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Influence of an Aziridine Precursor on the *In Vitro* Binding Parameters of Rat and Ovine Corticosteroid-Binding Globulin (CBG)

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ABSTRACT. Aziridines are highly reactive alkylating compounds used in cancer treatment. *Salsola tuberculatiformis* Botsch., which causes prolonged gestation in sheep and contraception in rats, contains a very labile hydroxy-phenylaziridine or its precursor. A less labile analogue, 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride (Compound **I**), was synthesized and has been shown to be contraceptive in rats and to be stabilized by corticosteroid-binding globulin (CBG). The current study compared the binding parameters of rat and ovine CBG and evaluated the effect of the aziridine precursor, Compound **I**, on these parameters. K_d and B_{max} values of 0.646 and 578 nM for corticosterone binding to rat CBG and 0.577 and 19.8 nM for cortisol binding to sheep CBG, respectively, were measured. In competitive binding studies with rat plasma, K_i values of 3.48 nM, 0.856 nM, 22.2 nM, 722 μM, and >1,000,000 μM for cortisol, corticosterone, progesterone, Compound **I**, and synephrine (Compound **II**), respectively, were found, while in sheep plasma the values were 0.409 nM, 1.78 nM, 5.28 nM, 594 μM, and >1,000,000 μM, respectively. Concentrations of Compound **I** equivalent to an effective pharmacological dose resulted in a significant ($P < 0.01$) decrease in CBG bound corticosterone and a significant ($P < 0.01$) increase in free corticosterone in rat plasma. In sheep, a similar effect was observed with cortisol. Progesterone binding, however, did not appear to be affected significantly by Compound **I** in either rat or sheep plasma. Compound **I** was found to be a competitive inhibitor of glucocorticoid binding to CBG. These results suggest that binding of Compound **I** to CBG with concomitant displacement of endogenous glucocorticoids, but not progesterone, may be part of the mechanism of action of these phenylaziridine compounds. BIOCHEM PHARMACOL **59**;2:167–175, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. corticosteroid-binding globulin; rat; sheep; aziridine; free steroids; glucocorticoids; progesterone

Aziridines or nitrogen mustards, are highly reactive *N*-tricyclic compounds that function as chemotherapeutic alkylating agents [1]. Most of these compounds are chemically unstable, and the more stable aziridine precursors are frequently used clinically. Cyclization and formation of the highly reactive aziridine intermediate constitute the initial reaction of nitrogen mustards. The aziridine ring and its protonated form, the aziridinium ion, are the reactive compounds that then react avidly with nucleophiles to form alkylated, ring-opened products [2]. Aziridines are seldom found in nature, due to their labile character, and mitomycins are among the few well-known naturally occurring aziridines [2]. Stabilization of the aziridine by the complex ring structure in mitomycins probably accounts for their presence in nature. Unmasking of the aziridine occurs *in vivo*, and mitomycins are used as antibiotics and antitumor agents.

Salsola tuberculatiformis Botsch., a Namibian shrub that causes prolonged gestation in sheep and contraception in rats [3], has been shown to contain an active but unstable aziridine derivative [4]. Chemical investigation of an active but labile HPLC fraction, S2, isolated from the shrub [5] indicated that the active component may be 2-(4-hydroxyphenyl)-1-methyl-aziridine or the corresponding open chain precursor [4]. The labile nature of the active component in S2 prompted the synthesis of a more stable but active chemical analogue, 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride (Compound **I**, Fig. 1), which cyclizes to the corresponding aziridine [2-(4-acetoxyphenyl)-1-methyl-aziridine], at physiological pH [6]. In an alkaline medium, both Compound **I** and S2 decompose to synephrine (Compound **II**, Fig. 1), which is biologically inactive [6].

Previous work established that *S. tuberculatiformis* and Compound **I** have a contraceptive effect in female rats, and that S2 and Compound **I** both inhibit sheep adrenal cytochrome P450c11 [6]. In addition, the cyclization of Compound **I** to the corresponding aziridine is retarded in sheep serum due to binding to CBG† and SHBG and in rat serum, which does not contain SHBG [7], due to binding to

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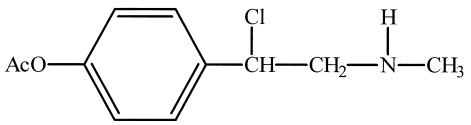
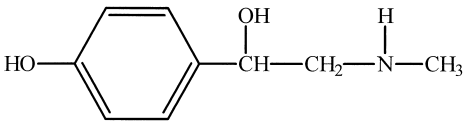
† *Abbreviations:* CBG, corticosteroid-binding globulin; SHBG, sex hormone binding globulin; and DCC, dextran-coated charcoal.



Compound **I**



Compound **II**

FIG. 1. Structures of Compound I [2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride] and Compound II [synephrine].

CBG [6]. Furthermore, a recent study in female rats demonstrated that administration of *S. tuberculatiformis* and Compound **I** results in an *in vitro* and *in vivo* displacement of corticosterone from rat CBG with a concomitant rise in free steroid, and suggests that this may be part of the mechanism of action of these substances [8]. Thus, although other probable sites of interaction of Compound **I** with glucocorticoid hormones, such as additional steroidogenic enzymes, the glucocorticoid receptor, or steroid-catabolizing enzymes, may certainly be present, previous experimental work strongly suggests a role for steroid-binding globulins.

In serum or plasma, steroid hormones are either free in solution or bound to albumin, CBG, and SHBG [9]. According to the currently accepted model of steroid action, the distribution of steroids in serum may influence the bioavailability of these hormones at different target tissues [10]. This distribution will depend on the binding properties and concentration of the serum-binding proteins and the relative concentrations of the endogenous steroids [11]. In addition, the administration of certain drugs, or the presence of certain exogenous compounds, may affect the transport and disposition of the endogenous steroids [12,13].

CBG was chosen specifically for investigation in the present study in an attempt to find a unifying mechanism of action that would reconcile the diverse effects, such as contraception in rats [6] and prolonged gestation in sheep [3], of the biologically active phenylaziridines. Rat plasma does not contain SHBG [7], and interaction of the phenylaziridines with CBG has been implicated as a contributing factor in the contraceptive effect on rats [8]. In addition, in fetal sheep it has been shown that the prepartum rise of cortisol does not result in negative feedback to the pituitary, and thus attenuation of the signal, because of a concomitant rise in CBG [14]; perturbation of this balance may influence the timing of parturition.

The binding parameters of CBG have been measured in several species [15]. Comparison of parameters across studies is difficult, however, as diverse methods and ligands have been used. Rats and sheep are the two species in which biological activity by *S. tuberculatiformis* has been demonstrated, and a direct comparison of the CBG binding parameters of these two species and the effect of the chemical analogue, Compound **I**, on these parameters would be valuable in elucidating the mechanism of action of the biologically active phenylaziridines. The glucocorticoids (cortisol or corticosterone) and progesterone were selected as the ligands to be investigated as these are quantitatively the most important steroid hormone ligands of CBG [9].

MATERIALS AND METHODS

Chemicals

Radioactive steroids ([1,2,6,7-^{3}H]cortisol, 74–80 Ci/mmol; [1,2,6,7-^{3}H]corticosterone, 82–88 Ci/mmol; [1,2,6,7-^{3}H]progesterone, 94–96 Ci/mmol; and [1-^{14}C]glucose, 55–59 mCi/mmol) were obtained from Amersham Life Sciences. Unlabeled steroids (cortisol, corticosterone, and progesterone) and synephrine were obtained from the Sigma Chemical Co. Compound **I** was synthesized as previously described [6], and the purity was confirmed with electrospray mass spectrometry. The effective pharmacological dose concentration range of Compound **I** was calculated to be between 0.396 and 6.34 mM based on results from a previous study [6] and the average blood volume of rats [16].

Blood Samples

Blood samples were collected from nulliparous female Wistar rats and young Merino ewes in heparin/lithium blood collection tubes, centrifuged at 3000 *g* for 15 min at 4°, pooled, and stored at −20° until used.

Saturation Binding Studies

The method of Hammond and Lähteenmäki [17] was used with modifications. In brief, rat or ovine plasma was incubated, in a ratio of 1:100, with DCC (0.5% Norit A and 0.05% Dextran T-70 in PBS) at room temperature for 30 min, while shaking gently. The DCC was removed by centrifugation at room temperature at 3000 *g* for 15 min. Ideally the concentration of binding sites should be around $0.1 \times K_d$ of the tracer ligand [15], and thus the ovine plasma was used directly at the dilution of 1:100, while the rat plasma was diluted further with PBS to a final dilution of 1:2000. For the saturation binding study in rat plasma, [^{3}H]corticosterone, the major glucocorticoid in rats, was used, whereas for ovine plasma, [^{3}H]cortisol, the major glucocorticoid in sheep, was added. Exponential dilutions of [^{3}H]steroid were made in the diluted serum to cover a range from 0.01 to 100 nM according to the method of Hulme and Birdsall [18]. The samples (300 μL) were incubated at room temperature for 2 hr and then were

transferred to an ice bath at 0° for 15 min. Ice-cold DCC (750 μL) was added to the samples, and the suspension was incubated for 10 min, after which the DCC was removed by centrifugation at 3000 *g* and 4° for 3 min. The supernatant was decanted into 6-mL plastic scintillation vials, 3 mL scintillant was added, the samples were vortexed, and the radioactivity was counted for 10 min in a liquid scintillation counter. The counting efficiency was 44%.

To investigate the mechanism of interaction of Compound **I** with rat or ovine CBG, saturation binding studies were repeated in the presence of 2 and 4 or 6 mM Compound **I** directly dissolved in the plasma.

Competitive Binding Studies

The same basic method as for the saturation binding studies was used, with the difference that a constant concentration of tritiated steroid (1 nM) was added, which was displaced by increasing concentrations of unlabeled (cold) ligands. Rat plasma was incubated with [^{3}H]corticosterone and sheep plasma with [^{3}H]cortisol. The unlabeled ligands tested were corticosterone (10^{-12} to 10^{-5} M), cortisol (10^{-12} to 10^{-5} M), progesterone (10^{-12} to 10^{-5} M), Compound **I** (10^{-8} to 10^{-1} M), and Compound **II** (10^{-8} to 10^{-1} M).

Determination of the Percentage of Free Steroids

The ultrafiltration-dialysis method of Hammond *et al.* [19] was used to determine the percentage of free steroid (corticosterone, cortisol, and progesterone) in rat and sheep plasma and the effect of concentrations of Compound **I** equivalent to an effective pharmacological dose (0.396 and 6.34 mM) on this percentage. In brief, plasma samples (450 μL), native or heated (1 hr at 60°), were incubated with 3×10^5 dpm [^{3}H]steroid and 12×10^3 dpm [^{14}C]glucose for 30 min at 37°. Compound **I** was dissolved directly into either the native or heated plasma. Two aliquots of 200 μL each of the incubate were pipetted into ultrafiltration vials, which were capped, and incubated overnight at 37° in a CO_2 (5%) incubator. The ultrafiltrate and an aliquot (30 μL) of the plasma retained by the ultrafiltration membrane were collected, and 350 μL water and 3 mL scintillant were added. The samples were vortexed, and the ^{3}H and ^{14}C were counted in a liquid scintillation counter. The ratio of [^{3}H]steroid/[^{14}C]glucose [20], the percentage of free steroid [19], and the distribution of steroid between free, albumin bound, and CBG bound were calculated [9].

Data Manipulation and Statistical Analysis

The Graph Pad Prism® program was used for data manipulations, graphical presentations, and statistical analysis. Ligand depletion was observed in the saturation binding experiments, and thus total binding was fitted as a function of added ligand as suggested by Swillens [21]. K_i values were calculated from the $K_{d_{app}}$ and $B_{max_{app}}$ as suggested by Cornish-Bowden [22] for the saturation binding studies, and according to the method of Cheng and Prusoff [23] for the competitive binding studies. One-way ANOVA and Dunnett's multiple comparisons test as post test were used for statistical analysis.

TABLE 1. CBG binding parameters in rat and ovine plasma determined by saturation binding studies

	K_d (nM)	B_{max}* (nM)
Rat†	0.646 ± 0.111	578 ± 30
Sheep‡	0.577 ± 0.124	19.8 ± 1.2

Data are means ± SEM of three duplicate determinations.
*B_{max} values were corrected for plasma dilution.
†Rat plasma was diluted 1:2000, and [^{3}H]corticosterone was used as the radioligand.
‡Sheep plasma was diluted 1:100, and [^{3}H]cortisol was used as the radioligand.

RESULTS

Binding Parameters of Rat and Ovine CBG

The binding affinities (K_d values) of corticosterone in rats and cortisol in sheep were very similar (Table 1). However, the binding capacities (B_{max} values) differed greatly, with rat plasma having a higher binding capacity for corticosterone than sheep plasma had for cortisol.

Competitive Binding of Test Compounds to Rat and Ovine CBG

For both species (rat and sheep), the major endogenous glucocorticoid, corticosterone and cortisol, respectively, appeared to have the highest affinity for CBG (Fig. 2). The IC_{50} values (Table 2) were similar and in the nanomolar range, and the K_i values calculated [23] compared well with the values obtained during saturation binding experiments (Table 1). The minor glucocorticoids, cortisol for rats and corticosterone for sheep, similarly had IC_{50} values in the nanomolar range. Progesterone, however, appeared to displace tritiated glucocorticoids to a much lesser extent. Compound **II**, the inactive breakdown product of Compound **I** [6] and the S2 fraction isolated from the shrub [4], did not appear to displace endogenous ligands from either sheep or rat CBG. Compound **I**, however, displaced tritiated glucocorticoids from rat and sheep CBG but only at relatively high concentrations, with IC_{50} values in the millimolar range.

Effect of Compound I on the Distribution of Steroids in Rat and Ovine Plasma

In rat and sheep plasma, Compound **I** caused a significant ($P < 0.01$ and $P < 0.05$, respectively, at 6.34 mM) concentration-dependent displacement of both glucocorticoids, corticosterone and cortisol, from CBG and a significant rise ($P < 0.01$ at 6.34 mM) in both free glucocorticoid levels (Fig. 3). A significant increase ($P < 0.05$) in albumin bound steroid was observed only in the case of cortisol in rat plasma.

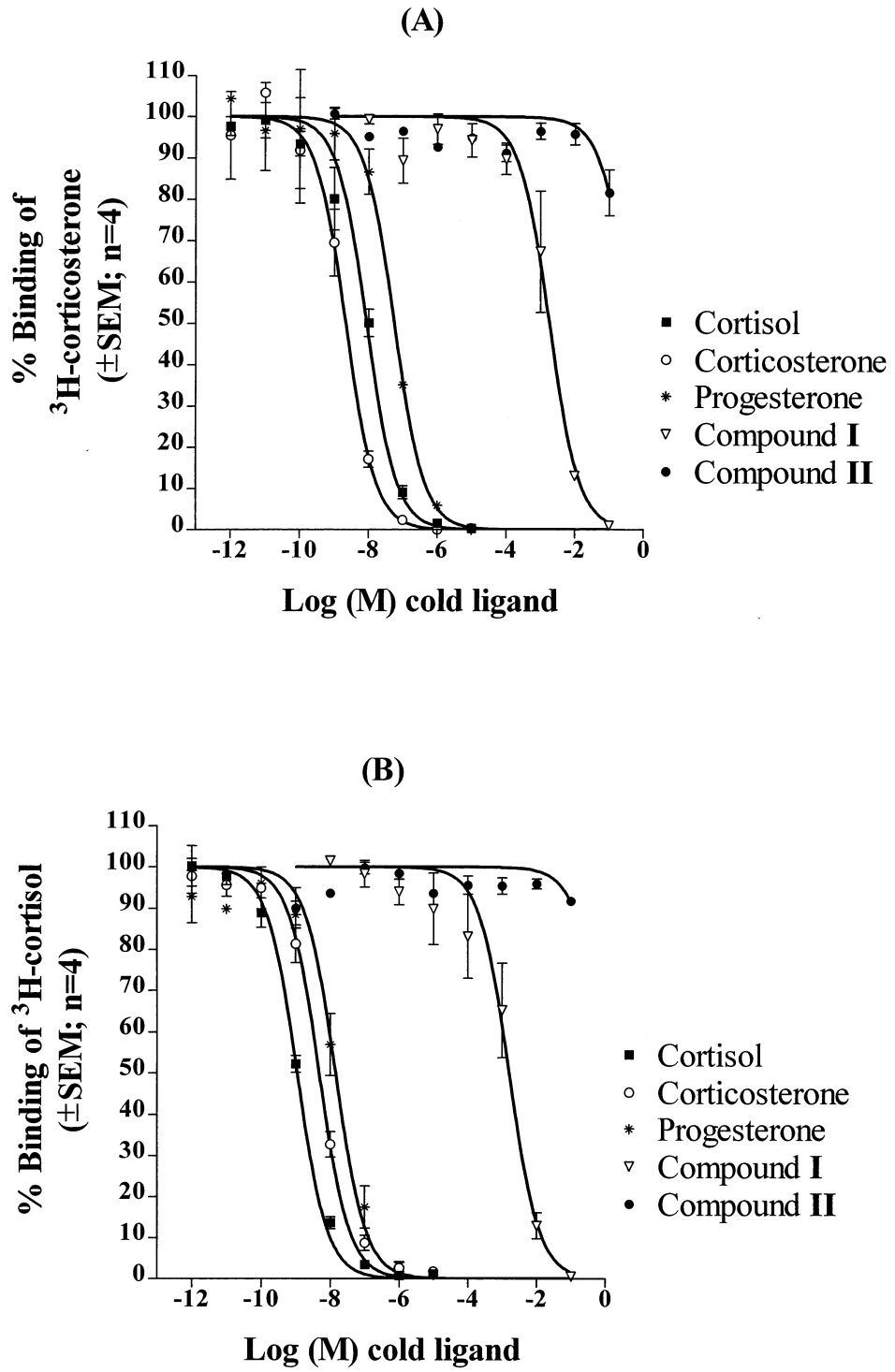


FIG. 2. Concentration-dependent inhibition of (A) 1 nM [^{3}H]corticosterone binding to rat plasma (diluted 1:2000), and (B) 1 nM [^{3}H]cortisol binding to sheep plasma (diluted 1:100) by unlabeled endogenous steroids (corticosterone, cortisol, and progesterone) and test compounds (Compound I and Compound II).

The distribution of progesterone binding in both rat and sheep plasma was not significantly influenced by the addition of Compound **I**. However, a slight increase in free and a concomitant decrease in CBG bound progesterone were observed in both rat and sheep plasma.

Mechanism of Interaction of Compound I with Rat and Ovine CBG

A comparison of the $K_{d_{app}}$ and $B_{max_{app}}$ values (Table 3) indicated that only the $K_{d_{app}}$ values, and not the $B_{max_{app}}$ values, were affected significantly ($P < 0.05$) by the addition of

TABLE 2. Inhibitory effect of test compounds on the binding of (a) [^{3}H]corticosterone (1 nM) to rat CBG, and (b) [^{3}H]cortisol (1 nM) to ovine CBG as determined by competitive binding studies

	IC_{50}* (μM)	K_i† (μM)
Rat		
Cortisol	0.00886 ± 0.0012‡	0.00348
Corticosterone	0.00218 ± 0.00045	0.000856
Progesterone	0.05650 ± 0.0093‡	0.0222
Compound **I**	1,840 ± 465‡	722
Compound **II**	>1,000,000‡	>1,000,000
Sheep		
Cortisol	0.00112 ± 0.000097	0.000409
Corticosterone	0.00485 ± 0.00063‡	0.00178
Progesterone	0.0144 ± 0.0025‡	0.00528
Compound **I**	1,623 ± 454‡	594
Compound **II**	>1,000,000‡	>1,000,000

Statistical analysis was done on log IC_{50} values comparing values with those of the major glucocorticoid, corticosterone in rats and cortisol in sheep.

*Values are means ± SEM of four duplicate determinations.

†K_i values were calculated using the K_d values from Table 1.

‡$P < 0.01$ in one-way ANOVA with Dunnett's multiple comparisons test as post test.

Compound **I**, and that Compound **I** thus acted as a competitive inhibitor of glucocorticoid binding to both rat and sheep CBG [18, 22]. The K_i values for Compound **I** determined from the $K_{d_{app}}$ and $B_{max_{app}}$ values [22] were 729 and 239 μM for rat and sheep CBG, respectively. The K_i value for rat CBG compared well with the K_i value determined using competitive binding studies [23], 722 μM (Table 2), but for sheep CBG the K_i value was lower than that determined by competitive binding, 594 μM.

DISCUSSION

The binding parameters of rat CBG for corticosterone measured during the current study (K_d: 0.646 nM; B_{max}: 578 nM) compared well with results from previous studies (K_d: 0.60 or 0.62 nM; B_{max}: 750 or 814 nM [24, 25]), despite different methods employed, and species of rats tested. For sheep CBG, however, the K_d obtained for cortisol (0.577 nM) was higher than measured previously (9.2 nM [26]), while the B_{max} (19.8 nM) was lower than reported previously (78 nM [26]; 46 nM [27]), which may be attributed to the difference in species of sheep tested or the methods employed [26]. We found that the binding affinities (K_d) for glucocorticoids of rat and sheep plasma were similar, but that the binding capacity (B_{max}) was higher in rats. This agrees with previous comparisons, with the exception that the extent of the difference (30×) in B_{max} was much greater in our study than the six times difference reported previously [7].

From the competitive binding studies it is clear that, of the endogenous steroids tested, the glucocorticoids bound with the highest affinity to CBG, and that the major endogenous glucocorticoid, corticosterone and cortisol respectively in rat and sheep, bound with a slightly higher affinity than the minor glucocorticoid. Although a previous study [7] comparing the corticol/corticosterone ratio in the sera of various species with affinities for the two glucocorticoids concluded that there was no correlation, a detailed examination of the results, specifically for rat and sheep sera, suggests that there is indeed a correlation, with rat sera displaying a higher affinity for corticosterone and sheep sera a higher affinity for cortisol [7].

Previous comparisons of binding affinities of corticosterone and progesterone in rats show that, unlike human plasma where the affinities are comparable, in rat plasma the affinity for corticosterone is higher than for progesterone [28]. The present study suggests that CBG does indeed have a significantly ($P < 0.01$) lower affinity for progesterone than for the major glucocorticoid in rats, but also in sheep, and that the affinity of rat plasma for progesterone is lower than that of sheep plasma.

Of the two test compounds studied, Compound **I** and Compound **II**, only Compound **I** appeared to have displacing activity in the effective pharmacological dose concentration range with similar binding affinities in rat and sheep plasma of 722 and 595 μM, respectively. A few studies have been done to determine the effect of endogenous or exogenous ligands on CBG binding parameters, and IC_{50} values ranging from 5 μM for Danazol [12] to 10 μM for unsaturated free fatty acids [13] have been reported. Similarly, for SHBG, another steroid transporter in plasma, IC_{50} values ranging from 10 to 50 μM for phytoestrogens [29], and 160 μM for hexachlorocyclohexane-γ [30] have been reported. Although the IC_{50} values described in our study for Compound **I** appear to be relatively high, studies with environmental xenobiotics [30], which also display weak hormonal activity and are present in low concentrations, have suggested several ways in which these compounds could nonetheless have a physiological function. These include accumulation in fatty tissues due to the lipophilic nature of the compounds, which would increase their concentrations and bioavailability, and the possibility of additive [31] and synergistic [32] effects of simultaneous exposure to more than one xenobiotic. Although Compound **I** is a lipophilic molecule, it is not known whether it accumulates in fat tissue. In addition, whether additive or synergistic effects pertain to additional compounds that may be present in the shrub *S. tuberculatiformis* is not known and will have to be investigated.

The value for percentage free cortisol (13.7%) in native sheep plasma obtained in this study compared well with the value (14%) obtained by Gayrard *et al.* [26] as does the percentage free corticosterone in rat plasma measured in our study (6.97%) relative to the 11% reported in a previous study [25]. The lower percentage free steroid of the major glucocorticoid, corticosterone in rats and cortisol in sheep, relative to the minor glucocorticoid may be ascribed to the higher binding affinities of these steroids for CBG (see Table 2).

Addition of concentrations of Compound **I** equivalent to an effective pharmacological dose to rat or sheep plasma resulted in a significant ($P < 0.01$ and $P < 0.05$,

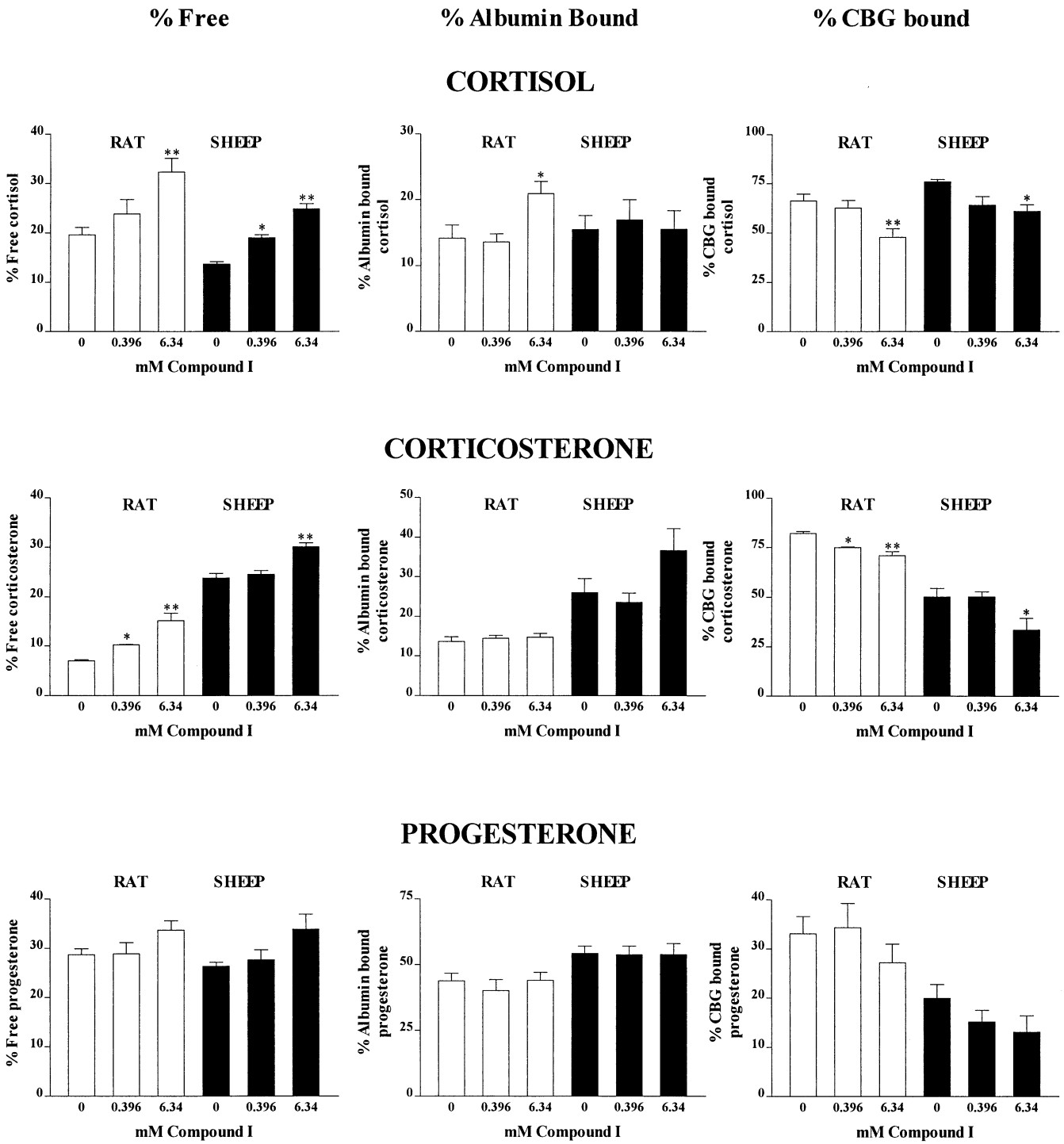


FIG. 3. Effect of concentrations of Compound I equivalent to an effective pharmacological dose on the percentage distribution of steroids between free, albumin bound, and CBG bound in rat and sheep plasma. The results are the means ± SEM of eight duplicate determinations. The results in the presence and absence of Compound I were compared, and an ANOVA followed by Dunnett's multiple comparisons test as post test was used for statistical analysis (*$P < 0.05$ and **$P < 0.01$).

respectively) displacement of glucocorticoids, but not progesterone, from CBG. Concomitantly, a significant ($P < 0.01$) increase in free glucocorticoids, but not progesterone, was observed. The results with progesterone may be explained by both the low concentration of progesterone relative to the glucocorticoids in sheep [33] and rat plasma [34], and by the low affinity of progesterone for rat and sheep CBG (present study). Calvano and Reynolds [28], in a study comparing glucocorticoid and progesterone binding in rats and humans, concluded that in rats there was little competition between corticosterone and progesterone for CBG binding sites. However, in humans, where progesterone and cortisol have similar affinities for CBG [28], high levels of progesterone, such as occur during pregnancy or

TABLE 3. Effect of different concentrations of Compound I on the binding parameters of CBG in rat and sheep plasma as determined by saturation binding studies

Compound I (mM)	$K_{d_{app}}$ (nM)	$B_{max_{app}}$* (nM)
Rat†		
0	0.65 ± 0.11	578 ± 30
2	2.77 ± 0.52‡	654 ± 58
6	6.55 ± 1.10§	628 ± 68
Sheep‖		
0	0.58 ± 0.12	19.8 ± 1.2
2	2.21 ± 0.92	21.5 ± 5.0
4	4.94 ± 1.53‡	18.5 ± 3.0

Values are the means ± SEM of three duplicate determinations.

*$B_{max_{app}}$ values were corrected for dilution of plasma.

†Rat plasma was diluted 1:2000 for binding studies, and the steroid used was [^{3}H]corticosterone.

‡,§Statistical analysis used was one-way ANOVA and Dunnett's multiple comparisons test as post test: ‡$P < 0.05$ and §$P < 0.01$.

‖Sheep plasma was diluted 1:100, and the steroid used was [^{3}H]cortisol.

estrogen administration, can displace cortisol from CBG due to competition for binding sites and result in a large increase in free cortisol [35]. The reverse, however, does not hold true, and an increase in cortisol concentration does not result in a great increase in free progesterone as the concentration of progesterone present is low and greater binding to albumin occurs.

Investigation into the mechanism of interaction of Compound **I** with rat and ovine CBG clearly showed that competitive inhibition of glucocorticoid binding occurred. This would suggest direct competition with the endogenous ligands, probably at the same ligand binding site [18].

The binding of Compound **I** to rat and ovine CBG, with concomitant displacement of endogenous glucocorticoids, may be part of the *in vivo* biological action of Compound **I** and possibly also of the shrub *S. tuberculatiformis*. Whether the *in vitro* increase in the concentration of biologically available glucocorticoids observed in this study also occurs *in vivo* remains to be determined. A recent study by us suggests that in female rats an *in vivo* increase in free glucocorticoid concentration is observed due to administration of Compound **I** or *S. tuberculatiformis* [8]. We suggest that the increase in free, biologically active glucocorticoids closely mimics a state of stress, either chronic [36] or acute [25]. Chronic social stress in rats with hyperstimulation of the hypothalamic–pituitary–adrenal (HPA) axis decreases CBG levels, resulting in an increase in free corticosterone levels and greater access to target organs despite no change in total corticosterone levels [36]. The conditions of the present study may suggest an acute rather than a chronic stress situation. Acute stressors in rats, however, also result in long-term increases in basal free corticosterone, due to decreases in CBG concentrations, exposing glucocorticoid-sensitive targets to high levels of free corticosterone for several days [25, 37]. Although it has been suggested that inhibition of reproductive function in the rat is mediated exclusively by prolonged or chronic stress [38], there have been reports of acute stressors influencing reproduction, at the level of endogenous opioids [39] and gonadal corticotropin-releasing factor (CRF) [40]. When considering the question of whether the compounds under investigation mediate an acute rather than a chronic stress response, it should be noted that binding to CBG has been shown to decrease the metabolic clearance rate (MCR) of compounds bound to it [41] and that previous work from this laboratory indicates that Compound **I** is stabilized by binding to steroid-binding globulins [6]. Thus, although the present study presents no data on the MCR of the biologically active aziridines, an aspect that will have to be addressed in the future, we would like to postulate that binding to CBG may extend and thus amplify the action of these compounds.

The biological effects of the shrub *S. tuberculatiformis* include not only contraception in rats but also prolonged gestation in sheep [3]. In an attempt to find a unifying motif for the action of the biologically active phenylaziridines we would like to start establishing a connection, albeit tenuously and by definition speculative at this stage, between the results of the current study and the phenomenon of prolonged gestation. In sheep, activation of the fetal HPA axis is required for parturition [42], and progressive increases in both ACTH and cortisol levels during the last 30 days of gestation, peaking 24–48 hr prepartum, have been observed [43]. The feedback relationship between the fetal pituitary and adrenal is established at this time [44], and between day 121 and day 131 the feedback is sensitive to very small changes in fetal plasma cortisol (1.6 ng/mL) [45]. Despite the prepartum rise in total cortisol, the percentage free cortisol does not change significantly during the last 20 days of gestation due to a concomitant progressive increase in plasma CBG [14, 46, 47]. CBG mRNA also has been detected in the fetal pituitary gland at day 125 and day 140, and the addition of CBG to cultured fetal pituitary cells has been shown to attenuate the inhibition of ACTH secretion by cortisol [47]. A role has been suggested for systemic CBG in modulating cortisol availability to the fetal pituitary and thus preventing or attenuating the negative feedback on ACTH secretion, with pituitary CBG additionally affecting negative feedback at a local level [14, 47]. Just before birth the absolute free cortisol levels rise significantly due to the rapid surge in total corticoid concentration and saturation of CBG binding sites [46, 47]. These high levels of fetal cortisol are required to increase 17α-hydroxylase activity to levels that would reduce maternal progesterone to concentrations where uterine activity is increased and labor ensues [48]. Although at present no information about the circulating levels of the phenylaziridines in sheep is available and, therefore, any claims to biological relevance are speculative, we would like to suggest that the results of the present study, in which Compound **I** bound to ovine CBG and displaced endogenously bound cortisol, may have a bearing on the syndrome of prolonged gestation found in sheep ingesting *S. tuberculatiformis* during the last 50 days of gestation [3]. We suggest a scenario whereby the increase in

free cortisol could stimulate fetal CBG synthesis. The stimulation of CBG synthesis by low dose corticoid infusion has been demonstrated amply in other studies [49, 50]. Even at day 100, when the fetal HPA axis is relatively unresponsive, low dose (1 mg/24 hr) infusion of cortisol stimulates CBG synthesis [51]. Although premature delivery may be induced after 48–72 hr by infusion of glucocorticoids [52–54] into fetuses of 100–121 days gestational age, these doses (50 mg cortisol/24 hr or 0.1 mg dexamethasone/24 hr, equivalent to 2.5 mg cortisol/24 hr) are much higher than those required for an increase in CBG synthesis. The postulated increase in systemic CBG levels could reduce the concentration of free steroid during the surge of total cortisol immediately prepartum. In addition, in the pituitary displacement of cortisol from pituitary CBG could increase local free steroid concentrations, resulting in negative feedback on ACTH secretion. Increased trophic drive from the hypothalamus, suggested by some to be due to reduced blood glucose levels [55], eventually would overcome these factors, and parturition, albeit delayed, will take place. It is interesting to note that an increased maternal ratio of cortisol to CBG has been identified as a marker for preterm labor in humans [56]. Although the above discussion is speculative, would require further experimental work to establish, and does not preclude other sites of action, it does suggest that the role of CBG, and specifically the displacement of endogenously bound glucocorticoids by Compound **I**, in contraception in rats and prolonged gestation in sheep, would be a fruitful avenue to explore.

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