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The Demonstration of Oestrogen, Androgen and Progestagen Receptors in the Cytosol Fraction of Canine Mammary Tumours

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Abstract—*The presence of cytoplasmic receptors in canine mammary tumours for oestrogens, androgens and progestagens has been demonstrated. The binding molecules showed low capacity and high affinity for each respective group of hormones with mean dissociation constants (and ranges) of* 3.9×10^{-9} M ($3.7 \times 10^{-10} - 9.9 \times 10^{-9}$) *for oestradiol-17β,* 1.37×10^{-10} M ($2.2 \times 10^{-10} - 3.6 \times 10^{-9}$) *for 5α-dihydrotestosterone and* 1.28×10^{-10} M ($3.4 \times 10^{-10} - 3.3 \times 10^{-9}$) *for R5020, a synthetic progestagen. The mean receptor site concentration (and ranges) were* 61.7 (13.4–129.1), 15.2 (3.5–30.0) *and* 30.6 (5.0–80.9) fmole/mg *cytosol protein respectively for these steroids.*

Sucrose density gradient ultracentrifugation analysis revealed that each receptor sedimented with a coefficient of 8S and that they were specific for their group of steroids.

INTRODUCTION

THE MAMMARY gland of the bitch is one of the most common sites for tumour development in this, or any, species, with a recorded incidence of 203.4 per 100,000 of the bitch population [1]. This is approximately three times higher than that of human breast cancer in the same geographical area. In women, such tumours may be shown to be hormone-dependent or -independent according to their response to endocrine therapy [2]. The incidence and behaviour of mammary tumours in the bitch would also appear to be influenced by hormones [3–5]. It is currently accepted that steroid hormones achieve their action in responsive organs by initially binding to protein molecules in the cytosol fraction of the cell. Absence of such proteins would, presumably, disallow further hormone activity. This cellular function is currently being used in the laboratory to forecast the behaviour of breast cancer in women [6–10] and mammary tumours in the bitch to various forms of endocrine manipulations [11, 12].

There is a group of women, however, whose breast tumours are found to be positive for oestrogen receptors and yet do not respond to anti-oestrogen therapy [13] leading to speculation that other trophic influences may be implicated. Such conjecture has been supported by the demonstration of androgen [14, 15] and progesterone [16, 17] receptors in breast tumours. Receptors for oestradiol-17β have been demonstrated in canine mammary tumours [11] and it was suggested then that anti-hormone therapy may be beneficial in these cases. Those original findings have subsequently been verified [12, 18, 19].

The present study has examined the receptor content and binding affinities of canine mammary tumours for these three classes of hormone with a view to obtaining information concerning the endocrine-dependence of these neoplasms which could be of value in subsequent therapy.

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MATERIALS AND METHODS

Dithiothreitol, monothioglycerol and bovine serum albumin (BSA) were obtained from the Sigma Chemical Company, London; Tris (hydroxymethyl) methylamine, ethylenediamine tetra-acetic acid (EDTA) and sucrose were obtained from Hopkin and Williams, England. Norit GSX and Dextran T70 were obtained from Hopkin and Williams, Chadwell Heath, Essex and Pharmacia Fine Chemicals, Uppsala, Sweden respectively. Diethylstilboestrol was obtained from Koch-Light Laboratories, Colnbrook, Bucks. Oestradiol-17α and oestradiol-17β were obtained from B.D.H. Chemicals Ltd., and oes-

triol, oestrone, androst-5-ene-3β,17β-diol, 5α-androstane-3β,17β-diol, cortisol, progesterone, 19-norethisterone and corticosterone from the Sigma Chemical Company, Kingston-on-Thames, Surrey. Testosterone, 5α-dihydrotestosterone and dehydroepiandrosterone (DHA) were purchased from Koch Light Laboratories, Colnbrook, Bucks: R5020 was kindly given by Dr. J. P. Raynaud, Roussel Uclaf, Paris, France, and 5α-androstane-3β, 17α-diol and 5α-androstane, 3α, 17α-diol were gifts from Schering Chemicals Ltd., Sussex; 5α-dihydroprogesterone was obtained from Mann Research Laboratories Inc., Liberty Street, New York.

The tritiated steroids 5α-dihydro-[1, 2, 4, 5, 6, 7 (*n*)-^{3}H]-testosterone (107 Ci/mmole) and [2, 4, 6, 7, (*n*)-^{3}H] oestradiol-17β (91 Ci/mmole) were obtained from the Radiochemical Centre, Amersham, Bucks. and R5020 (85 Ci/mmole) (17α, 21-dimethyl (17α-^{3}H methyl)-19-norpregna-4, 9-diene-3, 20-dione) was obtained from New England Nuclear, Boston, Massachusetts.

Mammary tumours were provided by local veterinary surgeons and transported to the laboratory on ice.

Steroid receptor assays

All procedures were carried out at 0–4°C unless otherwise indicated. Tissue was dissected free of fat and washed in saline to rid it of blood, blotted dry and weighed. The tissue was cut into small pieces, frozen in liquid nitrogen and then disintegrated with a stainless steel pulveriser gun. After addition of medium A (10 mM Tris–HCl; 1 mM EDTA; 10 mM monothioglycerol pH 7.4) for R5020 and 5α-dihydrotestosterone binding assays or medium B (10 mM Tris–HCl; 1 mM EDTA; 0.5 mM dithiothreitol pH 7.4) for oestradiol-17β, the tissue suspension (tissue to buffer ratio 1:3) was allowed to equilibrate at 4°C for 15 min. After homogenisation using a Teflon-glass homogeniser, the homogenate was filtered through a nylon net and centrifuged in a Beckman L2-65B ultracentrifuge in an SW 50.1 rotor at 114,000 ***g*** for 1 hr. Any fats were skimmed from the top of the cytosol extracts which were then vortexed with a pellet of dextran-coated charcoal prepared from a suspension of dextran-coated charcoal (0.5% Norit A; 0.05% Dextran and 0.1% gelatin) equivalent in volume to that of the cytosol. The mixture was incubated for 15 min at 4°C and then centrifuged at 800 ***g*** for 20 min. An aliquot of the charcoal-treated cytosol was used for protein determination by the method of Lowry *et al.* [20].

Estimation of steroid receptor binding in the cytosol was carried out by vortexing 250 μl aliquots of cytosol with equal volumes (in duplicate) of buffer A or B containing different concentrations of the tritiated steroid alone (0.1–10 nM), or with radioactive steroid plus 100 fold excess of the unlabelled ligand. Following incubation at 0°–4°C for 16–18 hr for [^{3}H] oestradiol-17β and 4–6 hr for [^{3}H] 5α-dihydrotestosterone and [^{3}H] R5020, 0.5 ml dextran-coated charcoal was added to each tube. Mixtures were vortexed and then allowed to stand for 15 min at 4°C. After centrifugation at 800 ***g*** for 15 min, 500 μl aliquots were taken for counting into scintillation vials and radioactivity measured in a Nuclear Chicago (Mark II) liquid scintillation spectrophotometer using external standardisation. The adsorption by dextran-coated charcoal was checked to be 98–99% efficient in each tube, by adding charcoal to a set of tubes containing the tracer in which cytosol was replaced by 250 μl buffer A or B. Estimation of the dissociation constant and binding capacity was achieved by the method of Scatchard (1949) [21].

Competition studies

Mammary tumour cytosol (250 μl) was incubated with an equal volume of buffer A or B containing 10 nM [^{3}H] oestradiol-17β, 1 nM [^{3}H] R5020 or 5 nM [^{3}H] 5α-dihydrotestosterone, and up to 1000 fold excess of unlabelled competitors for 16–18 hr at 0–4°C for [^{3}H] oestradiol-17β and 4–6 hr at 0–4°C for [^{3}H] R5020 and [^{3}H] 5α-dihydrotestosterone.

Sucrose gradient analysis of oestradiol-17β, R5020 and 5α-dihydrotestosterone binding in the cytosol

Aliquots of cytosol (1 ml) were incubated with 5 nM [H^3] steroid with and without a 100-fold excess of unlabelled steroid for 4 hr at 0–4°C and then an aliquot of 0.4 ml layered onto 5 ml of a 5–20% sucrose gradient prepared in buffer A or B with the aid of a former [22]. Before layering onto the gradient, the cytosol extracts were vortexed with a pellet of dextran-coated charcoal, equal in volume to that of the cytosol, and the charcoal then removed by centrifugation at 800 ***g*** for 10 min. The gradients were centrifuged at 114,000 ***g*** for 16–18 hr at 4°C in a Beckman L2-65B centrifuge using an SW 50.1 rotor in

association with a gradient containing a BSA standard at 1 mg/ml (4.6S marker). Fractions of 3 drops (approximately 200 μl) were obtained from the gradients by displacement and collected directly into scintillation vials and counted with 6 ml water-miscible scintillator (5 g PPO, 500 ml triton and 1500 ml toluene). The BSA aliquots were prepared with the addition of 1 ml water and the optical density read at 280 nM.

Evaluation of the nature of the competition for the binding of [^{3}H] *R*5020, [^{3}H] 5α-*dihydrotestosterone and* [^{3}H] *oestradiol*-17β *by the respective binding proteins*

Cytosol preparations from mammary tumour tissue were labelled with various concentrations of [^{3}H] oestradiol-17β, [^{3}H] 5α-dihydrotestosterone or [^{3}H] R5020 in the presence and absence of (1–100-fold) excess of various unlabelled steroids. From the titration data, double reciprocal plots were constructed [23] to evaluate the nature of the inhibition by these steroids.

RESULTS

Sucrose gradient analysis of specific binding for [^{3}H] *oestradiol*-17β, [^{3}H] *R*5020 *and* [^{3}H] 5α-*dihydrotestosterone*

Cytosol from mammary tumours, incubated with [^{3}H] oestradiol-17β, [^{3}H] R5020 and [^{3}H] 5α-dihydrotestosterone provided gradient profiles as shown in Fig. 1(a, b and c), respectively. The protein bound steroids all showed two components, sedimenting in the 4S, 8.5S; 4S, 7.8S; and 3.5S, 7S regions of the gradient respectively. The 7.8S bound radioactivity was completely displaced by a 100-fold excess of unlabelled steroid, indicating the binding proteins were of limited capacity.

Titrations of specific binding sites for [^{3}H] *R*5020, [^{3}H] *oestradiol*-17β *and* [^{3}H] 5α-*dihydrotestosterone*

Increasing concentrations of [^{3}H] R5020 were incubated with mammary tumour cytosol in each case in the presence and absence of 100-fold excess unlabelled R5020. Using dextran–charcoal to remove any free or loosely bound steroid, specific binding was calculated as the difference between total and non-specific binding. Saturation of specific binding sites in the cytosol occurred at approximately 2 nM. The mean (+ range) of Scatchard analyses of 4 tumours provided dissociation constants of 1.28×10^{-10} M (3.36×10^{-10}–3.3×10^{-9}) and 30.6 (5.0–80.9) fmole/mg cytosol protein of specific binding sites. A typical analysis is seen in Fig. 2(a) ($K_D = 1.88 \times 10^{-10}$ M and receptor site concentration of 45 fmole/mg protein).

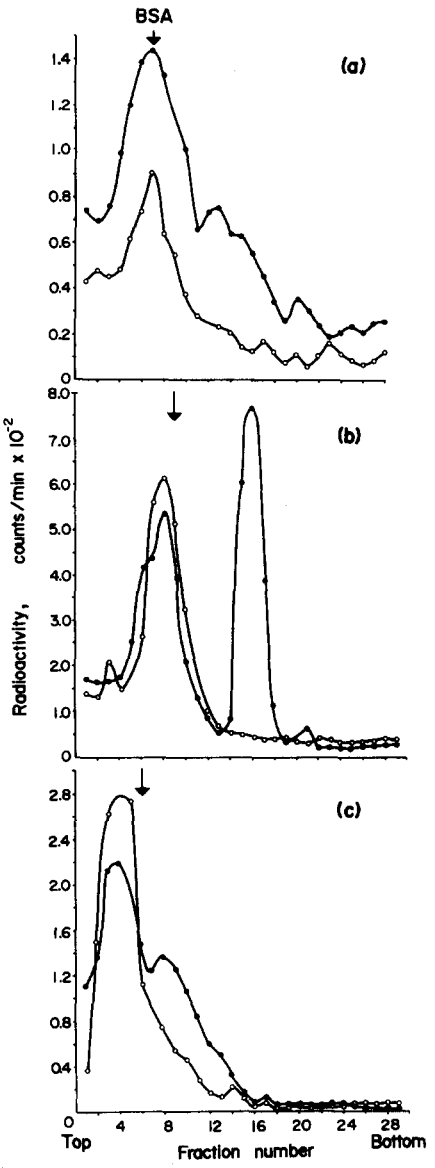

Fig. 1(*a*) *Radioactive profile of cytosol preparation from canine mammary tumour after ultra-centrifugation at* 114,000 *g for* 1 hr *at* 4°C. (●—●) *Profile from cytosol incubated with* [^{3}H]*oestradiol*-17β *alone,* (○—○) *profile from cytosol incubated with* [^{3}H]*oestradiol*-17β *in the presence of* 100-*fold unlabelled oestradiol*-17β.

(*b*) (●—●) *Profile from cytosol incubated with* [^{3}H]*R*5020 *alone,* (○—○) *profile from cytosol incubated with* [^{3}H]*R*5020 *in the presence of* 100-*fold unlabelled R*5020.

(*c*) (●—●) *Profile from cytosol incubated with* [^{3}H]5α-*dihydrotestosterone alone* (○—○) *profile from cytosol incubated with* [^{3}H]5α-*dihydrotestosterone in the presence of* 100-*fold unlabelled* 5α-*dihydrotestosterone.*

Saturation of the specific binding sites for oestradiol-17β occurred at approximately 5 nM. A typical Scatchard analysis is shown

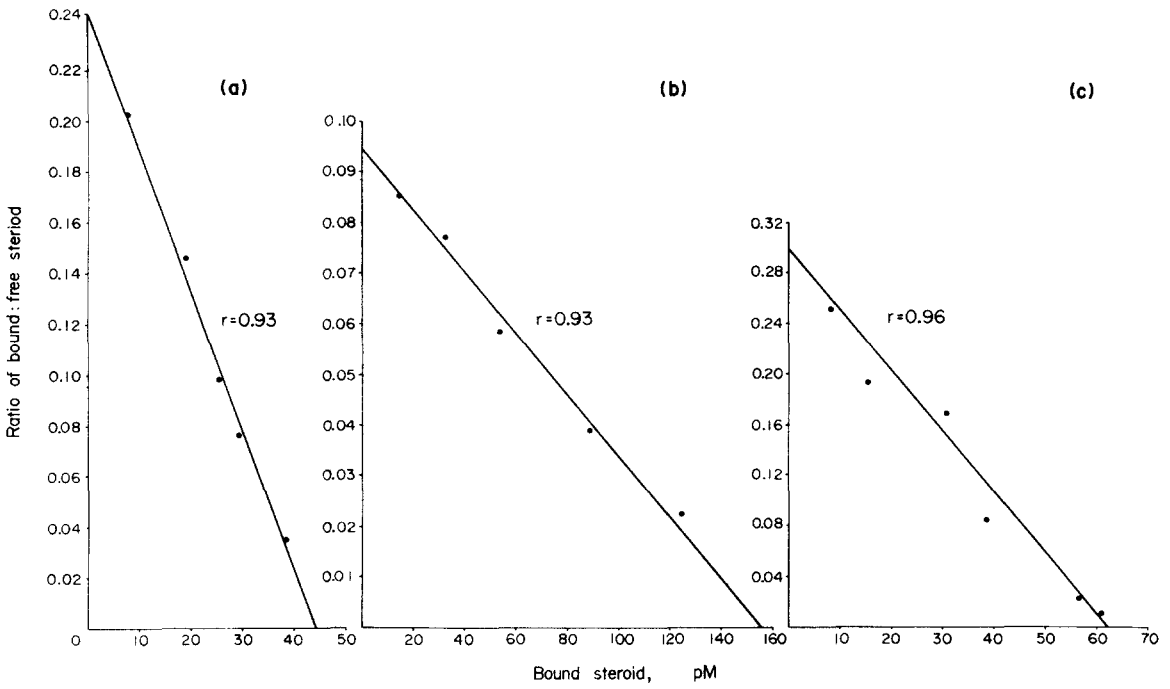

Fig. 2(a) Determination of the number of specific binding sites for R5020 in canine mammary tumour tissue. Cytosol fractions were incubated for 4–6 hr at 0–4°C with various concentrations of [^{3}H]R5020 in the presence or absence of a 100-fold excess of unlabelled R5020. Specific R5020-binding was analysed by the method of Scatchard (1949).

(b) Determination of the number of specific binding sites for oestradiol-17β in canine mammary tumour tissue. Cytosol fractions were incubated for 16–18 hr at 0–4°C with various concentrations of [^{3}H]-oestradiol-17β in the presence or absence of a 100-fold excess of unlabelled oestradiol-17β. Specific binding data were analysed by the method of Scatchard (1949).

(c) Determination of the number of specific binding sites for 5α-dihydrotestosterone in canine mammary tumour tissue. Cytosol fractions were incubated for 4–6 hr at 0–4°C with various concentrations of [^{3}H]-5α-dihydrotestosterone in the presence of a 100-fold excess of unlabelled dihydrotestosterone. Specific binding data were analysed by the method of Scatchard (1949).

in Fig. 2(b) indicating a single class of binding site with high affinity (K_D of 2.92 $\times 10^{-9}$ M) and a binding capacity of 56.1 fmole/mg cytosol protein. The mean from 7 tumour preparations gave a K_D of 3.9 $\times 10^{-9}$ M (3.7×10^{-10}–9.9×10^{-9}) and an average binding capacity of 61.7 (13.4–129.1) fmole/mg cytosol protein.

Certain of the mammary tumours were examined for the presence of androgen-binding proteins. The number of binding sites was found to be low. Scatchard analysis of one preparation showed a single class of binding site with high affinity and a K_D of 2.2 $\times 10^{-10}$M, Fig. 2(c). The binding capacity was found to be 18.5 fmole/mg cytosol protein. Saturation of specific binding sites occurred at approximately 5 nM. The mean values from four separate tumour preparations provided a K_D of 1.37×10^{-10} M (2.2×10^{-10} M – 3.57 $\times 10^{-9}$) and an average binding capacity of 15.2 (3.5–30.0) fmole/mg cytosol protein.

Examination of the specificity of the binding proteins

As shown in Table 1, oestradiol-17β was the most effective competitor for the [^{3}H]

Table 1. The relative ability of various steroids to cause a 50% reduction in the binding of [^{3}H]oestradiol-17β (nM) in the cytoplasm of a canine mammary tumour

Competitor	Concentration (nM) of competitor
Oestradiol-17β	280
Oestradiol-17α	1200
Oestriol	1100
Oestrone	720
Diethylstilboestrol	1500
Androst-5-ene-3β,17β-diol	1900
5α-Dihydrotestosterone	>20,000
Dehydroepiandrosterone	>20,000
5α-Androstane-3α,17α-diol	>20,000

oestradiol-17β binding sites, displacing 50% at only 50 fold excess of the labelled ligand, whereas to achieve the same effect oestradiol-17α, oestriol, oestrone, diethylstilboestrol and androst-5-ene-3β, 17β-diol were required at concentrations in excess of 100 fold. Other C_{19}-steroids 5α-dihydrotestosterone, dihydroepiandrosterone, and 5α-androst-3α,17α-diol failed to displace 50% of the total binding even at an excess of 20,000 nM (4000 fold excess, of the labelled ligand).

Table 2 shows the ability of various unlabelled steroids to displace 50% of the [^{3}H] R5020 from the "progesterone" receptor. Only R5020 and progesterone succeeded in displacing 50% of the label at less than 300 fold excess of the ligand. Corticosterone, cortisol, oestradiol-17β and 5α-dihydrotestosterone all failed to achieve this degree of displacement at up to 1000 fold excess as did 19-norethisterone.

Table 3 shows competitive binding studies for the [^{3}H] 5α-dihydrotestosterone binding protein. At a 10 fold excess of the labelled ligand, testosterone was able to reduce binding by 50% whilst 5α-dihydrotestosterone and 5α-androstane-3β, 17β-diol were required at approximately 30 and 100 fold excess respectively for the same effect. Androst-5-ene-3β, 17β-diol, progesterone and oestradiol-17β had no marked effect even at a 2000 fold excess.

Inhibition of progesterone, oestradiol-17β and 5α-dihydrotestosterone binding in cytosol

In these experiments cytosol preparations were titrated with various concentrations of tritiated [^{3}H] R5020, oestradiol-17β and 5α-dihydrotestosterone in the presence and absence of 100 fold excess of various unlabelled steroids (Fig. 3, 4, 5a, 5b). From the titration data, unlabelled progesterone, R5020 and 5α-dihydroprogesterone, demonstrated competitive inhibition for [^{3}H] R5020 specific binding sites, whilst corticosterone failed to compete (Fig. 3).

Similar experiments with the [^{3}H] oestradiol-17β binding protein indicated (Fig. 4) that oestradiol-17α, oestrone and oestradiol-17β competed for the same binding sites.

Inhibition of specific 5α-dihydrotestosterone binding sites by testosterone and 5α-dihydrotestosterone was of a competitive nature. Inhibition by 5α-androstane-3β,17β-diol was also competitive (Fig. 5a). In contrast, progesterone and oestradiol-17β showed non-competitive binding, (Fig. 5b).

DISCUSSION

Three classes of steroid binding proteins have been identified in mammary tumours of

Table 2. The relative ability of various steroids to cause a 50% reduction in the binding of [^{3}H]*R5020* (nM) *in the cytoplasm of a canine mammary tumour*

Competitor	Concentration (nM) of competitor
R5020	58
Progesterone	560
19-Norethisterone	2000
Cortisol	>2000
Corticosterone	>2000
Oestradiol-17β	>2000
5α-Dihydrotestosterone	>2000

Table 3. The relative ability of various steroids to cause a 50% reduction in the binding of [^{3}H]5α*-dihydrotestosterone* (nM) *in the cytoplasm of a canine mammary tumour*

Competitor	Concentration (nM) of competitor
Testosterone	80
5α-Dihydrotestosterone	180
5α-Androstane-3β,17β-diol	300
Androst-5-ene-3β, 17β-diol	>10,000
Progesterone	>10,000
Oestradiol-17β	>10,000

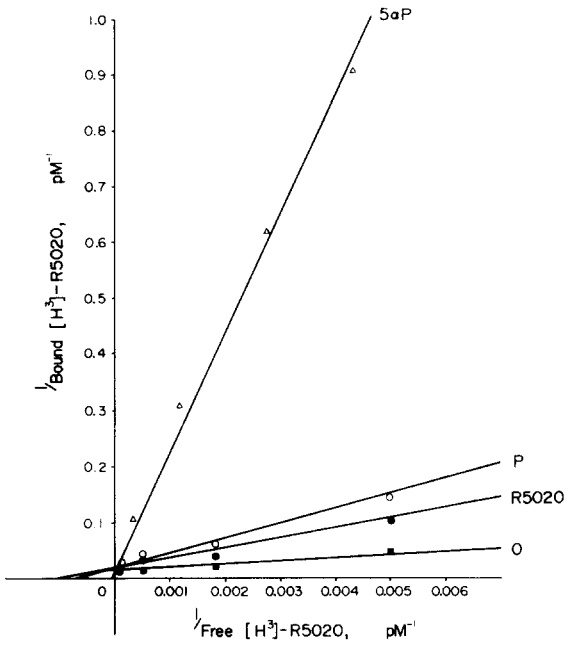

Fig. 3. Specific binding inhibition by various unlabelled progestagens for [^{3}H]*R5020 specific binding sites.* (■—■) *No competitor present.* (●—●) *R5020,* (○—○) *progesterone,* (△—△) 5α*-dihydroprogesterone.*

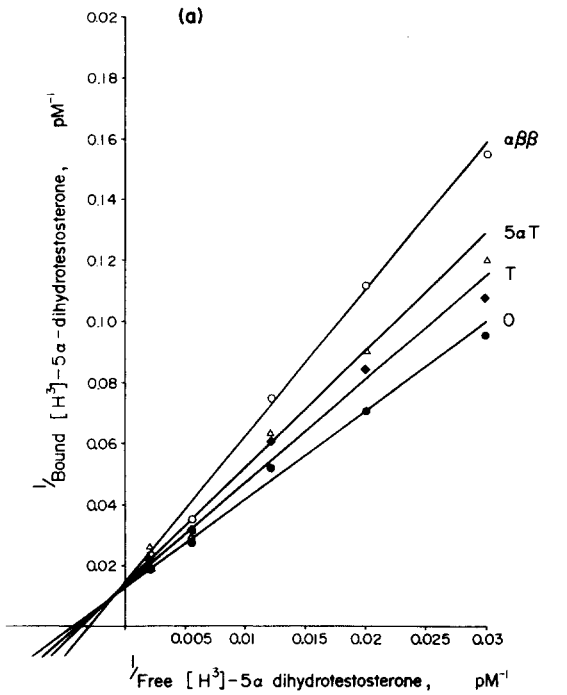

Fig. 5(a). Specific binding inhibition by various unlabelled androgens for [^{3}H]-5α*-dihydrotestosterone specific binding sites.* (●—●) *no competitor,* (◆—◆) *testosterone,* (△—△) 5α*-dihydrotestosterone,* (○—○) 5α*-androstane-3β, 17β-diol.*

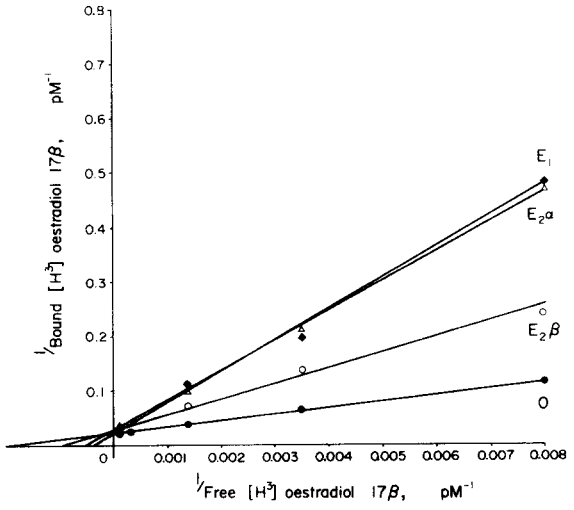

Fig. 4. Specific binding inhibition by various unlabelled oestrogens for [^{3}H]*oestradiol-17β specific binding sites.* (●—●) *no competitor,* (○—○) *oestradiol-17β,* (△—△) *oestradiol-17α,* (◆—◆) *oestrone.*

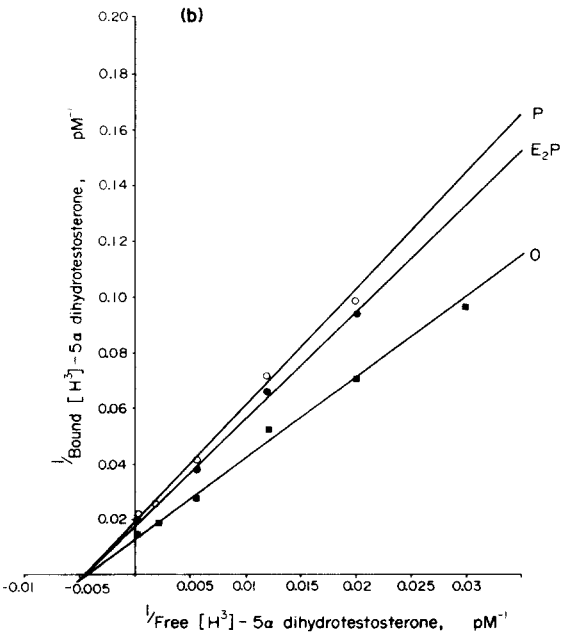

Fig. 5(b). Specific binding inhibition by various unlabelled steroids for [^{3}H]-5α*-dihydrotestosterone specific binding sites.* (■—■) *no competitor,* (●—●) *oestradiol-17β,* (○—○) *progesterone.*

the bitch sedimenting in the 4S and 7–8S regions of sucrose-density gradients in relation to bovine serum albumin (4.6S). In the presence of 100 fold excess unlabelled hormone, tritiated binding in the 7–8S region was eliminated, indicating low capacity binding. Gradient analysis of androgen, oestrogen and progesterone receptors from mammary glands of other species have shown similar findings [7, 17, 24].

Scatchard analysis of these steroid-receptor interactions showed linearity after correction for non-specific binding, and the dissociation constants obtained for the oestradiol-17β and 5α-dihydrotestosterone specific binding-proteins are in good agreement with data reported for other species [24–26]. Most information available concerning the assessment of the progesterone receptor has involved the use of [H^3]-progesterone. Dissociation constants for such preparations range from 1–10 nM [16, 27–29]. In this paper the synthetic progestagen R5020 was used to assess progesterone receptor affinity because it binds to a lesser extent to glucocorticoid binding protein and corticosteroid-binding globulins which may be present in relatively high proportions in mammary tissues and it also appears to stabilise the steroid-receptor complex. Scatchard results with R5020 gave low K_D values indicating high affinity binding.

Several problems have been encountered during the assaying of the [^{3}H] 5α-dihydrotestosterone binding protein. One is the assessment of minute quantities of receptor which, under the present assay conditions are difficult to assess accurately. Whilst the K_D is almost 20-fold lower than that of the [^{3}H] oestradiol-17β binding protein, the quantity of receptor is almost one quarter that of the oestradiol receptor. A second problem is that a significant amount of binding of 5α-dihydrotestosterone to contaminating plasma proteins occurs, which interferes in the charcoal assay. A final observation is the apparent lability of the receptor itself under the assay conditions. Specific binding is reduced by 50% after 6 hours incubation at 0–4°C. Lippman and Huff (1976) [25] compared the dextran–charcoal technique with a protamine sulphate precipitation method, designed to concentrate and free the receptor protein from plasma contaminants, and found the latter technique successful. Experimentation in this laboratory however shows no preference for this method. The present results indicate the presence of an androgen receptor protein with a K_D and sedimentation coefficient in good agreement with previous findings in mammary tissues from other species [24, 25].

As may be seen from competitive binding data inhibition by progesterone, R5020 and 5α-dihydroprogesterone for [^{3}H] R5020 binding was directly competitive, and corticosterone's failure to bind indicated distinction between the latter binding protein and transcortin (Fig. 3). R5020 is now widely used to assess the progesterone receptor and similar results have been found [16, 30]. [^{3}H] Oestradiol-17β, [^{3}H] 5α-dihydrotestosterone and [^{3}H] R5020 binding proteins were each seen to be distinct from one another. Further studies on the nature of the inhibition by certain of these unlabelled steroids suggested direct competition for [^{3}H] oestradiol-17β binding sites by the four (natural) oestrogens and also by the synthetic oestrogen, diethylstilboestrol (not shown) (Fig. 4). Inhibition of [^{3}H] 5α-dihydrotestosterone binding by 5α-dihydrotestosterone and testosterone was competitive, but interestingly oestradiol-17β and progesterone showed non-competitive characteristics (Fig. 5b).

These results indicate the complexity of the hormonal-dependence of canine mammary tumours and that specific antihormone therapy would be equally complex. The similarities, however, between these tumours and those of the breast in women bode well for comparison studies between the two species.

It should be emphasised that this report concerns tumours that were positive for the receptors examined and that there is a proportion in which one or more of the binding proteins are undetectable indicating a lack of hormone dependence as is the case in certain human breast cancers. The relative population of such neoplasms will constitute another communication.

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