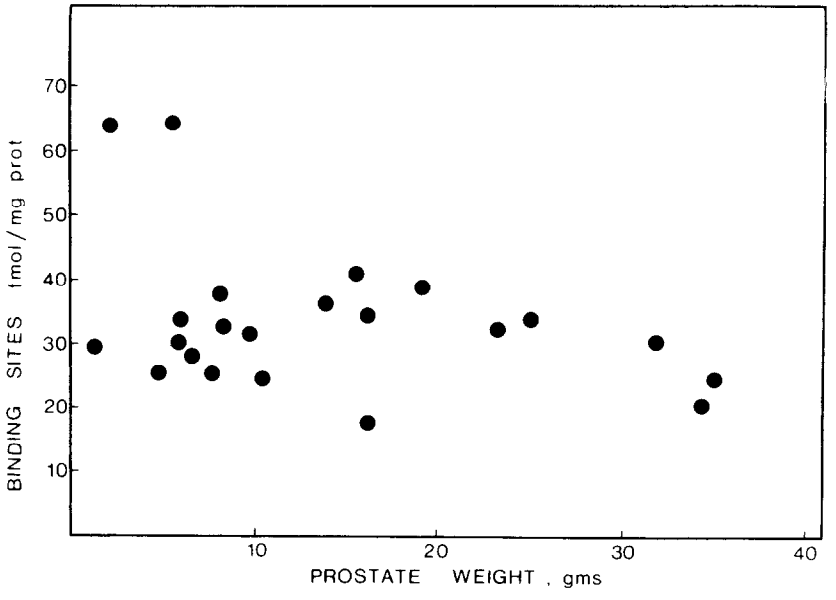

Fig. 8. The relation between prostate weight and progesterone receptor content in intact dogs. All individual determinations were obtained by Scatchard plot.

An interesting observation is that 5α-androstane-3β,17β-diol is bound with a relatively high affinity to the dog prostate estrogen receptor. These results combined with the DHT metabolism and binding studies reported in the previous paper [1] clearly indicate that after incubation of dog prostate cytosol with [^{3}H]-DHT, 5α-androstane-3β,17β-diol is produced and then is bound to the 4S estrogen receptor. This finding could have important implications in the pathogenesis of prostate hyperplasia since this steroid is a major metabolite of DHT during *in vivo* studies [13]. Furthermore, the studies of Rochefort and Garcia[21] have shown that high doses of DHT *in vivo* are effective in translocating estrogen receptor to rat uterus nuclei. In dog prostate, 5α-androstane-3β,17β-diol has much more affinity than DHT for the estradiol receptor and should then be more effective to promote nuclear translocation of the estrogen receptor. The studies of Walsh and Wilson[7] indicate that combined action of injected 5α-androstane-3α,17β-diol and estrogens are able to induce dog prostate hyperplasia. However, if we assume that 5α-androstane-3β,17β-diol is capable of some estrogenic action in dog prostate, pathologic changes in the production of this steroid in the prostate itself along with elevated levels of estradiol and estrone [8] could then be implicated in the development of hyperplasia. A recent study by Jacobi[22] show that cytosolic and microsomal 3α- and 3β-hydroxysteroid dehydrogenases increase dramatically with age and thus adds support to our hypothesis.

Our study also shows that progesterone receptors similar to those of the uterus of various mammalian species are present in dog prostate. In low salt gradients, these receptors sediment mainly in the 8S region and they are remarkably specific for natural and synthetic progestins. Another point of similitude with estrogen target tissues [17–19] is that prostate progesterone receptors can be induced by estrogens. However some differences in physicochemical characteristics are also apparent. The affinity constant (K_A) of the prostate receptor for R5020 is lower than in rabbit and guinea-pig uterus [23]. In common with the human prostate [9–11] and uterus [9], dog prostate cytosol appears to have similar affinity for progesterone and R5020. This situation complicates the determination of the number of binding sites, since R5020 is able to bind to transcortin and serum albumin [24]. Furthermore because of the relatively low affinity of the dog prostate cytosol for R5020, higher concentrations are needed to obtain a saturation. In this case, the non specific binding is high and can completely mask receptors in prostates containing low amounts of receptors. Furtunately, another synthetic steroid, R1881, is bound with a higher affinity than progesterone and R5020 to dog prostate cytosol and shows very low non specific binding. When unlabeled DHT is included in the incubation to saturate the androgen receptor, R1881 then measures only the progesterone receptor (Fig. 7). Using this procedure, progesterone receptors could be found in all dog prostates. No similar high affinity binding could be demonstrated in plasma (results not shown).

In the previous paper [1], we have also determined androgen receptor levels with [^{3}H]-R1881. Because of the large differences in affinity constants of androgen and progesterone receptors and in the concentration of binding sites in untreated animals, we can calculate that this procedure cannot overestimate androgen receptor levels by more than 5 to 10%. Such confidence can also be obtained by looking at competition experiments and at Scatchard plots showing a single class of high affinity binding sites in the experimental conditions used [1].

The question which arises naturally from our results concerns the role of estradiol and progesterone receptors in the prostate. Estradiol binding proteins have also been reported in rat [2], guinea-pig [3] and human [4, 5] prostates. Present evidence in the literature suggests that estrogens can possibly affect both prostatic stroma and glandular epithelium. In rat prostate organ culture, estradiol at various concentrations is able to counteract the inhibitory action of physiological concentrations of testosterone on the interstitial stroma [25]. In dog, the combined action of 5α-androstane-3α,17β-diol and estradiol produces striking epithelial hyperplasia [7] while estradiol alone shows histological pictures very similar to the one observed with the prostates of untreated castrated animals in which there is an absence of secretory epithelium. The nature of estradiol effects on dog prostatic epithelium remains obscure but it could act synergistically with androgens. In castrated rat prostate, daily injections of estradiol alone for 3 weeks can induce epithelial growth and secretory activity [26].

The culmination of hormonal effects observed in hyperplastic dog prostates does not appear to be mediated by pathological changes in either estradiol receptor affinity or concentration of binding sites as evidenced by receptor levels in 24 h castrated animals. However the progressive decrease of receptor sites with increasing prostate weight in intact dog could suggest an increased "estrogenic" stimulation resulting in depletion of cytoplasmic receptors with concurrent elevation of nuclear receptor levels. Menon *et al.*[12] have made the hypothesis that the presence of progesterone receptors in human prostate from aging males may indicate that estrogen is implicated in the growth and function of this organ since the progesterone receptor is usually under the control of estrogen. However, this hypothesis, at least in dog prostate, is not supported by the levels of progesterone receptors which remain low throughout life even though estradiol administration to dogs is able to increase progesterone receptor levels about 10-fold. Furthermore, it can be calculated from the concentrations of estradiol and estrone in dog prostate [8] and from our results on estradiol receptor levels that less than 10% of the prostate estradiol receptor is

occupied by estrogens even in hypertrophic glands. This observation agrees with our exchange assays at 23°C with [^{3}H]-estradiol in intact dog prostate and casts some doubts on the role of estrogens in the development of dog prostate hypertrophy. However cytosolic estrogen receptor levels decrease in hypertrophy. If we assume that this effect is explained by nuclear translocation of receptors, we must conclude indeed that androgens could be responsible for this transfer and for the estrogenic effects and as it is observed in rat uterus [21] and human breast cancer cell line MCF-7 [27]. Further studies will be required to ascertain this hypothesis.

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