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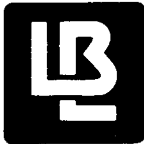

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Androgen and Estrogen Binding in Mouse Lung

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Running title: Sex Steroid Receptors in Mouse Lung

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ABSTRACT

Androgen and estrogen binding activities have been identified in cytosol fractions of adult male mouse lungs. These putative receptors exhibit specificity for the respective steroid and demonstrate high affinity and low capacity for the ligand. The binding activities sediment in the 8-9S region of sucrose gradients of low ionic strength containing sodium molybdate. The apparent Kd for the androgen "receptor" is 0.43 nM and for the estrogen "receptor" is 0.11 nM. Lung cytosol from intact adult male mice contains 14.11 ± 0.31 fmol androgen "receptor" and 3.43 ± 0.35 fmol estrogen "receptor" per mg cytosol protein. Castration results in an apparent transient reduction of androgen "receptor" content within the lung cytosol fraction, but values return to normal ranges at 18 hours post-castration. When quantitated 245 days post-castration, androgen binding was comparable to that observed in intact mice. Long term castration (245 days) resulted in a three fold increase in estrogen binding capacity.

INTRODUCTION

While studying the consequences of ozone toxicity in mice, it became apparent that sex-related differences were present when survival was quantitated (1). Intact female mice were more resistant to ozone toxicity than were intact male mice, and removal of the gonads reduced survival when compared to intact animals. However, within these groups of gonadectomized mice, castrate males tolerated the ozone environment much better than ovariectomized female mice. Hormone replacement in gonadectomized, ozone-exposed mice reduced mortality, and survival trends were normalized. Sex hormones, their presence or absence, modified the ozone response. The major biological interface with ozone is the lung, and after ozone exposure, a multitude of morphological and biochemical alterations occur within this tissue (2). Among these ozone induced changes are alterations in lipid enzymes and fatty acids of lung tissue (3, 4). Examination of endobronchial washings containing surfactant revealed ozone exposure caused major changes in the fatty acid composition of phosphatidylcholine (5). The phospholipid component of surfactant is approximately 80-90% phosphatidylcholine (6).

For some years, it has been recognized that glucocorticoids promote lung development, maturation and surfactant production in fetal animals (7, 8), and these principles have been applied clinically to reduce the incidence of respiratory distress syndrome (RDS), a surfactant deficiency disease of premature infants (9). Recent reports suggest infant males develop RDS more frequently than do infant females (10), and male fetuses at risk of premature birth respond to prenatal corticosteroid therapy less favorably than do female fetuses (11). Thus a sex-related difference appears to be emerging regarding

lung response in RDS.

Receptors for glucocorticoids have been identified in fetal and adult tissue of many species (12, 13, 14, 15) and in a variety of cultured lung cells of fetal and adult origin (16). Corticosteroid treatment stimulates phosphatidylcholine synthesis by cultured fetal lungs and evidence suggests that de novo protein synthesis mediated by glucocorticoid receptor mechanisms are necessary for phosphatidylcholine production (17). Recently, androgen receptors have been identified in fetal and adult rabbit lung (18), an animal model used to investigate the possible male disadvantage with respect to fetal lung development and resultant RDS. In addition, receptors for androgens and estrogens have been demonstrated in adult rat lung tissue (19).

The similarities of sex-related factors being important for the development of RDS and certain expressions of ozone toxicity, that fatty acid and surfactant production are affected, and that androgen receptors are present in adult rat and fetal and adult rabbit lung tissue, led us to investigate if the normal adult mouse, our model animal for ozone toxicity studies, contains specific receptors for androgens and estrogens in lung tissue.

MATERIALS AND METHODS

Steroids: The following steroids were used in this study: 17β-hydroxy-17α-methyl-4, 9, 11-estratrien-3-one (R1881, methyltrienolone); 17β-hydroxy-4-androsten-3-one (T, testosterone); 17β-hydroxy-5α-androstan-3-one (DHT, dihydrotestosterone); 1,3,5-(10)-estratriene-3,17β-diol (E_2, estradiol); 5α-andro-

stan-3α,17β-diol (3α-diol); 5α-androstan-3β,17β-diol (3β-diol); 4-pregnene-3, 20-dione (progesterone); 3,4-bis-(hydroxy-phenyl)-3-hexene (diethylstilbestrol); 1,2,5-(10)-estratriene-3-ol-17-one (E_1, estrone); 4-pregnene-11β,21-diol-3, 20-dione (compound B, corticosterone). [17α-Me-^{3}H]-R1881 (Specific activity: 87 Ci/mmol), radioenert R1881, [1,2,4,5,6,7,-^{3}H(N)]-DHT (Specific activity: 143 Ci/mmol), [2,4,6,7,16,17-^{3}H(N)]-E_2 (specific activity: 151 Ci/mmol), [methyl-^{14}C]methylated bovine serum albumin (specific activity: 0.016 mCi/mg) and [methyl-^{14}C]methylated gamma-globulins (specific activity: 0.0088 mCi/mg) were purchased from New England Nuclear Corp. (Boston, MA). Other radioenert steroids were purchased from Steraloids (Pawling, NY).

Tissue and cytosol preparation: Adult CD_1 male mice were anesthetized with Metophane (Pitman - Moore, Inc., Washington Crossing, New Jersey), the chest cavity opened, and the lungs were perfused with ice cold 0.01 M Tris-HCl, 0.0015 M Na_2EDTA, 0.01 M Na_2MoO_4, pH 7.4, at 4°C (TEM buffer) via the right ventricle until blood was cleared and the lung tissue was pale white. Perfused lungs were trimmed of adhering tissue and placed in TEM buffer (4°C) until homogenized. Lung tissue from 10-20 mice, depending upon experimental design was pooled, blotted on absorbant paper, weighed and homogenized in a volume of TEM so that the final cytosol concentration was 8-10 mg/ml. The tissue was homogenized in a glass homogenizer bathed in crushed ice with three up and down passes of a motor driven teflon pestle. Homogenates were centrifuged at 1200 x g for 10 min at 2°C to sediment nuclei and cellular debris. The supernatant was then centrifuged at 105,000 x g for 90 min or 189,000 x g for 60 min at 2°C in a Beckman SW 50.1 rotor to yield a cytosol fraction. Protein content of the cytosol was determined by the method of Bradford (20).

Saturation analysis: Cytosol (0.25 ml), containing 1-2 mg protein, was mixed with increasing quantities of radioactive steroid in 0.05 ml TEM buffer. For androgen receptors, $[^3H]$-dihydrotestosterone was the ligand and concentrations ranged from 10 pM to 5 nM. For estradiol receptors, the ligand was $[^3H]$-estradiol and concentrations ranged from 20 pM to 1.5 nM. The final incubation volume was 0.3 ml. A parallel series of tubes, identical except for the presence of 100 nM non-radioactive steroid was assayed simultaneously to quantitate non-specific binding. The samples were incubated overnight at 4^oC. At the end of the incubation period, bound and free steroid were separated by the addition of 0.2 ml charcoal solution (0.625% Norit A, 0.0625% Dextran T-70 in TEM), vortexed and maintained at 4^oC for 10 min. Samples then were centrifuged at 2,000 x g for 15 min and the tritium content of 0.2 ml of the supernate was quantitated. Data was plotted according to Scatchard (21). Studies showed that binding equilibrium was attained by 2 hr and remained constant through the next 18 hr for both androgen and estrogen binding at 4^oC.

Specificity studies: The specificity of ligand binding to lung cytosol receptor was examined by sucrose density gradient centrifugation. A cytosol fraction (89,000 x g for 1 hr) was prepared from lungs of 10 adult CD_1 mice which had been castrated 18 hr earlier. The cytosol fraction was diluted so that the protein concentration was 8-10 mg/ml. To study androgen receptor specificity, five incubation tubes were prepared which contained 18 nM $[^3H]$-R1881. One tube contained the radioactive ligand only. Other tubes in addition to the radioactive steroid contained 10 nM radioenert R1881, 100 nM radioenert R1881, 10 nM radioenert competitor steroid and 100 nM radioenert competitor steroid, respectively. The ethanol diluent for the non-radioactive steroids was evaporated at 45^oC under a stream of nitrogen. These tubes were

vortexed repeatedly after the addition of the 50 μl TEM containing the radioactive R1881 to insure solution of the radioenert steroids. Cytosol (250 μl) was added and the samples vortexed and incubated at 4°C. The final incubation volume was 0.3 ml. After an incubation period of 4 hr, the samples were briefly exposed to 0.2 ml charcoal solution (625 mg charcoal, 62.5 mg dextran T-70 in 100 ml TEM) and centrifuged at 4°C for 15 min at 2,000 x g. The supernatant sample was carefully removed from the pelleted charcoal. Samples (0.2 ml) were layered on the top of linear 10-30% sucrose gradients in TEM (4.8 ml). Upon the gradient in the sixth tube was layered either [^{14}C]-gamma globulin or testosterone antibody to which was bound [^{3}H]-testosterone. The sixth tube served as the sedimentation marker and it was found that no difference in sedimentation existed for gamma globulin or the antibody-bound testosterone. The latter appeared much more stable and was readily available. Samples were centrifuged in a SW 50.1 rotor at 2-4°C for 16 hr at 189,000 x g. After centriguation, the samples were fractionated by collecting drops from the bottom of each tube with a Hoefer fractionator. Twelve 10 drop fractions followed by three 20 drop fractions were collected in plastic counting vials. Distilled water (0.2 ml) and PCS scintillation fluid (4.5 ml) were added and the radioactivity in each sample was determined in a Beckman LS 9000 spectrometer. Quench was determined by H number in the data reduction system and the results were presented as DPM. Bound activity of the 8-9 S region was used to compute competition. The 100 nM R1881 values were arbitrarily set at 100% competition, and other competition was based on this comparison (22). Ten nM and 100 nM R1881 samples were included with each centrifugation specificity study. Specificity of estradiol receptors was done in a similar manner.

<u>Receptor Quantitation</u>: In addition to Scatchard analysis for receptor

quantitation, lung receptor content was estimated from sucrose gradient studies (fractionated as outlined in the previous section). Sedimentation markers of [^{14}C]-BSA and [^{14}C]-gamma globulin were included. The method of Shain and Barnea was used to quantitate the 8-9 S peak (23). Some studies of postlabeling of the estrogen and androgen receptors were completed (24). In these instances, the lung cytosol was layered upon 10-30% sucrose gradient in TEM and centrifuged at 2°C for 16 hr at 189,000 x g. The resultant samples were fractionated into 18 ten drop fractions, tritiated ligand was added and the samples were incubated at 4°C for 18 hr. Parallel tubes also contained 100 nM nonradioactive steroid to evaluate nonspecific binding. Samples then were exposed to dextran/charcoal for 20 min, centrifuged and radioactivity determined.

RESULTS

Binding of the male sex steroid, dihydrotestosterone, to a male mouse lung cytosol component was studied. Saturation analysis for this binding over a wide range of [^{3}H]-dihydrotestosterone concentrations (19 pM - 22 nM) results in a curve which, when analyzed by the method of Scatchard, reveals the presence of a single binding component (figure 1). This binding site has a high affinity with an apparent Kd of 0.43 nM and a low capacity, 8.6 fmol of receptors per mg cytosol protein. [^{3}H]-dihydrotestosterone binding to male mouse lung cytosol was examined by 10-30% linear sucrose gradients (figure 2). A radioactive peak of [^{3}H]-dihydrotestosterone was apparent in the 8-9 S region of the sucrose gradient. When large molar excesses of non-radioactive testosterone (144 nM) or dihydrotestosterone (144 nM) were included in the incubation, radioactive dihydrotestosterone was displaced from the 8-9 S peak.

Specificity of this androgen binding component of male lung cytosol was determined by sucrose density gradient centrifugation. The ligand for the specificity determinations was the synthetic androgen, [^{3}H]-methyltrienolone (R1881). These studies are summarized in table 1 and show that the only steroids which compete to any major degree are the androgens, R 1881, dihydrotestosterone and testosterone. Progesterone, androgen metabolites (3α-ol and 3β-ol), and estradiol showed minimal competition.

Estradiol binding by male mouse lung cytosol was examined (figure 2). Scatchard analysis of [^{3}H]-estradiol binding data reveal a single class of binding sites of high affinity and low capacity. The Kd was 0.11 mM and in this study, 4.87 fmol of estradiol receptor/mg lung cytosol protein were demonstrated. [^{3}H]-estradiol binding to this macromolecule was examined by sucrose density gradient centrifugation (figure 4). A peak of tritium activity sedimented in the 8-9 S region, and this activity was displaced when incubations included increasing quantities of non-radioactive estradiol.

Estradiol binding specificity of the cytosol component was examined by sucrose gradient centrifugation also. These data are summarized in Table 2. Only non-radioactive estradiol and diethylstilbestrol demonstrated significant competition with [^{3}H]-estradiol binding to this putative receptor. At 10 nM concentrations, dihydrotestosterone, corticosterone, progesterone and estrone failed to show significant competition with [^{3}H]-estradiol binding.

The effect of castration on steroid receptor content of the adult male mouse lung was examined and these results are summarized in Table 3. Cytosol

from lungs of intact mice contained 14.11 ± 0.31 (mean ± SE, n =7) fmol androgen receptor per mg cytosol protein. Androgen receptor content appeared to decline slightly when quantitated at 6 and 12 hrs post-castration. At 18 hr post-castration, a value of 13.38 ± 0.57 fmol/mg protein was observed when [^{3}H]-R1881 was the labeled ligand. Although this value is slightly greater than that observed with intact mice (14.11 ± 0.31), the difference is not statistically significant. After 245 days castration, there were 12.30 ± 1.43 fmol androgen receptor/mg cytosol protein. Receptor content was determined by sucrose gradient analysis of lung cytosol prelabeled with [^{3}H]-R1881. Two experiments in which androgen receptor content was estimated by post-labeling techniques yielded comparable results (16.90 and 14.52 fmol/mg protein, data not shown) at 18 hrs post-castration. Examination of receptor content 18 hrs post-castration when [^{3}H]-DHT was the ligand demonstrated 10.25 ± 0.79 fmol/mg protein determined from Scatchard analyses and was comparable to results from prelabelled gradient studies when [^{3}H]-DHT was the ligand (11.71 and 9.55 fmol/mg protein, data not shown). From these studies, it appears that [^{3}H]-DHT and [^{3}H]-R1881 yield different results when estimating androgen binding in these mouse lung cytosol samples.

Estrogen receptor content was estimated from sucrose gradient studies with [^{3}H]-E_2. Levels of estradiol binding were much lower than those observed for androgen binding. Intact male mice lung cytosol contained 3.43 ± 0.35 (mean SE, n = 3) fmol estradiol receptor/mg cytosol protein. The concentration of receptor content gradually increased as the post-castration period lengthened to 57 hr in these studies (Table 3). A statistically significant increase was observed after 245 days castration when compared to control values ($P < 0.005$). A single estimation of estradiol receptor content by post-labeling yielded 2.99

fmol/mg protein in intact male mice and was not different from those values observed when analysis was completed with prelabeled cytosols.

DISCUSSION

The presence of sex steroid binding activities in cytosol fractions of adult male mouse lung has been demonstrated. Estrogen and androgen binding to these putative receptors exhibited specificity for the respective steroid, and demonstrated high affinity and low capacity for the ligand. Binding activity sedimented in the 8-9 S region of sucrose gradients of low ionic strength containing sodium molybdate.

Quantitation of androgen binding activity in the present study revealed 14.11 ± 0.31 fmol receptor/mg cytosol protein in the intact adult male mouse lung, a value similar to that reported for lung tissue from 100 day old male rats (approximately 9-10 fmol/mg protein) when [^{3}H]-DHT was the labeled ligand (19). A recent report observed 12.0 fmol androgen receptor/mg cytosol protein in adult male rabbit lung tissue (18). In this latter study [^{3}H]-R1881 was the label. Our estimation of estrogen receptor concentration is 3.43 ± 0.35 fmol estradiol receptor/mg lung cytosol protein in intact adult male mice. This value is comparable to the 4-5 fmol/mg protein previously reported for 100 day old rat lung tissue (19).

In our studies, castration resulted in a transient decreased androgen binding in lung cytosol of male mice. Values observed at 6 and 12 hr post-castration were lowest and may represent partial depletion of cytosol receptor

by translocation to the nuclear compartment. Further studies are necessary to support this possibility. At 18 hr post-castration, the observed value of androgen receptor content of lung was slightly greater than that noted for intact male mice. This is in contrast to results observed in the rat in which levels of androgen binding in the lung increase as the post-castration time increases, reaching a maximum of 13-14 fmol/mg protein at 18 hours, thereafter declining to a value of approximately 9 fmol/mg protein (19).

Estrogen binding in lung cytosol from castrate male mice showed a gradual increase as the post-castration time lengthened. After 245 days castration, the estradiol receptor content of lung cytosol was three-fold greater than that observed in intact adult mice.

Comparison of Kd for androgen binding from our studies (0.43 mM) agrees favorably with those observed in adult rabbit lung (0.42 mM) (18), genital skin fibroblasts (0.56 mM) (25) and samples of human benign prostatic hyperplasia (0.61 nM) (26). The adult rat lung value of 1.85 nM is slightly greater than that for the mouse. Kd's for the estrogen binding component in mice (0.11 nM, this study) and rats (0.1 nM) (19) are comparable.

From our specificity studies, we find that R1881 binds with a greater affinity to the lung receptor than does DHT, and this corresponds to observations in other systems of other investigators (25, 26, 27, 28, 29). However, we find that quantitation of androgen binding sites with [^{3}H]-R1881 in sucrose gradients yields a significantly greater value (13.38 fmol/mg) than obtained when binding is estimated by Scatchard analysis (10.25 fmol/mg) using [^{3}H]-DHT as the ligand. Comparison of [^{3}H]-DHT and [^{3}H]-R1881 in human genital

skin fibroblast cultures resulted in comparable values for binding sites when analyzed by Scatchard analysis (21).

The presence of sex steroid binding in a specific and saturable manner to these putative receptors within lung tissue does not identify the lung as a target tissue, but it suggests that sex steroids may be exerting specific biochemical influences. The observations of sex related responses in pathophysiological processes involving the lung, i.e., respiratory distress syndrome (10, 11) and ozone toxicity (1, 30), sex differences in lung scleroprotein concentrations (31), differences in enzyme activities (32), all suggest that sex hormones may be regulating particular biochemical events within this tissue and possibly through steroid receptor mechanisms. Much evidence suggests glucocorticoid action in the developing lung is receptor mediated and involves _de novo_ protein synthesis, and several recent reports support this possibility (17, 33).

Our interest in lung steroid receptors was aroused by the sex related differences in mice when ozone toxicity was studied. We have demonstrated sex steroid binding components in normal mouse lung cytosol and these binding components exhibit many properties of steroid receptors. It is uncertain if the sex related differences observed in ozone toxicity are directly related to sex steroid action within the lung; however, it has been demonstrated that estrogen stimulates phosphatidylcholine content of fetal rabbit lung and enzymes related to surfactant production (36). Ozone exposure alters lipid composition of endobronchial washings containing surfactant (5) and lung tissue lipid enzyme activities are affected (4). Preliminary experiments from our laboratory show a 50% reduction of androgen receptor content in cytosol from

lungs of mice exposed to ozone. It is possible that certain aspects of ozone toxicity and the additional risk of RDS expressed by the male fetus share common mechanisms which are influenced by steroid hormones.

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LEGENDS FOR FIGURES

Figure 1. [^{3}H]-dihydrotestosterone binding to male mouse lung cytosol analyzed by the method of Scatchard (21). Mice were castrated 18 hr prior to cytosol preparation. Lung cytosol (250 µl containing 1.4 mg protein) were incubated overnight at 4°C with increasing quantities of [^{3}H]-dihydrotestosterone. Incubation volume was 0.3 ml. Bound and free steroid were separated by charcoal/dextran and radioactivity in an aliquot of the supernatant was determined. Values were corrected for non-specific binding. Saturation data are represented in the insert. This experiment represents 8.6 fmol dihydrotestosterone mg cytosol protein and the apparent Kd is 0.43 nM. (B/F, bound ligand / free ligand).

Figure 2. Sucrose denisty gradient analysis of dihydrotestosterone binding by lung cytosol which was prepared from twenty adult male mice castrated 18 hr previously. Cytosol (250 µl containing 3.2 mg protein) was incubated with 10 nM [^{3}H]-dihydrotestosterone in the absence (○) or presence of 144 nM nonradioactive dihydrotestosterone (●) or 144 nM nonradioactive testosterone (□). Samples were treated as indicated in figure 1. Arrows indicate sedimentation markers (^{14}C-IgG, 7 S, ^{14}C-BSA, 4.6 S).

Figure 3. [^{3}H]-estradiol binding to male mouse lung cytosol analyzed by the method of Scatchard (21). Mice were castrated 18 hr prior to

cytosol preparation. Lung cytosol (250 μl containing 2.0 mg protein) were incubated for 4 hr at $4^{o}C$ with increasing quantities of $[^{3}H]$-estradiol. The incubation volume was 0.3 ml. Bound and free steroid was separated by charcoal/dextran and radioactivity in an aliquot of the supernatant was determined. Values are corrected for non-specific binding. Saturation data are represented in the insert. This experiment represents 4.88 fmol estradiol bound/mg cytosol protein and the apparent Kd is 0.11 nM.

Figure 4. Sucrose density gradient analysis of estradiol binding by lung cytosol which was prepared from ten male mice castrated 18 hr previously. Cytosol (250 μl) was incubated with 5 nM $[^{3}H]$-estradiol in the absence (○) or presence of non-radioactive estradiol (□ = 10 nM estradiol, △ = 50 nM estradiol and ● = 100 nM estradiol). After a 4 hr incubation, the samples were treated with 200 μl charcoal/dextran solution for 3 min. The samples were centrifuged and 200 μl of the supernatant was layered on a 10-30% continuous sucrose gradient in TEM and centrifuged for 18 hr at 185,000 x g.

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Table 1

Specificity of ^{3}H-methyltrienolone binding to mouse lung cytoplasmic androgen receptor

Competitor	Percent competition 10 nM	100 nM
Methyltrienolone	87	100
Dihydrotestosterone	69	82
Testosterone	48	76
Estradiol	18	35
3α-Diol	12	32
3β-Diol	8	20
Progesterone	7	13

Specificity of the androgen binding component of male mouse lung cytosol was determined by sucrose gradient centrifugation studies. The synthetic androgen, [^{3}H]-R1881, was the radioactive ligand in these studies. Non-radioactive steroid concentrations were 10 nM and 100 nM. Specific binding in the 8-9 S region of 10-30% linear sucrose gradients in TEM was quantitated. Competition by 100 nM non-radioactive R1881, which was included with each centrifugation study, was arbitrarily established as 100%, and competition generated by other competitor steroids was based on this value. Results from several experiments are summarized in this table.

Table 2

Specificity of ^{3}H-estradiol binding to mouse lung cytoplasmic estrogen receptor

Competitor	Percent competition 10 nM	100 nM
Estradiol	47	100
Diethylstilbestrol	--	80
Dihydrotestosterone	18	40
Corticosterone	22	39
Estrone	13	37
Progesterone	5	11

Specificity of the estrogen binding component of male mouse lung cytosol was determined by sucrose gradient centrifugation studies. [^{3}H]-estradiol was the radioactive ligand in these studies. Non-radioactive steroid concentrations were 10 nM and 100 nM. Specific binding in the 8-9 S region of 10-30% linear sucrose gradients in TEM was quantitated. Competition by 100 nM non-radioactive estradiol, which was included with each centrifugation study, was arbitrarily established at 100%, and competition generated by other competitor steroids was based on this value. Results from several experiments are summarized in this table.

Table 3

Sex Steroid Receptor Content in Adult Male Mouse Lung Cytosol[a]

	fmol/mg cytosol protein (mean ± SE)
Androgen[b]	
Intact (7)	14.11 ± 0.31[e]
6 hr castrate (3)	9.67 ± 1.21
12 hr castrate (6)	6.87 ± 0.57[e]
18 hr castrate (9)	13.38 ± 0.57[f]
245 day castrate (4)	12.30 ± 1.43
Androgen[c]	
18 hr castrate (4)	10.25 ± 0.79[f]
Estrogen[d]	
Intact (3)	3.43 ± 0.35[g]
18 hr castrate (7)	3.43 ± 0.27
24 hr castrate (3)	4.65 ± 0.68
57 hr castrate (4)	5.32 ± 0.72
245 day castrate (3)	11.69 ± 0.12[g]

[a] Statistical differences were determined by one-way analysis of variance (33,34). Numbers within parentheses indicate number of determinations within each group.

[b] $[^3H]$-R1881 was the ligand and receptor content was quantitated from sucrose gradients (23).

[c] $[^3H]$-DHT was the ligand and receptor content was quantitated by Scatchard analysis (21).

[d] $[^3H]$-E2 was the ligand and receptor content was quantitated from sucrose gradients (23).

[e] $P<0.005$

[f] $P<0.005$

[g] $P<0.005$

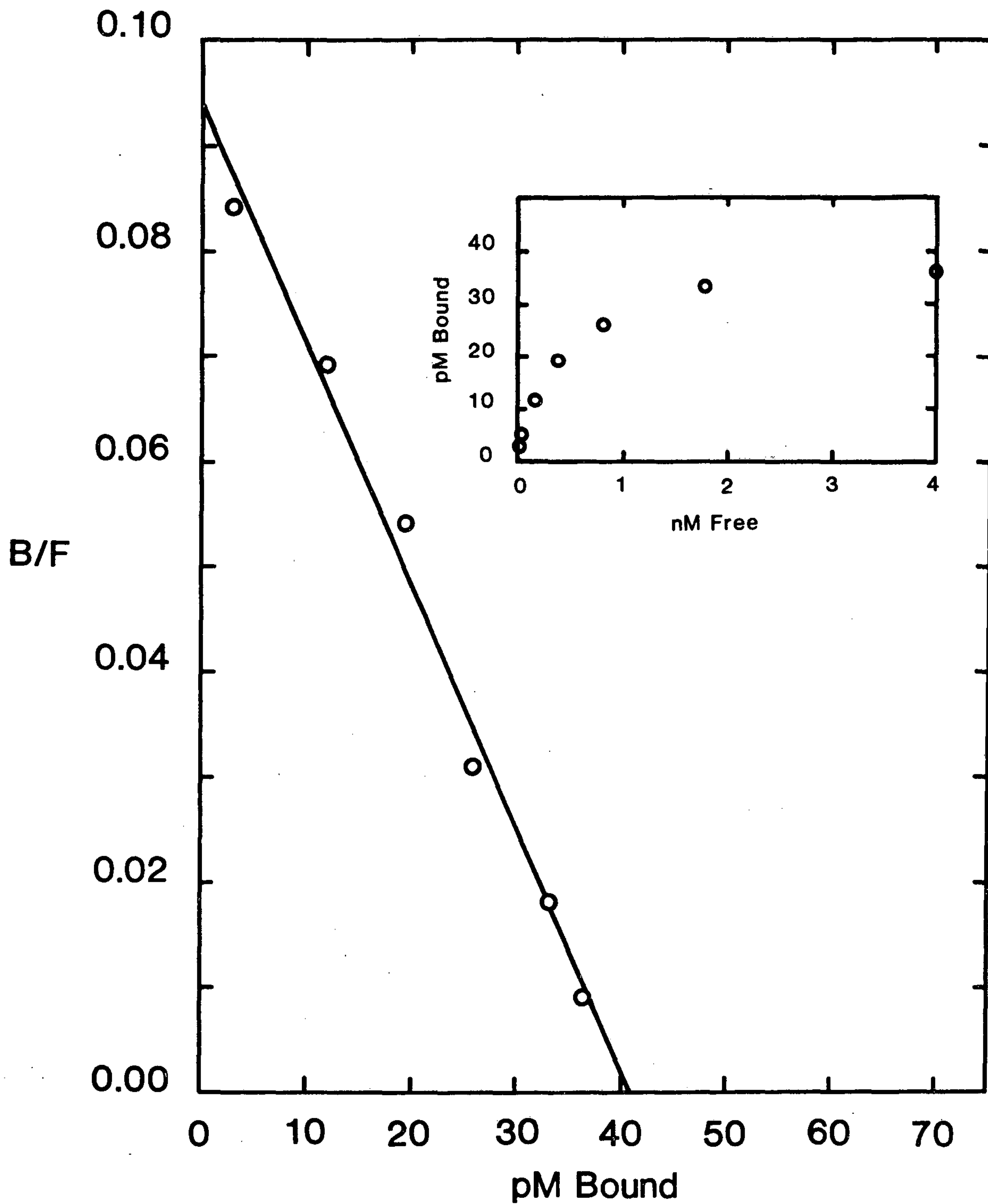

Figure 1 Connell & Carr

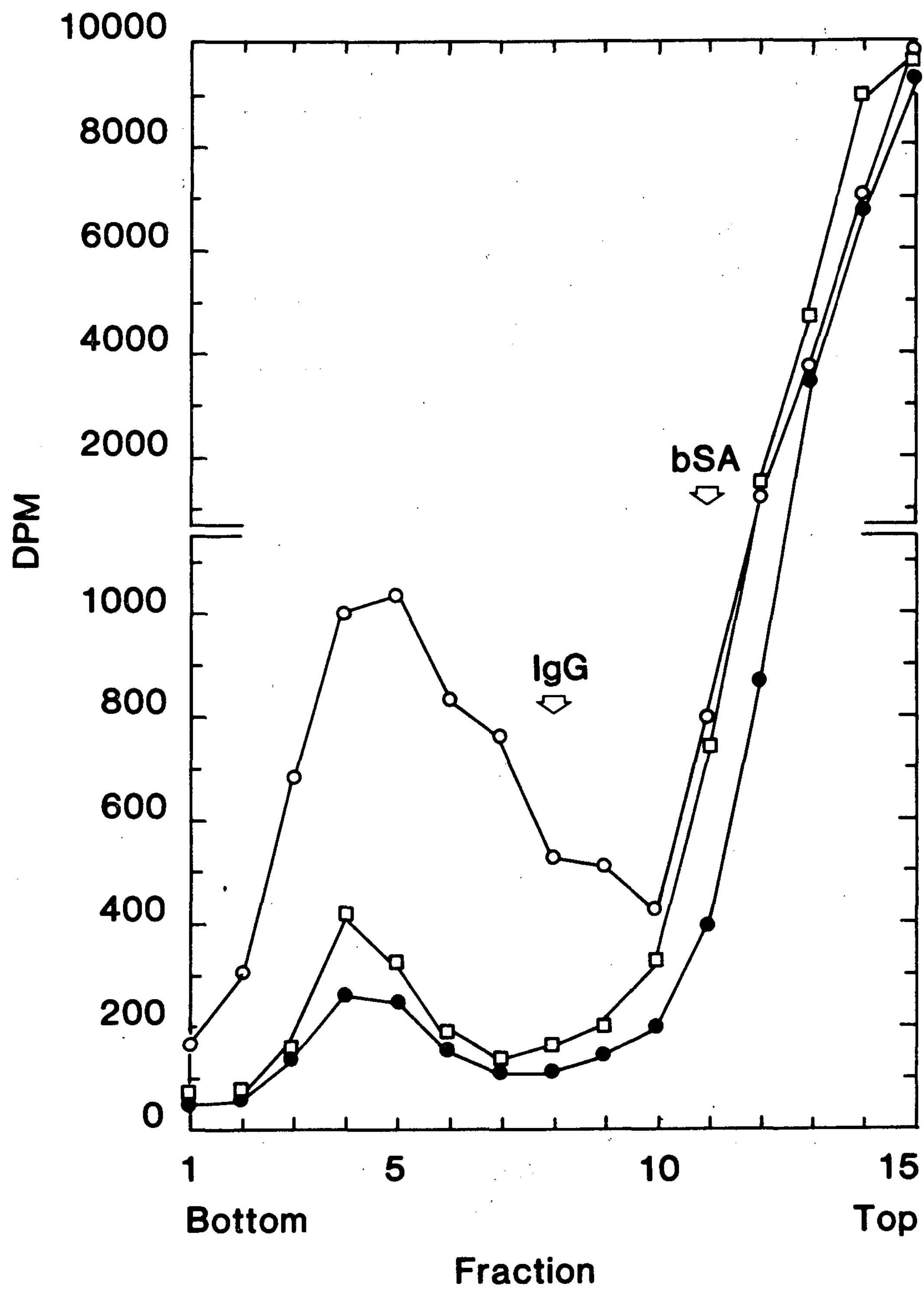

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Figure 2 Connell & Carr

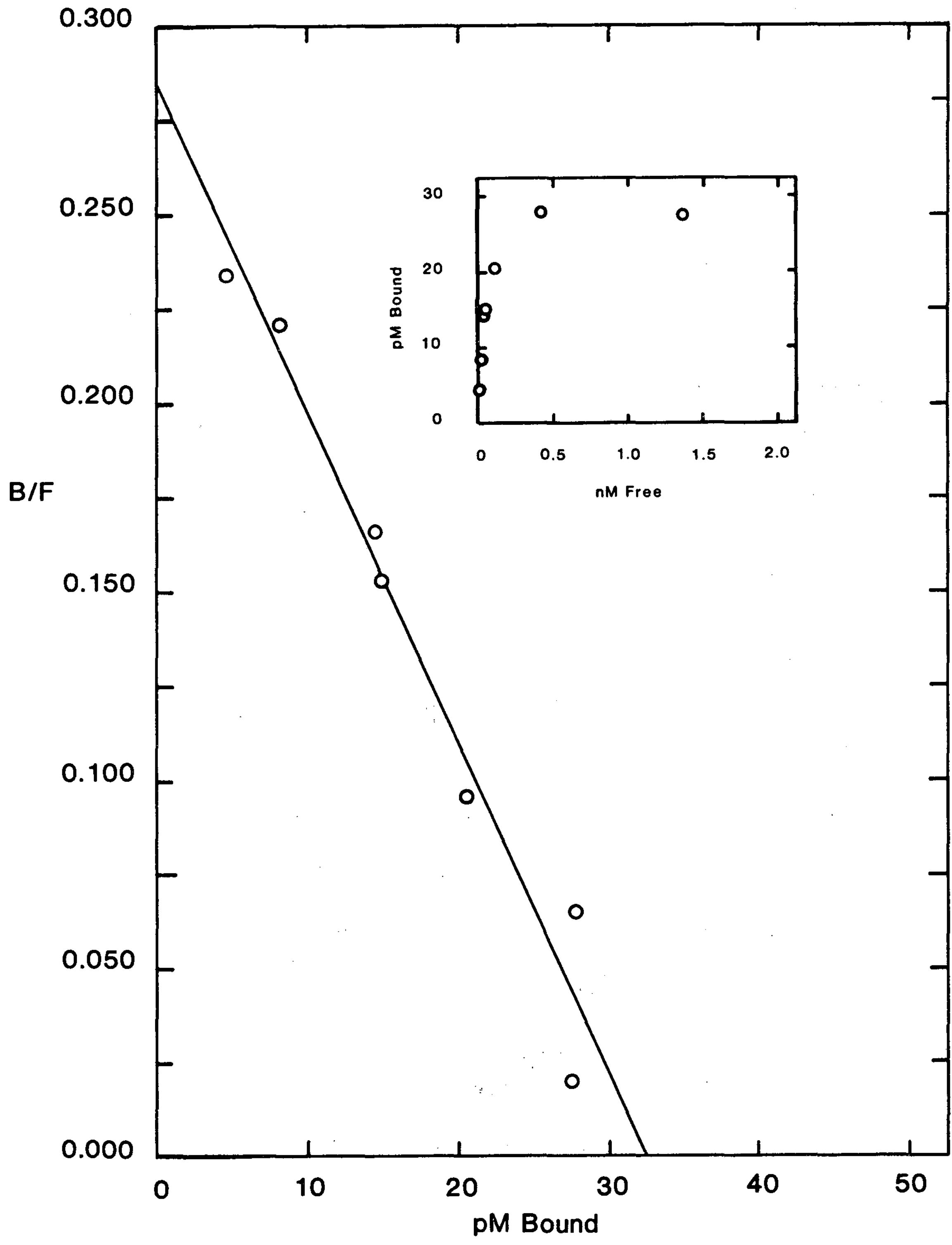

Figure 3 Connell & Carr

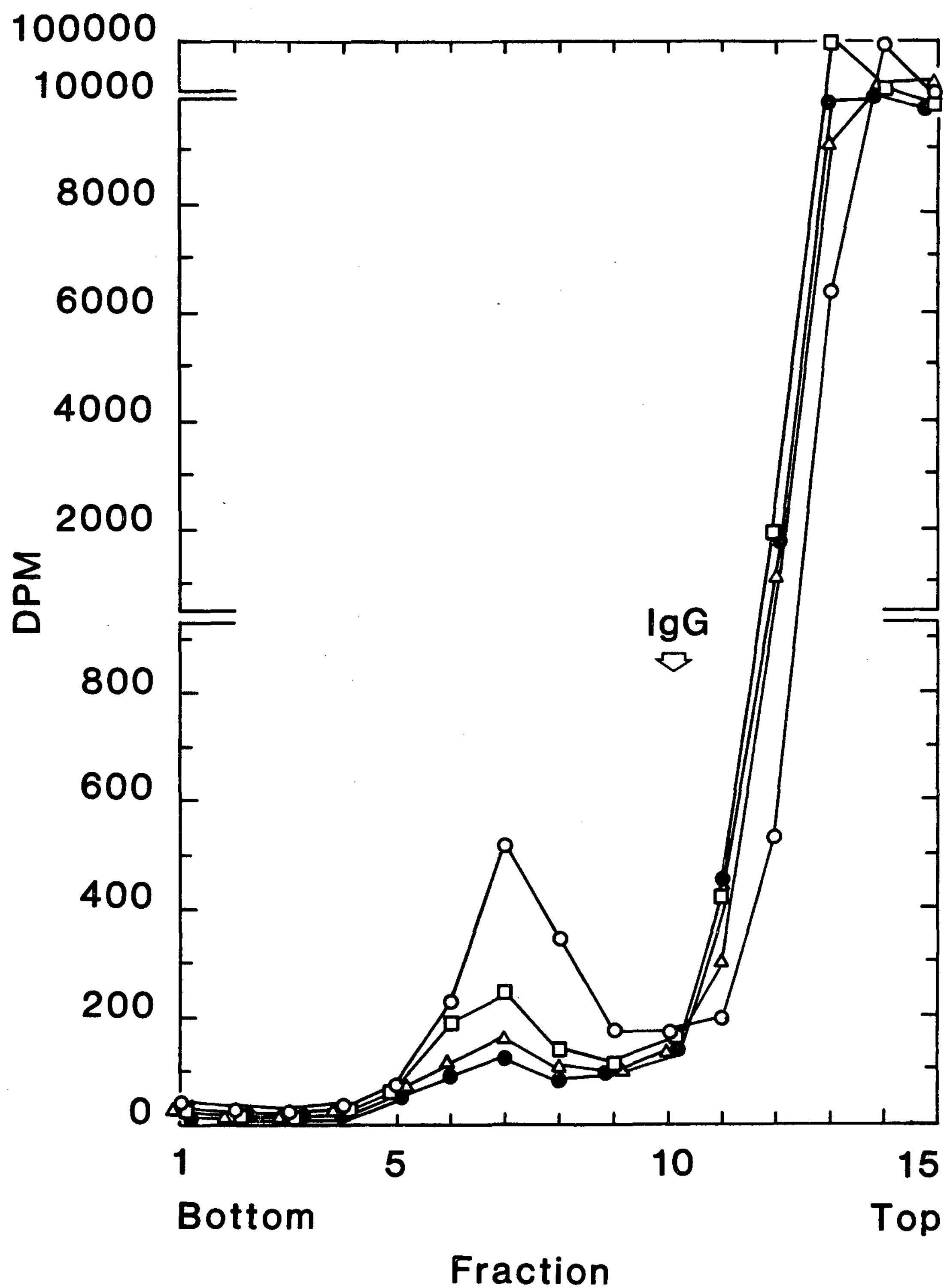

Figure 4 Connell & Carr

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