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CORTICOSTEROID BINDING PROTEIN IN THE PLASMA OF *MACACA NEMESTRINA**

HARVEY S. SCHILLER†, JAMES W. LANGLEY and PHILIP H. PÉTRA
Departments of Laboratory Medicine, Obstetrics and Gynecology and Biochemistry, University of Washington, Seattle, WA 98195, U.S.A.

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SUMMARY

A specific cortiscosteroid binding protein (nCBG) was identified in the plasma of *Macaca nemestrina*. The properties of this protein are similar but not identical to those of the corticosteroid binding globulin described in human plasma (hCBG or transcortin). The similar features include identical electrophoretic mobility in polyacrylamide gels, high affinity and low capacity for corticosteroids, relatively broad specificity, and no difference in molar concentration of CBG between the sexes. Also, corticosterone, cortisol, and progesterone compete for the same binding site of nCBG. The dissimilar features are as follows: the binding site of nCBG accommodates a 17 α-hydroxyl group poorly, the equilibrium constant of association (K_A) of corticosterone for nCBG is 5–10 times higher than that of cortisol, and the molar concentration of CBG is higher in *M. nemestrina* than in humans. These findings indicate that *Macaca nemestrina* is a good animal model to study the physiological role of CBG.

INTRODUCTION

In recent years steroid binding proteins in the blood of numerous species have been extensively studied (see Westphal[1] for review). One such protein identified in man was shown to have high affinity and low capacity for corticosteroids [2–4]. The protein was shown to bind cortisol, corticosterone, progesterone and a few closely related steroids [5, 6]. Although CBG has been extensively studied in humans, relatively few studies have been reported in nonhuman primates. The physiological significance of this protein has not yet been established. The purpose of these studies is to develop an animal model system closely related to humans for studying the function of CBG. In this report, we demonstrate the presence of a steroid binding protein in the plasma of *Macaca nemestrina* (*M. nemestrina*) which has many properties similar to those of CBG characterized from human plasma.

MATERIALS AND METHODS

Reagents and chemicals. Radioinert steroids were purchased from Steraloids Inc. or Sigma Chem. Co. [1,2-^{3}H]-corticosterone (S.A. 54.5 and 8.0 Ci/mmol) and [1,2-^{3}H]-cortisol (S.A. 40 Ci/mmol) were purchased from New England Nuclear Corp. DEAE cellulose filter paper disc (DE81, 2.3 cm diameter) were purchased from Reeve Angel and Co.

Monkey plasma. Venous blood was collected in heparinized glass tubes from healthy adult *M. nemestrina* housed at the University of Washington Primate Center. The plasma was stored at −20°. In every instance endogenous steroids were initially removed by gently shaking the plasma at 25° for 0.5 h with Norit A charcoal (50 mg/ml) as described by Heyns and DeMoor [7]. The suspension was centrifuged twice at 2000 *g* for 10 min, and the plasma was stored at 4° until use. Plasma was diluted with 10 mM Tris–Cl (pH 7.4, 4°) immediately before each experiment.

Polyacrylamide gel electrophoresis. Analytical disc gel electrophoresis was performed according to Davis [8] as modified by Shuster [9]. Five percent gels, prepared at pH 8.9 in the presence [10] or absence of tritiated steroid, were cast into tubes (100 mm × 5 mm i.d.) and allowed to polymerize at 25° for 2 h. The gels were cooled to 4° and preelectrophoresed for 2 h at constant current (2 mA/gel). Samples were prepared by mixing 10 μl of plasma (pretreated with charcoal) with 170 μl of pH 8.9 gel buffer and 20 μl of glycerol and incubated with steroid overnight at 4°. Five microliters of 0.05% bromophenol blue solution in glycerol was added to the top of the gel and mixed with 50 μl aliquots of the sample applied to the gel. The samples were electrophoresed for 2.5 h at 4° until the tracking dye had migrated approximately 7 cm into the gel. Gels were sliced at the dye front, frozen on dry ice, cut transversely into 1.3 mm slices, and counted. Gels were also stained with Buffalo Black to compare the migration of albumin and other proteins with CBG.

Determination of CBG concentration in plasma. The concentration of CBG in the plasma of the *M. nemestrina* was determined as previously described [11]

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† Address correspondence to this author at the Department of Laboratory Medicine, SB-10.

with the following modifications. The "efficiency" of the filter to retain the nCBG–corticosterone and nCBG–cortisol complexes was 82%. The "efficiency" of the filter assay was determined under conditions where 97–99% of the steroid was bound to nCBG. The charcoal treated plasma was diluted 100 fold and 0.5 ml was incubated overnight with 70 pmol of [^{3}H]-corticosterone (S.A. 8.0 Ci/mmol). Under these conditions 95–98% of the nCBG binding sites are saturated with [^{3}H]-corticosterone (as determined by the law of mass action); no correction was made for this underestimation of total binding sites. Non-specific binding was determined in the presence of the [^{3}H]-corticosterone and a 150-fold molar excess of radioinert corticosterone. The concentration of nCBG was calculated from the binding capacity assuming mol/mol stoichiometry of binding as has been shown for hCBG [12, 13]. The concentration of nCBG was expressed as μg corticosterone bound/100 ml plasma or as μM. Radioactivity was measured as previously described [14].

Specificity studies. The relative ability of various steroids to compete with [^{3}H]-corticosterone for the binding site of nCBG was determined by the method of Vermeulen and Verdonck [15]. A standard curve was obtained by incubating 0.5 ml of 200-fold diluted plasma from a female monkey with 0.3 pmol [^{3}H]-corticosterone (S.A. 54.5 Ci/mmol) and increasing concentration of radioinert corticosterone (0–0.03 nmol).

Determination of equilibrium constants of association (K_A). The K_A's of nCBG for cortisol and corticosterone were determined by the method of Scatchard [16] at 4° in 10 mM Tris-Cl buffer, pH 7.4. The K_A's were determined from the Scatchard plots by linear regression analysis, calculated by the least squares method. The separation of bound and unbound steroid to nCBG was carried out either by the filter assay method or by equilibrium dialysis [15, 17]. The filter assay method was performed as described above. The samples containing various amounts of radioactive steroid were equilibriated for 48 h at 4°. The amount of steroid bound to nCBG was calculated from the radioactivity bound to the filter after correcting for both the "efficiency" of the filter assay (82%) and the efficiency of counting tritium. The unbound steroid was determined by subtracting the bound steroid from the total. Equilibrium dialysis was performed by adding various amounts of radioactive steroid and 15 ml of buffer to 20 ml capacity scintillation vials, and then placing dialysis bags, each containing 1 ml of 100-fold diluted plasma, into the vials. The vials were allowed to equilibrate for 48 h at 4°.

The equilibrium constant of association of nCBG for cortisol was also determined by incubating diluted plasma with various amounts of [^{3}H]-corticosterone necessary to generate a Scatchard plot and with a fixed amount of radioinert cortisol ("competitive" Scatchard analysis). The K_A can be calculated from the equation [18]: $K_P = K_D\ (1 + K_I[I])$ where K_P = equilibrium constant of dissociation in the presence of the competing steroid, K_D = equilibrium constant of dissociation of corticosterone, $[I]$ = concentration of competing radioinert steroid and K_I = equilibrium constant of association of the competing steroid. The intersection of both lines on the abscissa represents a case of competitive inhibition and indicates that the radioinert steroid and corticosterone bind at the same site on the protein.

Determination of dissociation rates. Dissociation rates were determined as previously described using the filter assay [14]. Charcoal treated plasma samples were diluted 100 fold and incubated overnight with either 17.5 nM [^{3}H]-corticosterone or 3 nM [^{3}H]-cortisol. Five hundred-fold molar excess of the corresponding radioinert steroid was added at zero time. Aliquots (100-μl) were removed at timed intervals and the amount of CBG–steroid complex was measured. Binding was also measured after 24 h when dissociation of the trace should essentially be completed; the value was used to correct for nonspecific binding. The rate constant, k, is calculated from the slope of the line obtained by plotting the log of the remaining CBG bound radioactivity as a function of time.

RESULTS

Equilibrium constants of association of nCBG. We determined the equilibrium constants of association

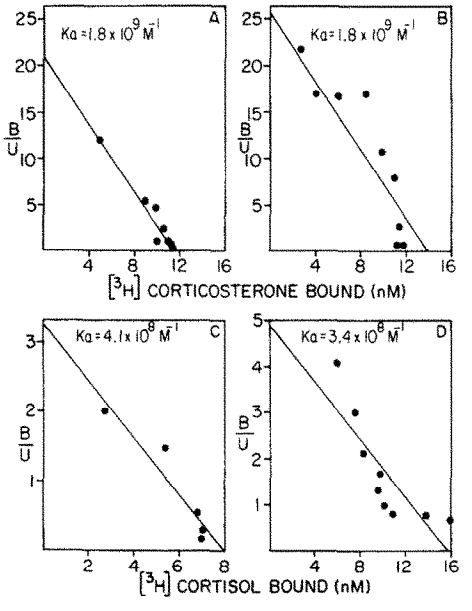

Fig. 1. Equilibrium constants of association of nCBG for corticosterone and cortisol determined by the filter assay and equilibrium dialysis techniques. The procedures were performed as described in the "Methods" on a single plasma sample from a female *M. nemestrina*. The radioactive steroid was either [^{3}H]-corticosterone (S.A. 8.0 Ci/mmol) in A and B or [^{3}H]-cortisol in C and D. Equilibrium dialysis on the left (A and C); filter assay method on the right (B and D). Each Scatchard analysis was performed on a different day. The plasma sample was freshly diluted 100 fold on the day of analysis.

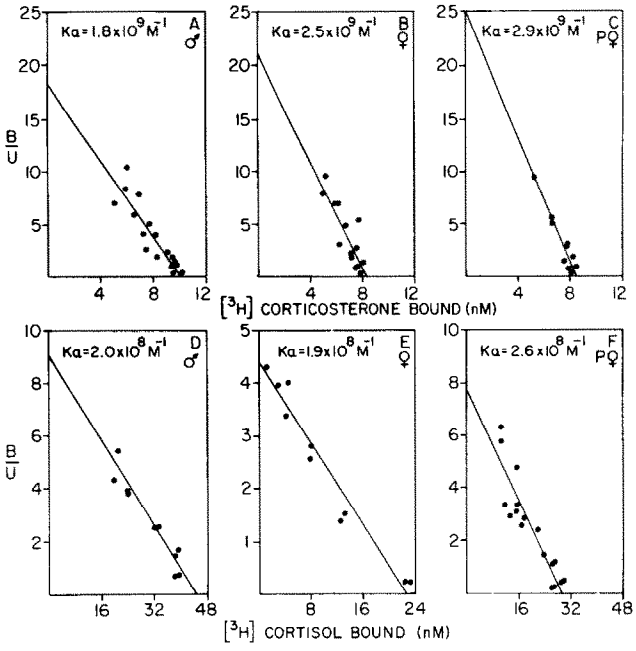

Fig. 2. Equilibrium constants of association of nCBG for corticosterone and cortisol in plasma from a male and a pregnant and another nonpregnant female. The procedures were performed as described in Fig. 1 with the filter assay technique. Male (♂), A and D; female (♀), B and E; and pregnant female (P♀), C and F. The radioactive steroid was either [^{3}H]-corticosterone (A, B, C) or [^{3}H]-cortisol (D, E, F). Plasma was diluted 200 fold for A, B and C, 50 fold for E and F and 35 fold for D.

of nCBG (at 4° and pH 7.4) for corticosterone and cortisol in the same sample using both the DEAE cellulose filter paper assay and equilibrium dialysis techniques to separate bound and unbound steroid (Fig. 1). The K_A for corticosterone binding to nCBG in diluted plasma from a nonpregnant monkey was 1.8×10^9 M^{-1} for both the filter assay and equilibrium dialysis techniques. The K_A for cortisol binding to nCBG in the same sample was 3.4 and 4.1×10^8 M^{-1} for the filter assay and equilibrium dialysis techniques, respectively.

We also determined these equilibrium constants of association in diluted plasma from a male, a pregnant and another nonpregnant female (Fig. 2). The respective K_A's of nCBG for corticosterone were 1.8, 2.9 and 2.5×10^9 M^{-1}. The respective K_A's of nCBG for cortisol were 2.0, 2.6 and 1.9×10^8 M^{-1}.

Competition of corticosterone, cortisol and progesterone for the binding site of nCBG. "Competitive" Scatchard analyses were performed as described in the "Methods" with plasma from a nonpregnant female (same sample used as in Fig. 1). Conversion of both lines at the abscissa in Figs 3A and 3B indicates that corticosterone, cortisol and progesterone compete for the same binding site on nCBG. The equilibrium constants of association for cortisol and progesterone calculated from these plots are 3.0×10^8 M^{-1} for both. The cortisol constant agrees well with that determined above (see Figs 1C and 1D).

Specificity studies. The relative ability of various steroids to displace [^{3}H]-corticosterone from nCBG in plasma is shown in Table 1. In the case of nCBG, corticosterone and 11-deoxycorticosterone displace [^{3}H]-corticosterone best while cortisol competes rather poorly. This is in sharp contrast to hCBG but similar to CBG in *Macaca mulatta*. The presence of a 17α-hydroxyl group on other steroid molecules also reduces their binding to nCBG, e.g. progesterone vs 17α-hydroxyprogesterone, and prednisone or prednisolone.

Polyacrylamide gel electrophoresis. *M. nemestrina* plasma was electrophoresed in 5% polycrylamide gels. When [^{3}H]-corticosterone was preincubated with the plasma before electrophoresis only one major peak of radioactivity with an R_F of 0.5 was observed (Fig.

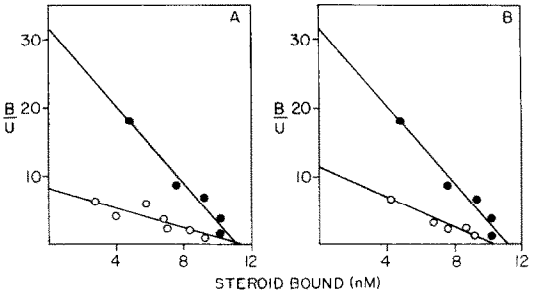

Fig. 3. "Competitive" Scatchard analyses. The procedure is described in the "Methods". The same plasma sample as in Fig. 1 was diluted 100 fold and incubated with [^{3}H]-corticosterone (solid circles) and with 10 nM radioinert cortisol (open circles, A) or 5 nM radioinert progesterone (open circles, B). The equilibrium constant of association (upper line) of nCBG for corticosterone is 2.8×10^9 M^{-1} as compared with 1.8×10^9 M^{-1} determined in Fig. 1.

Table 1. Relative Binding of Steroids to CBG in Plasma*

Steroid	Relative binding (%)		
	M. nemestrina†	*M. mulatta*‡	human§
corticosterone	100	100	100
11-deoxycorticosterone	96		50
11-dehydrocorticosterone	90		
progesterone	72	43	55
17α-hydroxyprogesterone	30	7	60
cortisol	23	20	100
11-deoxycortisol	21	17	70
testosterone	12	6	20
20α-hydroxy-5-pregnen-3-one	9		
dihydrotestosterone	8		
prednisone	7		
cortisone	5	4	25
aldosterone	1	2	10
prednisolone	<1		100
dexamethasone	<1		
pregnenolone	<1		
4-androstene-3,17β-diol	<1		
estradiol-17β	<1		4
estrone	<1		

* Determined as described in the "Methods". † This work. ‡ Ref. [19]. § Ref. [20].

4A). In the presence of 150-fold molar excess of radioinert cortisol (Fig. 4B) or testosterone (Fig. 4C) this peak was greatly diminished but not abolished. However, the peak can be abolished if the plasma is incubated with [^{3}H]-corticosterone in the presence of either 150-fold molar excess of radioinert corticosterone or 3700-fold excess of cortisol before electrophoresis (data not shown).

In the next series of experiments gels were prepared with or without [^{3}H]-cortisol [10]. Samples incubated with [^{3}H]-cortisol and 70-fold molar excess of radioinert cortisol gave a peak at R_F = 0.5 (Fig. 5A, solid line), but no peak was observed when gels did not contain [^{3}H]-cortisol (Fig. 5A, dotted line). Similar results were obtained when samples were incubated with radioinert testosterone (Fig. 5B). The same R_F of 0.5 was obtained for hCBG (Fig. 5C).

Rates of dissociation of CBG–steroid complexes. The rate constants of dissociation were calculated from the data of Fig. 6. The data suggest that the mechanism of dissociation follows first-order kinetics, and the rate constants were obtained from the slope of the line calculated by the method of least squares. These were as follows: nCBG–corticosterone

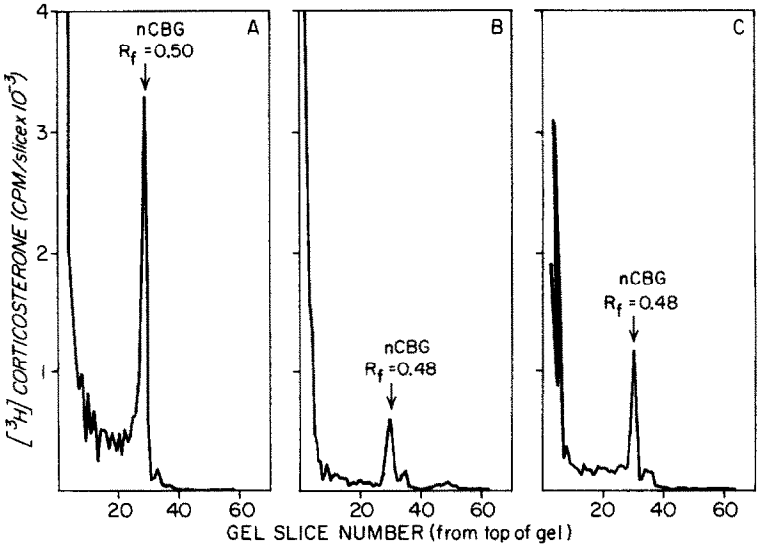

Fig. 4. Analytical polyacrylamide gel electrophoresis of plasma from a female *M. nemestrina*. The procedure is described in the "Methods". The gels were prepared without radioactive steroid. Plasma was incubated with: 0.7 μM [^{3}H]-corticosterone, S.A. 8.0 Ci/mol, (A); [^{3}H]-corticosterone, and a 150-fold molar excess of radioinert cortisol (B) or [^{3}H]-corticosterone and a 150-fold molar excess of radioinert testosterone (C). Albumin R_F = 0.66.

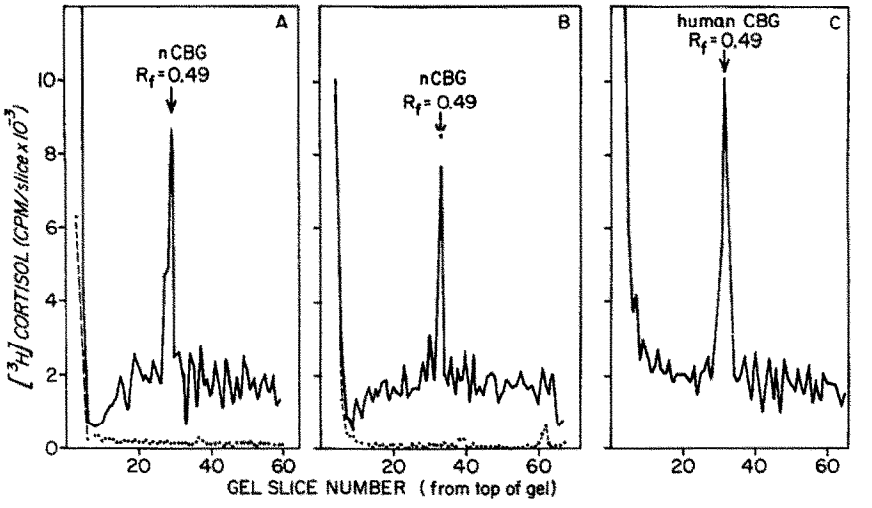

Fig. 5. Analytical polyacrylamide gel electrophoresis of plasma with [^{3}H]-cortisol. The procedure is described in the "Methods". The gels were prepared either with (solid lines) or without (dotted lines) [^{3}H]-cortisol. *M. nemestrina* plasma was incubated with 0.4 nM [^{3}H]-cortisol and a 70-fold molar excess of either radioinert cortisol (A) or radioinert testosterone (B). Human plasma (C) was incubated with 0.4 nM [^{3}H]-cortisol. All samples were electrophoresed simultaneously.

$k = 0.020\ \text{min}^{-1}$ ($t_{\frac{1}{2}} = 35.4$ min); nCBG–cortisol, $k = 0.161\ \text{min}^{-1}$ ($t_{\frac{1}{2}} = 4.3$ min); hCBG–corticosterone, $k = 0.062\ \text{min}^{-1}$ ($t_{\frac{1}{2}} = 11.1$ min); hCBG–cortisol, $k = 0.076\ \text{min}^{-1}$ ($t_{\frac{1}{2}} = 9.1$ min).

Concentration of CBG. The values for the concentration of CBG in monkey plasma are shown in Fig. 7. The mean binding capacities for male, pregnant and nonpregnant females were 45.6, 40.4 and 46.0 μg corticosterone bound/100 ml plasma, respectively.

DISCUSSION

This report demonstrates the presence of a steroid binding protein in the plasma of *M. nemestrina* which is similar but not identical to human CBG. The similarities include the following: identical electrophoretic mobility in polyacrylamide gels, high affinity and low capacity for corticosteroids, and relatively broad specificity. In this regard the nCBG binding site recognizes a variety of corticosteroids and progestins.

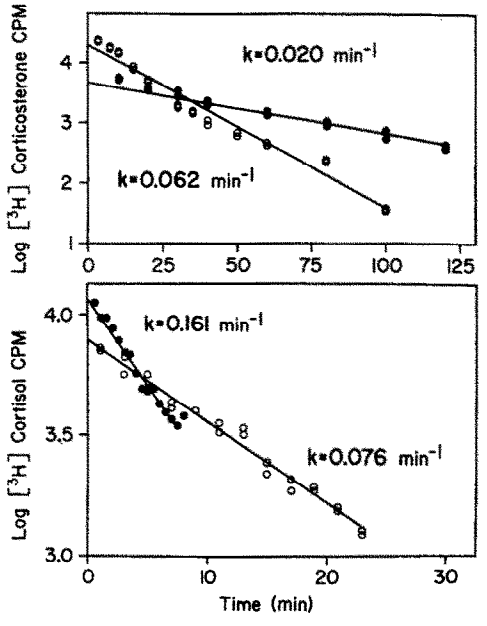

Fig. 6. Dissociation rates of CBG–steroid complexes. The procedure is described in the "Methods". One hundred-fold diluted *M. nemestrina* (closed circles) or human (open circles) plasma was incubated with 17.5 nM [^{3}H]-corticosterone (top) or 3 nM [^{3}H]-cortisol (bottom). Five hundred-fold molar excess of the corresponding radioinert steroid was added at zero time. Rate constant = k.

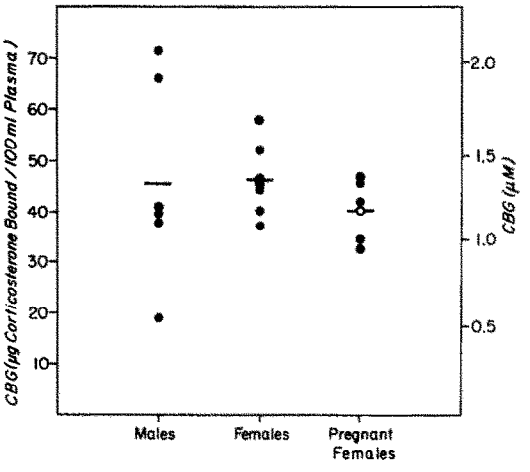

Fig. 7. Concentration of CBG in the plasma from male and pregnant and non-pregnant female *M. nemestrina*. 0.5 ml of 100-fold diluted plasma was incubated overnight at 4° with 70 pmol of [^{3}H]-corticosterone in the presence and absence of a 150-fold molar excess of radioinert corticosterone. The procedure and calculations are described in the "Methods". Mean values are depicted with horizontal bars. Plasma was drawn at 112 days of pregnancy except for the one sample (empty circle) which was drawn at 130 days of pregnancy. Values are expressed either as μg of corticosterone bound/100 ml plasma or as μM, assuming mol/mol stoichiometry of binding between corticosterone and nCBG.

Like hCBG, testosterone binds less well and estrogens do not bind at all. Furthermore, cortisol, corticosterone and progesterone compete with each other for the same binding site.

The main differences are primarily in the relative values of the K_A's for corticosterone and cortisol binding and in the total concentration of the protein in plasma. The K_A of nCBG is 5–10 times higher for corticosterone than for cortisol. This differs from hCBG where the values are similar or slightly higher for corticosterone [1, 21, 22]. Other steroids also having a 17α-hydroxyl group such as prednisolone and 17α-hydroxyprogesterone do not bind well to nCBG (Table 1) in contrast to hCBG. Reduction of the C-20 of C-3 ketone groups also reduces binding, e.g. corticosterone vs 20α-hydroxy-5-pregnen-3-one and testosterone vs 4-androstene-3, 17β-diol. Furthermore, elimination of the 11-hydroxyl group appears to be of less importance for nCBG binding than for hCBG, e.g. corticosterone vs 11-deoxycorticosterone and cortisol vs 11-deoxycortisol. Whether or not all of these differences in binding properties between nCBG and hCBG are due to specific structural differences in the binding sites of these proteins is yet to be determined.

The data on the rates of dissociation for the CBG–steroid complexes in conjunction with gel electrophoresis data reveal additional information about the specificity of binding. For example we show that the rate of dissociation of the nCBG–cortisol complex is markedly faster than that of the nCBG–corticosterone complex. This finding is consistent with all the equilibrium data (K_A and relative binding affinities) as well as the electrophoretic data of Figs 4 and 5. That is, the faster the rate of dissociation of nCBG–steroid complex, the lower the equilibrium constant of association and the lower the relative binding affinity with respect to corticosterone. This observation is further supported by data relating hCBG and nCBG. In this regard cortisol binds less with nCBG than hCBG as compared with corticosterone binding (Table 1). The K_A for corticosterone is slightly higher ($1.8–2.5 \times 10^9$ M^{-1}) for nCBG than $0.8–1.0 \times 10^9$ M^{-1} reported for hCBG [21, 23], and the dissociation rate is slower with nCBG than hCBG (nCBG, $t_{\frac{1}{2}} = 35.4$ min vs hCBG, $t_{\frac{1}{2}} = 11.1$ min). Furthermore, the K_A for cortisol is lower for nCBG than that reported for hCBG [1], and the dissociation rate is faster with nCBG than hCBG (nCBG, $t_{\frac{1}{2}} = 4.3$ min vs hCBG, $t_{\frac{1}{2}} = 9.1$ min). These results strongly suggest that factors which control the rate of dissociation are important in the determination of specificity [14, 24].

Our findings in *M. nemestrina* are in agreement with those who have studied CBG in other species of monkey. Murphy reported only 20% relative binding of cortisol to CBG in *M. mulatta* as compared with the binding of corticosterone [19]. The equilibrium constant of association of CBG in *M. mulatta* for cortisol was reported to be 3.0×10^8 M^{-1} at 4° [22]. The K_A for cortisol at 4° in *M. nemestrina* is comparable. At 37° the K_A for corticosterone binding in *M. mulatta* is 1.4×10^8 M^{-1} which is about five times that for cortisol binding [22]. These data suggest that CBG is similar in the two species of macaques. The binding capacity of nCBG in *M. nemestrina* is similar for males and females, as is the case in humans [21], but the values are higher in *M. nemestrina*: 43 μg/100 ml for nCBG vs 24 μg/100 ml for hCBG [25, 26]. Seal and Doe reported the cortisol binding capacity of CBG in monkey sera (species not noted) to be 36.8 μg/100 ml [27]. They also studied the corticosteroid binding capacity in various species of monkeys [28]. The binding capacity of CBG for cortisol was similar for man and the old world monkeys studied, *M. mulatta* (Rhesus) and *Cercopithecus aethiops sabeus* (green monkey), but was distinctly lower (1.4–5.8 μg/100 ml) in several new world monkeys. The binding capacity of CBG for corticosterone was slightly higher (32.4 μg/100 ml) in *Cercopithecus aethiops sabeus* but was not reported in *M. mulatta*. Subsequently, the cortisol binding capacity in nonhuman primates at 4° has been reported by others to be: 34 μg/100 ml in *M. mulatta* [21]; 33.4 μg/100 ml in female *Papio papio* (baboon) [29]; 24.1 μg/100 ml in males, and 25 μg/100 ml in females and 18.1 μg/100 ml in pregnant female *M. mulatta* [30].

It should be pointed out from our results that the determination of the binding capacity of steroid binding proteins should be performed with the ligand of highest affinity and lowest dissociation rate. This is true for equilibrium as well as nonequilibrium methods. In the case of nCBG, if one attempts to saturate nCBG with [^{3}H]-cortisol, the binding capacity may be underestimated using either method as a consequence of the low K_A and high rate of dissociation. This problem can be circumvented by using the high binding and slow dissociating ligand corticosterone. Furthermore, a steroid binding protein may not be detected if nonequilibrium methods are used in conjunction with a ligand having a rapid rate of dissociation. Such is the case for the sex steroid binding protein which was reported to be absent in dog [31] but recently found using steady-state gel electrophoresis (Tabei, Mickelson and Pétra, to be published).

Finally, we also report similar equilibrium constants of association for male and pregnant and nonpregnant females. This suggests that the same protein exists during pregnancy in *M. nemestrina*. Similar conclusions have been made regarding *P. papio* [29] and when comparing human maternal and fetal CBG [32]. Recently we have identified a sex steroid binding protein in *M. nemestrina* [14]. These data taken together with those reported here make this species of monkey ideal for studying the physiological role of both proteins in the same animal.

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