Journal of Steroid Biochemistry, 1978, Vol. 9, pp. 983–988. Pergamon Press. Printed in Great Britain

SEX STEROID BINDING PROTEIN (SBP) IN DOG PLASMA*

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(*Received* 18 *July* 1977)

SUMMARY

A specific sex steroid-binding protein (SBP) is demonstrated in the plasma of mongrel dogs. For mature females (5 yrs old) the equilibrium constants of association (K_a) at 4°C are $1.44 \pm 0.26 \times 10^8\ M^{-1}$ for 5α-dihydrotestosterone (DHT)‖, $4.20 \pm 2.90 \times 10^7\ M^{-1}$ for testosterone (T), and $7.72 \pm 5.29 \times 10^6\ M^{-1}$ for oestradiol-17β (E_2). For mature males, the K_a for DHT is $1.22 \pm 0.34 \times 10^8\ M^{-1}$. Electrophoresis in 5% polyacrylamide gels containing [^{3}H]-DHT showed the presence of an active molecular species with R_F 0.32. Dehydroepiandrosterone, oestrone, androstenedione, progesterone, and cortisol do not significantly compete with DHT for the steroid binding site. The rate constant of DHT dissociation at 4°C is $0.009\ s^{-1}$ ($t_{1/2} = 77$ s). The concentration of SBP (μg DHT bound/100 ml plasma, mean ± S.D.) in mature females (9.0 ± 1.3) is significantly higher ($P < 0.01$) than in mature males (6.2 ± 0.8). No sex difference was observed in immature dogs, the values are in the range of mature females (8.7 ± 1.4 for males and 8.2 ± 0.4 for females). The results indicate that the dog should be a valuable animal model for studying the physiological role of SBP.

INTRODUCTION

Sex steroid hormones are bound to a specific protein (SBP) present in the β-globulin fraction of plasma in many species including man [1, 2]. The physiological role of SBP is still unknown; however, it has been suggested that the protein is involved in the mechanism of steroid hormone action by keeping steroid in the circulation and therefore controlling the availability of the unbound hormone for target tissues. For instance, an inverse relationship between the metabolic clearance rate of testosterone and the SBP levels in various species including man [3, 4] and Rhesus monkey [5] suggests that the protein regulates the concentration of unbound steroid available for target organs. In order to study the general physiological role of SBP, various animal model systems are required. Some years ago, it was reported that SBP could not be detected in dog plasma [6] and that testosterone and androstenedione were metabolized at the same rate in males and females [7]. However, in a more recent publication [8], the existence of an androgen-binding protein with no affinity for 17β-estradiol was demonstrated in male dog plasma by a two-phase equilibrium partition system. In order to clarify these contradictory reports and also to establish the dog as a proper model system for studying the physiological role of SBP, we have re-examined the properties of dog plasma. In this paper, we present evidence for the existence of dog SBP and we describe its binding properties.

MATERIALS AND METHODS

Animals. Healthy adult mongrel dogs of both sexes (12 of each) weighing between 15 and 20 kgs, and puppies (3–12 months, 5 males and 4 females) were used. Blood samples were obtained by puncture of a leg vein. The plasma was prepared by centrifugation at 1500 rpm for 10 min and stored at −20°C.

Reagents and chemicals. [1,2-^{3}H-5α-dihydrotestosterone (30–40 Ci/mmol) was purchased from New England Nuclear and Research Product International Corp. DEAE-cellulose filter paper discs (DE-81, 2.3 cm diameter) were purchased from Reeve Angel & Co., and acrylamide was obtained from Eastman Kodak Co. All other chemicals were reagent grade.

Radioactivity measurements. Aqueous samples or filter discs were added to vials containing 10 ml of scintillant (5 gm PPO/liter toluene) and counted in a Beckman LS-100C scintillation counter. Gels were sliced transversally into 1.3 mm slices, added to 10 ml of scintillant, and left overnight at room temperature

* This work was supported by USPHS grant HD-07190 (P.H.P.).

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‖ Abbreviations. SBP, sex steroid-binding plasma protein; dSBP, dog SBP; hSBP, human SBP; nSBP, *Macaca nemestrina* SBP; cSBP, cat SBP; rSBP, rabbit SBP; DHT, 5α-dihydrostestosterone; T, testosterone; E_2, 17β-oestradiol.

before counting to extract all radioactive steroids into the scintillant. Tritium counting efficiency was 45%.

Polyacrylamide gel electrophoresis. Gel electrophoresis was carried under steady-state binding conditions as previously described [9, 13] at 4°C and pH 9.5. Analytical disc gel electrophoresis was performed by the method of Davis[10] as modified by Shuster[11]. Polymerization of the 5% acrylamide gels (100 mm × 5 mm inside diameter) was performed at room temperature in the presence of 1 nM [^{3}H]-DHT. The gels were pre-electrophoresed at 4°C for 2 h at a constant current (2 mA/gel). Aliquots (5 μl) of plasma samples were diluted to 50 μl with 10 mM Tris buffer containing 10% glycerol and 1 nM [^{3}H]-DHT. Samples were incubated for 30 min at 25°C and cooled to 0°C. One microliter of 0.05% bromophenol blue solution was added and the sample was applied to the gel and electrophoresed until the tracking dye had migrated approximately 7 cm (2.5 h at 4°C, 2 mA/gel). Gels were severed at the dye front and sliced transversely into 1.3 mm thick slices and were counted.

Determination of SBP concentration. The concentration of SBP was measured with the filter assay according to procedures previously described [12, 13]. The filter efficiency was determined to be 59 ± 3% (S.D.) after correcting to 100% bound steroid in the assay using $K_a = 1.44 \pm 0.26 \times 10^8\ M^{-1}$. All values were corrected for the 41% loss of DHT–dSBP complex occurring during the standard assay procedure. This procedure consists of incubating 0.5 μl aliquots of plasma 50-fold diluted with 10 mM Tris-Cl, pH 7.4, with 25 nM [^{3}H]-DHT in the presence or absence of 100-fold molar excess of radioinert DHT at 25°C for 30 min and cooled to 0°C. Aliquots of 100 μl are applied onto DEAE-cellulose filter paper discs followed by ten 1-ml washes. SBP concentration was calculated from the binding capacity and expressed either as μg DHT bound/100 ml plasma or nM, assuming mole/mole stoichiometry of binding. In view of the low value of K_a (DHT), only 78% saturation of dSBP was achieved under standard assay conditions (25 nM [^{3}H]-DHT). All values were corrected to 100% saturation of dSBP.

Rate of dissociation The rate of dissociation of the DHT–SBP complex was measured by the ammonium sulfate method as described by Heyns and DeMoor[14]. 1.5 ml plasma was added to 5 ml of 10 mM Tris buffer containing 0.14 M NaCl and 8 ng ^{3}H-DHT (10^5 c.p.m./ng) and incubated for 1 h at 4°C. At zero time, 1 ml of buffer containing 8 μg of radioinert DHT was added. Aliquots (0.5 ml) were removed at 15 s intervals and immediately mixed with 0.5 ml of a saturated ammonium sulfate solution for 5 s using a Vortex mixer. After the last aliquot was taken, the tubes were centrifuged at 6000 *g* for 10 min at 4°C. An aliquot (0.4 ml) of the supernatant was removed and added to counting vials containing 10 ml of scintillation fluid. Non-specific binding was corrected by the amount of steroid not precipitated by ammonium sulfate 8 h after the addition of radioinert steroids. The quantity of radioactivity precipitated at the various 15 s time intervals was subtracted from this value to obtain the specifically bound steroid. The rate constant and $t_{1/2}$ were obtained by plotting the log of SBP activity remaining as a function of time.

Specificities studies. A standard DHT-binding curve was determined as described previously [13]. Two different concentrations of the competing steroid were used to determine the relative binding affinity with respect to DHT. Standard steroid solutions were prepared by dissolving in benzene–ethanol (9:1). Evaporation of aliquots was carried out at room temperature under N_2.

Determination of equilibrium constants. Scatchard analyses were carried out with [^{3}H]-DHT in the presence or absence of a potentially competing radioinert steroid as previously described [13, 15]. The equilibrium constant of the competing steroid is obtained from the following equation:

$$K_p = K_d(1 + K_I[I]$$

where: K_p = apparent equilibrium constant of dissociation in the presence of competing steroid. K_d = apparent equilibrium constant of dissociation of DHT–SBP complex. $[I]$ = competing steroid. K_I = apparent equilibrium constant of association of competing steroid.

Intersection of Scatchard plots at abscissa indicates competition at same binding site. The equilibrium constants were calculated by linear regression analysis of Scatchard plots. No difference in K_a's were observed when the data were fitted using either the linear regression analysis of Scatchard plots or the Newton–Raphson non-linear treatment of the observation equation as previously shown [13]. The ± values represent the upper and lower limits of the slope from a single Scatchard plot at the 95% confidence level.

The competitive Scatchard method for the determination of $K_a(T)$ and $K_a(E_2)$ proved very valuable in these studies. Normally, these constants can be directly determined using the corresponding radioactive steroids. However, because of the relatively low values of the equilibrium constants of association and interference with nonspecific binding, the Scatchard plots were not linear and difficult to interpret. The use of the competitive method not only resolved this problem but also provided evidence about the specificity of the binding site. Both methods, however, have been shown to yield identical constants for hSBP and rSBP [20].

RESULTS AND DISCUSSION

Polyacrylamide gel electrophoresis

Electrophoresis of plasma samples in gels containing [^{3}H]-DHT produced two major radioactive peaks

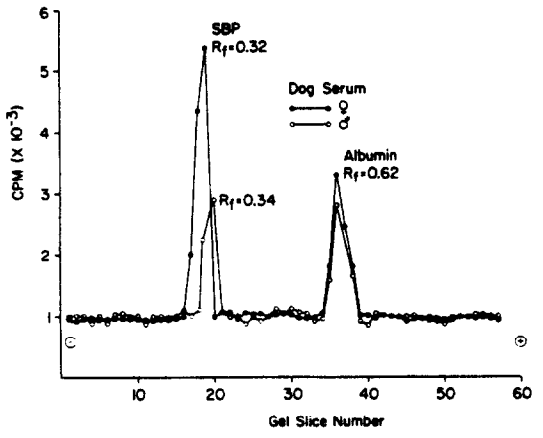

Fig. 1. Polyacrylamide gel electrophoresis of dog plasma. 5 μl aliquots of plasma were diluted to 50 μl with 10 mM Tris buffer, pH 7.4, containing 10% glycerol and 1 nM [^{3}H]-DHT. Samples were incubated for 30 min at 25°C and cooled to 0°C. One microliter of 0.05% bromophenol blue solution was added, and the sample was applied to pre-electrophoresed gels. When the tracking dye had migrated approximately 7 cm (2.5 h at 4°C, 2 mA/gel), the gels were cut at the dye front, sliced, and counted.

as shown in Fig. 1. The fast-moving peak is albumin as shown by its R_F of 0.62 corresponding to albumin when gels are stained. The same R_F is obtained for human serum albumin (unpublished results) and *Macaca nemestrina* albumin [13] under the same conditions. The slow migrating peak of R_F 0.32 in female plasma and 0.34 in male plasma is defined as SBP. The slight variation in R_F between the sexes results from the slicing procedure and does not reflect the presence of two different binding proteins. Similar R_F values were obtained for other SBP's when electrophoresed in the same way: hSBP, $R_F = 0.30$ [16]; nSBP, R_F 0.34 [13]; cSBP, $R_F = 0.34$ [16]. When [^{3}H]-DHT is omitted from the gel during the polymerization step, no radioactive peak can be detected when dog and cat sera pre-incubated with [^{3}H]-DHT are electrophoresed, explaining why Corvol and Bardin [6]have reported the absence of SBP in these animals. In the light of our results, other species such as donkey, chick, pigeon, duck and hamster should be re-examined using steady-state gel electrophoresis.

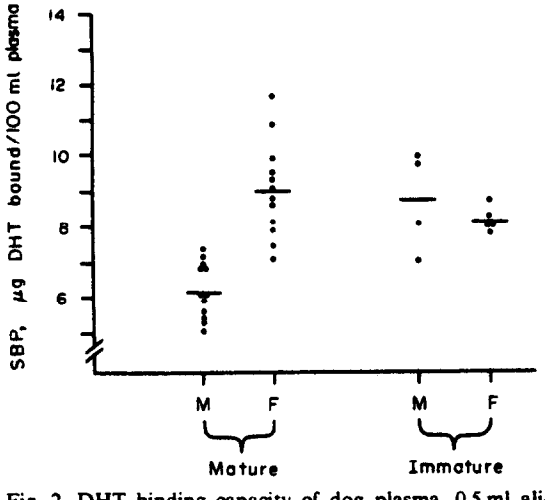

Fig. 2. DHT binding capacity of dog plasma. 0.5 ml aliquots of plasma, 50-fold diluted with 10 mM Tris–Cl, pH 7.4, were incubated with 25 nM [^{3}H]-DHT in the absence or presence of 2500 nM of radioinert DHT. Aliquots of 100 μl were applied onto DEAE-cellulose filter paper discs followed by ten 1-ml washes. Values were corrected for a 59% filter "efficiency" and normalized to 100% saturation of SBP (see methods).

Concentration of SBP is dog plasma

Figure 2 shows the mean concentration (± S.D.) of SBP expressed as μg DHT bound/100 ml plasma for 12 mature females, 9.0 ± 1.3, and twelve mature males, 6.2 ± 0.8. The female values are significantly higher than the males ($P < 0.01$). Immature dogs have relatively high levels of SBP in the range of the mature female, but no sex differences are observed (8.7 ± 1.4 for males, and 8.2 ± 0.4 for females). The DHT binding capacity of plasma from male and female dogs is in the range of the *Macaca nemestrina* [13] but both are much higher than that of human plasma. Our reported mean value of 6.2 ± 0.8 in the adult male dog is higher than that reported by Carstensen *et al.*[8], 3.88 ± 0.67. Variation in methodology could perhaps explain this difference.

Steroid binding affinity and specificity

Table 1 describes the relative binding affinity of various steroids to dSBP with respect to [^{3}H]-DHT.

Table 1. Specificity of Dog SBP

Steroids	Relative binding* affinity (%)
DHT	100.00
Testosterone	25.5 31.9
Estradiol-17β	6.8 5.2
Estrone	1.2 1.9
Progesterone	1.8 1.6
DHA	1.60 1.40
Androstenedione	< 0.1 < 0.1
Cortisol	< 0.1 < 0.1

* A standard DHT binding curve was determined by incubating 0.5 ml samples of a 50-fold diluted plasma with 3 nM [^{3}H]-DHT and increasing concentrations of radioinert DHT (2 nM to 20 nM). The relative binding affinity was estimated by incubated samples in the presence of two different concentrations of radioinert competing steroid and comparing to the standard curve. The two values reported for each competing steroid represent relative binding affinity at the two different concentrations of competitor.

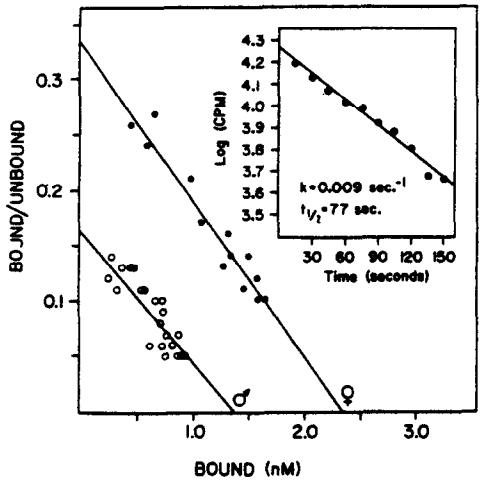

Fig. 3. Determination of equilibrium and dissociation rate constants. 0.5 ml samples of 100-fold diluted plasma were incubated with [^{3}H]-DHT (2 nM to 20 nM) for 30 min at 25°C and cooled to 0°C. 100-μl aliquots were counted and applied to filters. Values were corrected for 59% filter "efficiency". (O——O) male plasma; (●——●) female plasma. Inset, rate of dissociation of the DHT–dSBP complex at 4°C. The ammonium sulfate method of Heyns and deMoor[14] was used.

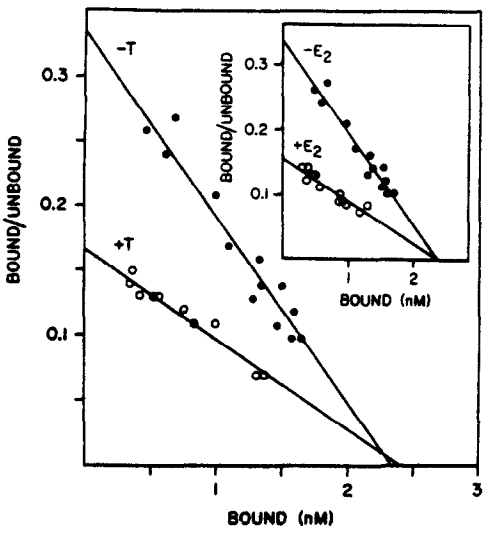

Fig. 4. Determination of equilibrium constants of association of T and E_2, to dSBP by the competitive Scatchard method. Same conditions as described in Fig. 3. (●——●) [^{3}H]-DHT; (O——O) [^{3}H]-DHT plus 30 nM radioinert T in every tube. Inset. (●——●) [^{3}H]-DHT; (O——O) [^{3}H]-DHT plus 169 nM radioinert E_2 in every tube.

The results indicate that dSBP is mainly an androgen binding protein although 17β-estradiol is shown to significantly displace [^{3}H]-DHT. This is further substantiated in Figs 3 and 4 from which the following equilibrium constants of association (K_a) determined by the filter assay can be calculated. For female plasma, K_a(DHT) = 1.44 ± 0.26 × 10^8 M^{-1}, K_a(T) = 4.20 ± 2.90 × 10^7 M^{-1}, and $K_a(E_2)$ = 7.72 ± 5.29 × 10^6 M^{-1}. For male plasma, K_a(DHT) = 1.22 ± 0.34 × 10^8 M^{-1}. The value of K_a(DHT) at 4°C (100-fold diluted plasma) determined by equilibrium dialysis is 2.98 ± 0.90 × 10^8 M^{-1} indicating that both methods yield similar values. The competitive Scatchard plots (Fig. 4) reveal that DHT, T, and E_2 compete for the same binding site. A good correlation exists between the displacement studies shown in Table 1 and the equilibrium data of Figs 3 and 4. For example, displacement suggests that DHT binds 3.5 times better than T and 17 times better than E_2 which is in agreement with the K_a's; K_a(DHT) is 3.4 times greater than K_a(T) and 19 times greater than $K_a(E_2)$. Table 2 compares dSBP with other SBP's examined in our laboratory. The data indicate that although dSBP is an androgen binding protein, its affinity for DHT and T is much less than hSBP, nSBP and rSBP, and its value is much lower than that reported previously [8].

* dSBP is apparently unstable *in vitro* at 37°C. It was not possible to determine the K_a for DHT and T by equilibrium dialysis under our present conditions at 37°C. The value of 1.33 × 10^7 M^{-1} for K_a(T) is calculated by taking 1/3 of the K_a(T) at 4°C (Table 2). We have found a threefold decrease in K_a(T) for hSBP in raising the temperature from 4°C to 37°C (unpublished results). Similar results have been published by others [see Ref. 1].

Chapdelaine[7] has suggested that the high conversion of blood testosterone to androstenedione in dog plasma is due to low binding of testosterone. Using an average concentration of 11 nM testosterone in normal male dog plasma [8], a mean concentration of 300 nM (6.2 μg DHT bound/100 ml plasma) for dSBP in the male dog (Fig. 2), and assuming K_a(T) for dSBP of 1.33 × 10^7 M^{-1} at 37°C* as well as an albumin concentration similar to man with the same testosterone binding affinity (6.1 × 10^{-4} M, K_a(T) = 2.02 × 10^4 M^{-1} at 37°C, [ref. 18]), we calculate (see Appendix) that only 23% of testosterone is bound to dSBP under these conditions. However, when we perform a similar calculation for human male plasma ([SBP] = 6.3 × 10^{-8}, K_a(T) at 37°C = 7.8 × 10^8 M^{-1}) at 11 nM T or 34 nM T [18], we find that 76% and 69% of T is bound to hSBP respectively. Therefore, even though the concentration of SBP is approximately 5 times higher in the male dog as compared to the human male, the specific binding of testoster-

Table 2. Equilibrium and dissociation rate constants at 4°C

	DHT		T	E_2
	K_a × 10^{-9} (M^{-1})	k (min^{-1})	K_a × 10^{-9} (M^{-1})	K_a × 10^{-9} (M^{-1})
hSBP	2.76§	0.009‡	1.20*	0.67‡
nSBP†	2.04	0.013	0.41	0.24
rSBP[c]	2.15	0.239	0.33	0.003
dSBP	0.14	0.533	0.04	0.008

* Lebeau *et al.*[17].
† Pétra and Schiller[13].
‡ Mickelson[16].
§ Mickelson and Pétra[12].

one to SBP is much lower. This is caused by the low value of K_a(T) in dog plasma. In addition, Chapdelaine[7] also found that the metabolic clearance rates of testosterone and androstenedione are not significantly different in dogs in contrast to humans. Although we find that testosterone binds about 300 times better than androstenedione to dSBP (Table 1), the binding difference is probably not expressed physiologically again because of the low value of K_a(T). Our data are therefore consistent with the results and interpretations of Chapdelaine.

The difference in DHT affinity between dSBP and hSBP or nSBP is in part due to the first order rate constant of dissociation of the DHT–nSBP and DHT–hSBP complexes [13, 16]. However, the rate of association of the steroid must be greater in rabbit SBP since the DHT–rSBP complex displays a high rate of dissociation while its K_a(DHT) has also a high value. The explanation of this phenomenon will undoubtedly be important in the understanding of the steroid binding specificity of SBP in relation to its biological function. We believe that the study of the structure–function relationship of these various SBP's will be helpful in achieving this goal. To this end we have already purified and chemically characterized hSBP [15, 19] and rSBP [20]. Finally, it can be noted that all of these proteins bind 17β-estradiol to varying degrees at the same site as T and DHT [13, 16, and Fig. 4]. This property is probably due to an inherent physico-chemical characteristic of the androgen binding sites of these proteins which can more or less accomodate 17β-estradiol. Whether or not this general estradiol binding property will have physiological meaning in the case of a particular SBP will inevitably depend upon the magnitude of the rate constants of association and dissociation of the estradiol–SBP complex in that particular species. Since the affinity of sex steroids to SBP in dog plasma is markedly different from that of other species studied, this animal model should prove valuable in deciding on whether SBP has a common physiological function in all the species which comprise it, or has a unique role in each species.

Acknowledgements—The authors wish to thank Dr. W. L. Heinrichs for his support and encouragement. The authors also thank the vivarium at the University of Washington for providing dog plasma.

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APPENDIX

T binding to SBP is described by

$$K_A = \frac{[ST]}{[S][T]},$$

and T binding to albumin by

$$K_B = \frac{[AT]}{[A][T]}.$$

where:

K_a = equilibrium constant of association of T to SBP
[ST] = bound T to SBP
[S] = unbound SBP
[T] = unbound T
K_b = equilibrium constant of association of T to Alb.
[AT] = bound T to Alb
[A] = unbound Alb
[T] = unbound T

The conservation equations are:

$$[S] = [S]_t - [ST]$$
$$[A] = [A]_t - [AT]$$
$$[T] = [T]_t - [ST] - [AT]$$

where:

$[S]_t$ = total SBP
$[A]_t$ = total Alb
$[T]_t$ = total T

[ST] is the meaningful solution to the equation

$$A[ST]^3 + B[ST]^2 + C[ST] + D = 0$$

where:

$$A = -K_A^2 + K_A K_B$$
$$B = [S]_t(2K_A^2 - K_A K_B) + [T]_t(K_A^2 - K_A K_B) + [A]_t K_A K_B + K_A - K_B$$
$$C = [S]_t[T]_t(-2K_A^2 + K_A K_B) - K_A^2[S]_t - K_A - K_A K_B[A]_t$$
$$D = K_A^2[S]_t^2[T]_t$$