

The Hypothalamic–Pituitary–Adrenal Axis of Prairie Voles (*Microtus ochrogaster*): Evidence for Target Tissue Glucocorticoid Resistance

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Basal plasma corticosterone levels in prairie voles (*Microtus ochrogaster*) are extremely high, in the absence of any apparent negative consequences of glucocorticoid excess. We tested the hypothesis that prairie voles are a novel rodent model of target tissue resistance to glucocorticoids. Prairie voles had a significantly higher adrenal-to-body weight ratio, 5- to 10-fold greater basal plasma corticosterone, and 2- to 3-fold greater basal plasma ACTH concentrations than montane voles (*Microtus montanus*) and rats. While plasma corticosterone binding globulin (CBG) was 2-fold higher in prairie voles than in rats, both estimated and directly measured plasma free corticosterone were significantly higher in prairie voles than in rats. Plasma corticosterone levels in prairie voles were responsive to both circadian cues and a stressor, but were resistant to suppression by the synthetic glucocorticoid, dexamethasone (DEX). Western blots of brain and liver protein extracts, using a glucocorticoid receptor (GR) antibody, revealed the presence of a ~97 kDa immunoreactive band, the expected size for GR. Binding assays revealed significantly lower DEX affinity of corticosteroid receptors (CR) in cytosol of prairie vole

brain and liver than that in the same tissues in rats. We conclude that prairie voles are a novel rodent model of glucocorticoid resistance, and that decreased affinity of CR for ligand might be partially responsible for this phenomenon. © 1997 Academic Press

Elevated plasma concentrations of glucocorticoids are often used as an index of stress (Chrousos and Gold, 1992). Chronic stress and the attendant chronic hypersecretion of glucocorticoids have been linked to many different pathologies (Chrousos, 1995). Relatively high levels of glucocorticoids, however, are not always indicative of chronic stress. Decreased target tissue sensitivity to these steroids, known as glucocorticoid resistance, is also associated with “compensatory” hypersecretion, in the absence of stigmata of glucocorticoid excess (Chrousos *et al.*, 1993).

The pathologic syndrome of generalized glucocorticoid resistance has been reported in several human families (Vingerhoeds *et al.*, 1976; Chrousos *et al.*, 1982b, 1993). While these patients exhibit no symptoms of glucocorticoid excess, compensatory alterations in other components of the hypothalamic–pituitary–adrenal (HPA) axis may result in hyperandrogenism and hypermineralocorticoidism. Interestingly, several spe-

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cies, including guinea pigs (Claman, 1972; Kraft *et al.*, 1979; Keightley and Fuller, 1996) and some New World primates (Yamamoto *et al.*, 1977; Chrousos *et al.*, 1982a), exhibit nonpathologic glucocorticoid "resistance" as part of their normal physiology. Their HPA axes appear to be regulated at a higher set-point, resulting in high basal plasma glucocorticoid levels.

High basal plasma corticosterone levels have been reported in some New World species of the rodent genus *Microtus* (Seabloom, 1965; Shapiro and Insel, 1990; Boonstra and Boag, 1992; McDonald and Taitt, 1982; DeVries *et al.*, 1995). For example, prairie voles (*Microtus ochrogaster*) have very high basal corticosterone levels compared to rats (DeVries *et al.*, 1995), while montane voles (*Microtus montanus*) do not (Taymans, unpublished data). Those *Microtus* species with elevated basal corticosterone levels apparently suffer no adverse glucocorticoid-induced effects (Dieterich and Preston, 1977; Klein *et al.*, 1996). Therefore, we hypothesized that prairie voles could provide a unique rodent model of glucocorticoid resistance.

Prairie voles have been studied extensively by ecologists (Getz *et al.*, 1993), and are notable for demonstrating a social system based on monogamy. Some glucocorticoid-resistant New World monkey species also exhibit monogamy (Johnson *et al.*, 1996a). In comparison to voles, the study of primates, some of which are endangered, is more difficult. Prairie voles provide a convenient small rodent in which to study glucocorticoid resistance and its potential behavioral consequences, in the context of natural history and an unusual mammalian social system (monogamy). Here, we report evidence that prairie voles, in comparison to both montane voles and Sprague-Dawley rats, exhibit target tissue glucocorticoid resistance, in addition to having decreased ligand binding affinity of target tissue corticosteroid receptors (CR).

MATERIALS AND METHODS

Animals

Voles were the laboratory-bred F₃ generation of wild stock captured near Urbana, Illinois (prairie voles) and Jackson's Hole, Wyoming (montane voles; stock provided by Dr. Pat Berger, University of Utah). Young

adult voles (60–90 days old, ~40 g) and Sprague-Dawley rats (~250 g, Taconic Farms, Germantown, NY) were used in all experiments. Rats were acclimated for 7–10 days before experimental use. Animals were group-housed in polycarbonate cages (12 × 18 × 28 cm for voles; 20 × 25 × 45 cm for rats) on pine chip bedding, and maintained under long-day lighting (14:10 light:dark (L:D) for voles, 12:12 L:D for rats; lights on at 0600 hr EST). Food (Purina rat and rabbit chow for rats and voles, respectively, St. Louis, MO) and water were available *ad libitum*.

Because gonadal hormone changes, such as those associated with the estrus cycle, can affect HPA axis parameters (Carey *et al.*, 1995), research on adrenal function has typically used males. To facilitate comparisons with the existing literature on corticosterone sensitive animals, only males were used, with the exception of female prairie voles used to study the variation of plasma corticosterone levels with time of day.

Animal Procedures

All animal procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Blood and tissue collection. Animals were removed from their cages individually, and taken to another room. Blood was collected following cervical dislocation and decapitation, except after swim tests, when it was collected from the periorbital sinus (Riley, 1960) under Metofane anesthesia (Pitman-Moore, Mundelein, IL). Less than 1 min elapsed between removal of the animal from its cage and completion of blood collection. Unless stated otherwise, blood collection took place from 0900 to 1200 hr. Blood was collected into tubes containing EDTA and stored on ice until centrifugation. Plasma was stored at -70° until assayed. Tissues were dissected immediately postmortem. Adrenal glands were trimmed of nonadrenal tissue before weighing. Tissues for GR assay and Western blot were rinsed thoroughly in isotonic saline, frozen on dry ice, and stored at -70° until use.

Time of day and corticosterone secretion. Blood was collected from independent groups of male and female prairie voles at 0800, 1400, 2000, and 0200 hr ($n = 12-15$ per group, approximately equal numbers of males and females in each group). Due to the large

number of prairie voles needed in 1 day, females were included. The sexes were not expected to differ, because of a general reduction of sexual dimorphism in prairie voles (Dewsbury *et al.*, 1980; Jacobs *et al.*, 1990), and because female prairie voles do not have a spontaneous estrus cycle (Sawrey and Dewsbury, 1985). In a separate study, plasma samples were collected from females accidentally housed under conditions of continuous light (LL) due to a broken timer. LL samples were collected at 0800, 1400, and 2000 hr ($n = 17, 8,$ and $10,$ respectively). During the dark phase, dim red light was used to aid the investigators. Total plasma corticosterone was measured as described in general methods.

Swim test. Male prairie voles were assigned to one of five swim groups, or to the control condition ($n = 8-10$ per group). Controls remained undisturbed in their home cages. For the swim test, prairie voles were placed individually in polycarbonate tanks ($20 \times 25 \times 45$ cm) filled with water ($32 \pm 1^\circ$) to 15 cm, preventing climbing out, or touching the bottom. After 3 min, animals were returned to their home cages. Between swim tests, the tanks were emptied and cleaned to reduce exposure to alarm substances potentially released by the previous swimmer (Abel, 1991). Independent periorbital sinus blood samples were collected 5, 15, 30, and 60 min and 24 hr after the onset of swimming. Swim test onset times were staggered so that all blood samples (including controls) were collected at ~ 1400 hr.

Dexamethasone suppression test. Stock dexamethasone (DEX) suspension (6.4 mg/ml) was made by mixing dexamethasone sodium phosphate salt (Steroids, Wilton, NH) and Tween-80 (Fluka, Inc), then homogenizing the mixture in phosphate-buffered saline (PBS). The control suspension was made identically, but without DEX. Stock suspensions were stored at 4° until further dilution in PBS for injection. Final suspensions were prepared to optimize injection volume and DEX dose for each animal based on body weight [0.25 and 0.5 cc for the average prairie vole (40 g) and rat (250 g), respectively]. Prior to intraperitoneal (ip) injection at 0800 hr, suspensions were warmed to 37° and thoroughly vortexed. Plasma was collected 3, 6, or 12 hr later. For the dose-response curve, male prairie voles were randomly assigned to one of six treatment groups: 10, 40, 100, 400, or 1000 $\mu\text{g}/\text{kg}$ of

DEX ($n = 8-10$ per group), or CTL suspension ($n = 14$). Animals remained undisturbed until plasma collection 12 hr later. Using the same protocol, male rats were injected with 100 $\mu\text{g}/\text{kg}$ of DEX or CTL ($n = 8$ each) to serve as "positive controls."

Adrenalectomy. Bilateral adrenalectomies were performed under pentobarbital anesthesia (65 mg/kg). A dorso-medial incision provided a clear view of the adrenal gland. During removal, gentle suction was applied to the adrenal gland, as it was teased away from the kidney capsule. Postsurgically, animals were allowed *ad libitum* access to both water and 3% saline. Tissues were collected 24 hr postadrenalectomy. Effectiveness of adrenalectomy was verified by corticosterone radioimmunoassay (RIA) of plasma samples taken at the time of tissue collection, when plasma corticosterone concentrations were less than 2% of controls (Taymans, unpublished data).

Assays

Prior to the studies described here, the assays for corticosterone and ACTH were validated for use with prairie vole plasma, using the criteria of parallelism to the standard curve, biological agreement, repeatability, and cross-reactivity (Taymans, unpublished).

Corticosterone. Total plasma corticosterone was measured using a specific radioimmunoassay (RIA) kit (ICN, Costa Mesa, CA). Plasma was diluted in assay buffer as necessary to give results reliably within the linear portion of the standard curve (1:2121 for prairie voles, 1:1111 for montane voles, and 1:200 for rats). Samples were run in duplicate. Coefficients of variation (CVs) were less than 7 and 10%, within and between assays, respectively. Cross reactivity with other steroids totals less than 1% (as reported by ICN).

ACTH. ir-ACTH was measured by specific RIA using a 1:24,000 final dilution of antibody DP-6, as previously described (Estivariz *et al.*, 1992). Prior to assay, prairie vole plasma samples were pooled to obtain 500 μl . Samples were extracted (Sep-Pak C18 cartridges, Waters, Milford, MA), lyophilized, and reconstituted in assay buffer. Samples were assayed in duplicate. CVs were less than 8 and 13%, within and between assays, respectively. Cross reactivity, as assessed by HPLC, was negligible.

Maximum corticosterone binding globulin (CBG) capacity. CBG capacity was measured by the non-equilibrium method of Hammond and Lähteenmäki (1983), to facilitate comparisons with other *Microtus* species (Boonstra and Boag, 1992; McDonald and Taitt, 1982). Preliminary studies established the optimal procedures, which were subsequently used (Taymans, unpublished). Plasma was stripped of endogenous steroids (98.8%) by incubation with dextran-coated charcoal (DCC) for 1.5 hr at 32°. Stripped plasma, diluted in PBS (1:700 for prairie voles and 1:500 for rats), was incubated with 6.7 or 36 nM [³H]corticosterone ([1,2,6,7-³H]corticosterone, 87 Ci/mmol; Amersham, Arlington Heights, IL), in the presence (nonspecific binding, NSB) or absence (total binding, TB) of 16.7 μM unlabeled corticosterone (Research Biochemicals Inc., Natick, MA) at room temperature for 1 hr and at 4° for 15 min. Previous studies had shown that under these conditions, 6.7 nM [³H]corticosterone saturated specific binding in stripped plasma from both rats and prairie voles (Fig. 1). TB and NSB were measured in duplicate. The addition of cold DCC, followed by centrifugation (5 min at 3000g, 4°), separated bound and free corticosterone. The supernatant was counted in 10 ml of Cytoscint (ICN). Specific binding (SB = TB - NSB) in disintegrations per minute (DPM) was converted to maximum corticosterone binding capacity (MCBC; nM), using the specific

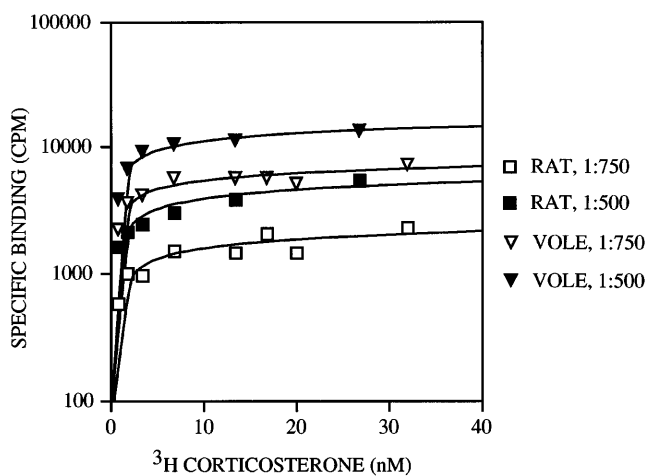


FIG. 1. Saturation of specific binding of [³H]corticosterone to different dilutions of steroid-free plasma from rats and prairie voles. Specific binding is saturated at about 6.7 nM of [³H]corticosterone. A log scale was used for specific binding. CPM, counts per minute.

activity of the [³H]corticosterone (1 mmol/87 Ci; 1 Ci = 2.2 × 10¹² DPM). Intra- and interassay CVs were 9.54 and 9.70%, respectively. Cross-reactivity with sex hormone-binding globulin, as indicated by the displacement of SB by testosterone, was negligible.

Free corticosterone. Concentrations of free corticosterone were estimated using the data from the CBG assay, and were also measured directly. Estimations of plasma free corticosterone were performed using both Barsano and Baumann's equation (Barsano and Baumann, 1989) and Tait and Burstein's equation (Tait and Burstein, 1964; McDonald and Taitt, 1982; Boonstra and Boag, 1992) (see Appendix for equations). Because it is a nonequilibrium binding method, Hammond and Lähteenmäki's procedure cannot be used to determine the affinity of CBG for corticosterone. Instead, we used previously published constants. In the rat it was calculated using constants from adult male Sprague-Dawley rats ($K_t = 2.81 \times 10^7 M^{-1}$, $K_a \Sigma P_a = 6.2$; Perrin and Forest, 1975); while in prairie voles it was calculated using those previously determined in the closely related *M. pennsylvanicus* ($K_t = 6.32 \times 10^7 M^{-1}$; $K_a \Sigma P_a = 2.05$; R. Boonstra, personal communication).

To measure free corticosterone directly, an ultrafiltration device was used (according to the manufacturer's instructions; Centrifree, Amicon, Beverly, MA) to separate bound and free corticosterone in another group of plasma samples. This device retains both CBG and albumin-bound corticosterone while allowing free corticosterone to flow through. The corticosterone concentration of the undiluted ultrafiltrate was measured by the RIA described earlier.

Dexamethasone assay. To meet volume requirements, plasma samples from prairie voles were pooled according to DEX dose and corticosterone concentration. DEX was measured by an extraction/chromatography RIA (Corning-Hazelton, Vienna, VA), as previously described (Evans *et al.*, 1985).

Glucocorticoid receptor binding assay. All procedures were carried out at 4°, using ice-cold solutions. Cytosol from liver (~80 μg/100 μl) and whole brain (~500 μg/100 μl) from 24 hr postadrenalectomy male prairie voles and rats ($n = 5$ each) was prepared by homogenization and centrifugation (105,000g, 1 hr) in cytosol buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, 20 mM Na molybdate (Mb), 1 mM DTT). Protein content was measured by the method of

Bradford (1976), using BSA as the standard. Cytosol was incubated with 0.3–10 nM [³H]dexamethasone ([1,2,6,7-³H]dexamethasone, 83 Ci/mmol, Amersham), in the presence (NSB) or absence (TB) of 16 μM unlabeled dexamethasone (Sigma, St. Louis, MO) for 24 hr. TB and NSB were measured in duplicate. Steroids and DCC were prepared in assay buffer (10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 5% glycerol, 20 mM NaMb, 2 mM DTT). DCC addition terminated incubation. Samples were centrifuged (1500g, 15 min) and aliquots of the supernatant were pipetted into 10 ml of Cytoscint. SB (in dpm) was calculated as the difference between the means of TB and NSB. LIGAND (Munson and Rodbard, 1980), a computerized, nonlinear least-squares curve-fitting analysis, calculated receptor affinity and concentration. A one-binding-site model was assumed. Receptor concentration was corrected for cytosol protein content. This assay did not differentiate between type I and type II corticosteroid receptors (CR) in the brain, both of which bind DEX (Spencer *et al.*, 1990).

Western Blot

Brain and liver from 24-hr postadrenalectomy male prairie voles and rats ($n = 3$ each) were homogenized and centrifuged (200g, for 15 min) in ice-cold buffer (0.25 M sucrose, 3 mM imidazole, pH 7.5, 2 μg/ml aprotinin, 2 mM phenylmethylsulfonyl fluoride, and 50 μg/ml leupeptin), and the pellets were discarded. Protein content was measured using BCA protein assay reagent (Pierce, Rockford, IL), with BSA as the standard. Eighty-five-microgram aliquots of the protein extracts were treated with Laemmli buffer (Laemmli, 1970) containing 100 mM DTT, and heated to 95° for 3 min before loading on a precast 8% polyacrylamide gel. Prestained markers (including proteins from 4 to 205 kDa; Novex, San Diego, CA) were electrophoresed in parallel. Proteins were transferred to a nitrocellulose membrane by electroelution at 30 V for 75 min. To decrease NSB, the membrane was preincubated in wash buffer (500 mM NaPO₄, 1 M NaCl, 0.05% Tween 20) with 5% nonfat dry milk for 1 hr. For immunoblotting, the membrane was incubated overnight at 4° in wash buffer with 0.0025% BSA and 1.75 μg/ml of anti-human GR antibody (clone 57, Catalog No. PA1-511, Affinity Bioreagents Inc., Golden, CO). This antibody recognizes an N-terminal epitope

of the human, rat, and mouse GR. After washing, the blot was incubated for 1 hr with peroxidase-conjugated anti-rabbit immunoglobulin antibody (Dako, Carpinteria, CA) at 1:4000 dilution. The blot was washed again before exposure to a chemiluminescence solution (ECL kit, Amersham) for 1 min. After film development (X-OMAT AR, Eastman Kodak, Rochester, NY), the blot was stained with India ink (1 μl/ml) for 1 hr to verify loading equivalency.

Statistics

Unpaired *t* tests or ANOVAs were used as appropriate. Significant factorial ANOVAs ($P < 0.05$) were followed by corrected least significant difference tests (LSD; $0.05/n$, where n = number of post hoc comparisons), so experiment-wise error was $P < 0.05$ for each ANOVA.

RESULTS

Cross-Species Comparison of Total Plasma Corticosterone and ACTH Concentration, and Adrenal-to-Body Weight Ratio

The primary characteristic of glucocorticoid resistance is elevated basal HPA axis function (Cassorla *et al.*, 1982; Chrousos *et al.*, 1993). Therefore, unstimulated total plasma corticosterone and ir-ACTH concentration, and the ratio of adrenal-to-body weight in male prairie voles, were compared to those of male montane voles and male Sprague-Dawley rats (experiment 1).

Corticosterone concentration differed significantly by species ($F_{2,27} = 24.63$, $P < 0.0001$; Table 1). In prairie voles, unstimulated corticosterone titers were 5- and 10-fold greater than those in montane voles and rats (LSD = 260.96 and 248.16, respectively; $P < 0.0167$). Corticosterone concentration was not significantly different between montane voles and rats (LSD = 255.28, $P = 0.57$). Similarly, ACTH varied by species ($F_{2,20} = 7.44$, $P = 0.0038$; Table 1), with prairie voles having 2- to 3-fold higher ir-ACTH than montane voles and rats (LSD = 36.99 and 32.76, respectively; $P < 0.0167$), but ACTH levels were not significantly different between montane voles and rats (LSD = 34.33, $P = 0.40$).

TABLE 1
Cross-Species Comparison of Hypothalamic–Pituitary–Adrenal Axis Indices

Species	AD/BW (mg/100 g)	Total B (ng/ml)	Free B (ng/ml)	MCBC (nM)	ACTH (pg/ml)
Prairie vole	32.4 ± 2.64* (n = 9)	701 ± 117.7* (n = 10)	nd	nd	76 ± 15.0* (n = 7)
	nd	631 ± 62.5** (n = 9)	37 ± 9.9** (n = 9)	1553 ± 182.7** (n = 9)	nd
Montane vole	17.0 ± 2.14 (n = 7)	130 ± 33.8 (n = 9)	nd	nd	26 ± 5.7 (n = 6)
Rat	14.2 ± 0.73 (n = 11)	72 ± 8.5 (n = 11)	nd	nd	37 ± 5.3 (n = 10)
	nd	71 ± 5.9 (n = 9)	9 ± 1.3 (n = 9)	799 ± 123.0 (n = 9)	nd

Note. AD/BW, adrenal-to-body weight ratio; B, plasma corticosterone; MCBC, maximum corticosterone binding capacity of CBG; nd = not done. Number of animals per group is indicated in parentheses. Data are expressed as mean ± standard error of the mean. For prairie voles and rats, the top and bottom rows are results from Experiments 1 and 2, respectively.

* Experiment 1; significantly different from corresponding measurements from rats and montane voles ($P < 0.05$).

** Experiment 2; significantly different from corresponding measurements in rats ($P < 0.05$).

The ratio of adrenal-to-body weight differed significantly by species ($F_{2,24} = 28.39$, $P < 0.0001$; Table 1). Male prairie voles had ~two-fold greater ratio of adrenal-to-body weight than both montane voles and rats (LSD = 7.27 and 6.49, respectively; $P < 0.001$). The adrenal-to-body weight ratio was not significantly different between montane voles and rats (LSD = 6.98, $P = 0.31$).

Plasma Maximum Corticosterone Binding Capacity and Estimated and Directly Measured Free Corticosterone Concentration in Prairie Voles vs Rats

It is thought that only free glucocorticoids (not bound to CBG) are biologically active (Brien, 1981; Ekins, 1990). To determine the biological relevance of the 10-fold corticosterone excess in prairie voles, we measured total plasma corticosterone, (MCBC of CBG) and calculated the estimated levels of free plasma corticosterone in male prairie voles and rats (experiment 2), using both the Tait and Burstein (1964) and Barsano and Baumann (1989) equations. Free corticosterone (not bound to CBG or albumin) was also measured directly. Again, total plasma corticosterone was significantly greater in prairie voles than in rats ($t_{16} = 8.93$, $P < 0.0001$). Using 6.7 nM [3 H]corticosterone, the MCBC was ~2-fold greater in prairie voles than in rats ($t_{16} = 3.42$, $P = 0.0035$); however, the estimated free corticosterone levels were still ~4-fold

higher in prairie voles than rats when using the Tait-Burstein equation ($t_{16} = 2.86$, $P = 0.0114$; Table 1), and ~10-fold higher in prairie voles than in rats when using the Barsano and Baumann equation ($t_{16} = 2.78$, $P = 0.0133$; data not shown). These results were replicated using 36 nM [3 H]corticosterone. Again, MCBC and estimated free corticosterone were significantly higher in prairie voles than in rats by both calculation methods (for MCBC, $t_{10} = 4.75$, $P < 0.05$; and for estimated free corticosterone, $t_{10} = 2.56$, $P < 0.05$ by Tait-Burstein, and $t_{10} = 2.41$, $P < 0.05$ by Barsano and Baumann).

When the plasma concentration of free corticosterone was directly measured, prairie voles had 10-fold higher levels than rats (8.7 ± 2.6 vs 0.9 ± 0.3 ng/ml, $t_{14} = 2.9$, $P < 0.05$).

Time of Day and Fluctuations in Corticosterone Concentration in Prairie Voles

Maintenance of a circadian rhythm of glucocorticoid secretion is consistent with glucocorticoid resistance (Vingerhoeds *et al.*, 1976; Chrousos *et al.*, 1993). The purpose of this experiment was to determine if total plasma corticosterone levels vary with time of day in prairie voles.

ANOVA of plasma corticosterone concentrations in prairie voles at different times of the day was significant ($F_{3,51} = 4.91$, $P = 0.0045$). At 0800 hr, corticoste-

rone was significantly lower than that at both 1400 hr (LSD = 493.96, $P = 0.0011$) and 2000 hr (LSD = 467.57, $P = 0.0025$) (Fig. 2). There was an approximately two-fold difference in corticosterone levels between peak (at 1400 hr) and trough levels (at 0800 hr). Separate analysis of LD males and females revealed that although females had higher plasma corticosterone concentrations than males, the same pattern of an early morning corticosterone trough and a midafternoon peak was present in both sexes. However, the variation in plasma corticosterone levels with time of day was statistically significant in females ($F_{3,20} = 3.09$, $P < 0.05$), but not in males ($F_{3,27} = 1.79$, $P = 0.17$). Corticosterone concentration did not vary significantly in LL females (housed in continuous light) ($F_{2,32} = 0.3$, $P = 0.7425$) but remained at "peak" levels (greater than 1000 ng/ml) at all time points (data not shown).

Plasma Corticosterone Response to a Mild Stressor in Prairie Voles

Increased secretion of glucocorticoids in response to a stressor is found in glucocorticoid resistance (Chrousos *et al.*, 1993). To induce stress, male prairie voles

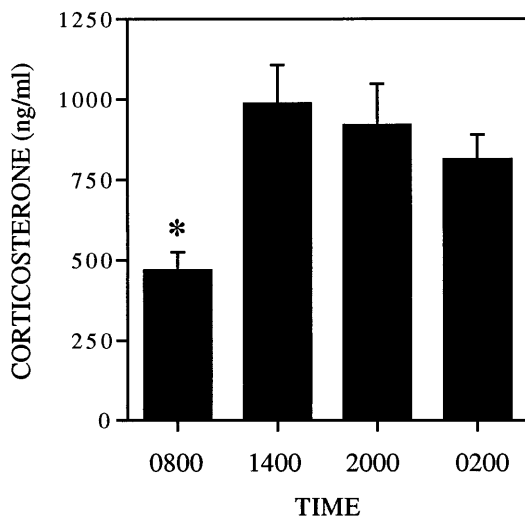


FIG. 2. Changes in plasma corticosterone concentration with time of day in male and female prairie voles. Blood samples were collected from independent groups at 0800, 1400, 2000, and 0200 hr ($n = 12$ –15 per group). Bars represent mean \pm SEM. *At 0800 hr, corticosterone was significantly lower ($P < 0.0167$) than at 1400 and 2000 hr.

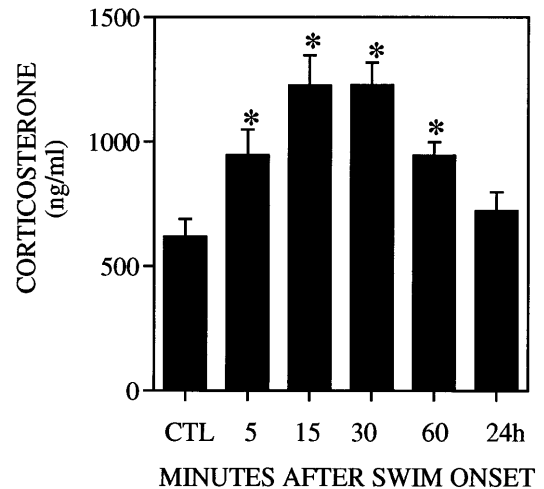


FIG. 3. Male prairie voles' corticosterone response to a 3-min swim test (mean \pm SEM), at various times after onset of swimming ($n = 8$ –10 per group). *At all time points except 24 hr, plasma corticosterone levels were significantly higher in swim-tested animals than in undisturbed controls.

were given a 3-min swim test. Significant elevations in corticosterone concentration were present 5, 15, 30, and 60 min after the onset of swimming ($F_{5,52} = 7.892$, $P = 0.001$; Fig. 3). At 24 hr postswim, corticosterone had returned to baseline levels.

Dexamethasone Dose Response in Prairie Voles

The dexamethasone suppression test is used widely for the diagnosis of abnormal feedback inhibition of glucocorticoids (Chrousos *et al.*, 1993). The goal of this experiment was to determine if prairie voles require more DEX than do corticosterone sensitive rats in order to achieve the same level of glucocorticoid suppression.

Plasma corticosterone levels from eight DEX-treated rats were below the lowest corticosterone assay standard (5 ng/ml). For five of these, extrapolation was possible; the remaining three were arbitrarily assigned a value of 1 ng/ml. All of the CTL rat samples, and all prairie vole samples, were well within the detection limits of the assay.

Doses ranging from 10 to 1000 $\mu\text{g}/\text{kg}$ of DEX failed to significantly suppress corticosterone concentrations in male prairie voles 12 hr postinjection ($F_{5,53} = 0.36$, $P = 0.87$; Fig. 4), while in male rats, corticosterone declined significantly 12 hr after 100 $\mu\text{g}/\text{kg}$ DEX as

compared to CTL (17 ± 5.6 and 144 ± 23.5 , mean \pm SEM, respectively; $t_{14} = 5.23$, $P = 0.0001$, data not shown).

Plasma Levels of Dexamethasone in Rats and Prairie Voles

To eliminate variation due to species differences in DEX absorption or metabolism, we compared the plasma level of DEX, rather than the injection dose, necessary for suppression of corticosterone in male prairie voles and rats.

Plasma DEX concentration was significantly lower in male prairie voles ($n = 9$ pools) than in male rats ($n = 8$) 6 hr after injection of $400 \mu\text{g}/\text{kg}$ of DEX ($7 \pm 0.4 \text{ ng}/\text{ml}$ vs $57 \pm 3.4 \text{ ng}/\text{ml}$, mean \pm SEM; $t_{15} = 15.7$, $P < 0.0001$). These results suggest that rapid clearance of DEX might contribute to the lack of corticosterone suppression in prairie voles, 12 hr after ip injection of up to $1000 \mu\text{g}/\text{kg}$ DEX. To obtain a range of similar plasma levels of DEX, samples collected 6 hr after injections of $100 \mu\text{g}/\text{kg}$ of DEX in male rats ($n = 9$), and 3 or 6 hr after injection of 400 or $1000 \mu\text{g}/\text{kg}$ of DEX in male prairie voles ($n = 5$ pools). In samples with similar plasma DEX concentrations, there was greater relative suppression of corticosterone levels in rats than in prairie voles (Fig. 5).

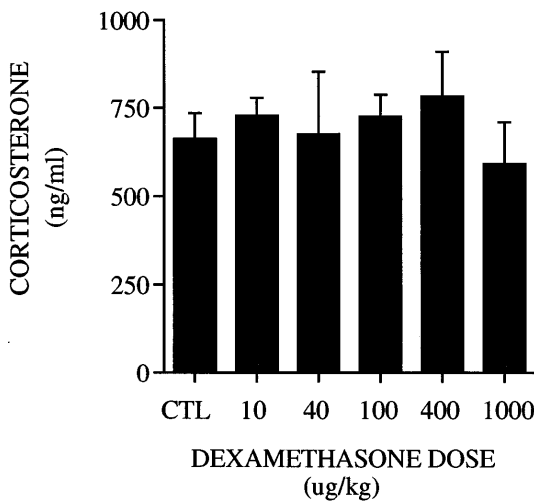


FIG. 4. Corticosterone response to DEX in male prairie voles (mean \pm SEM). Corticosterone was measured in plasma samples collected 12 hr after ip injection of DEX or CTL solution ($n = 14$ CTL; 8–10 per DEX group). DEX doses of up to $1000 \mu\text{g}/\text{kg}$ did not significantly suppress corticosterone.

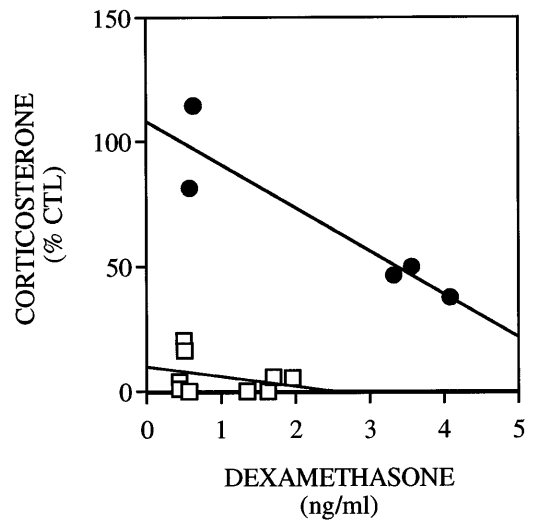


FIG. 5. Effect of comparable plasma levels of DEX on corticosterone concentrations in male prairie voles (●) vs male rats (□). DEX and corticosterone were measured by RIA, in samples taken 6 hr after ip injection of $100 \mu\text{g}/\text{kg}$ of DEX in rats, and 3 or 6 hr after ip injection of 400 or $1000 \mu\text{g}/\text{kg}$ of DEX in prairie voles. Plasma corticosterone is expressed as the percentage mean plasma corticosterone concentration of time-matched, CTL-injected animals.

Characterization of Prairie Vole GR: Immunologic Analysis, Abundance, and Affinity

To determine if glucocorticoid resistance in prairie voles is due to gross GR defects, protein extracts from prairie vole and rat liver and brain were subjected to Western blot analysis, using a specific antibody directed to an epitope in the N-terminal portion of the GR. In both prairie vole and rat brain and liver protein preparations, the major band detected by the anti-GR antibody was $\sim 97 \text{ kDa}$, the expected GR size (Fig. 6). Other detected bands may represent degradation products of the GR (Hollenberg *et al.*, 1985).

Reduced glucocorticoid signal transduction, due to decreased abundance of target tissue GR or low affinity of GR for its hormonal ligand, could cause glucocorticoid resistance. Therefore, the abundance and ligand binding affinity of prairie vole and rat GR were compared using protein binding assays. While the GR concentration (B_{max}) was significantly lower in liver cytosol from male prairie voles than male rats (28.8 ± 2.61 and $69.9 \pm 2.98 \text{ fmol}/\text{mg}$, respectively; $t_8 = 10.38$, $P < 0.0001$), the concentrations of CR (which includes both types I and II) in brain cytosol did not differ in the two species (Fig. 6; 35.5 ± 5.35 and

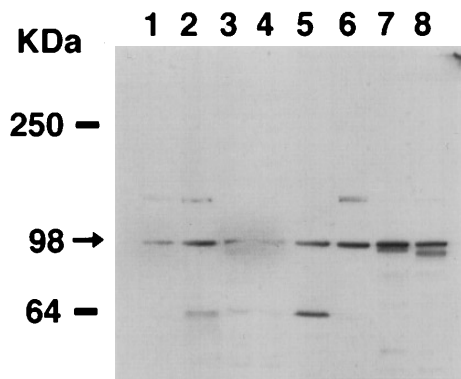


FIG. 6. Western blot of brain and liver cytosol (85 μ g protein) from prairie voles and rats ($n = 2$ each). Blots were probed with an anti-human GR antibody directed to an epitope in the amino-terminal domain of the protein. The expected size of the GR is \sim 97 kDa. Lanes 1 and 2, prairie vole liver; lanes 3 and 4, prairie vole brain; lanes 5 and 6, rat liver; lanes 7 and 8, rat brain.

34.2 ± 7.56 fmol/mg, respectively; $t_8 = 0.144$, $P = 0.89$). GR affinity for DEX in liver and CR affinity in brain cytosol were significantly lower in prairie voles than in rats (Fig. 7; K_d liver: 0.73 ± 0.005 vs 0.43 ± 0.003 nM; $t_8 = 4.87$, $P = 0.0012$; K_d brain: 1.2 ± 0.007 vs 0.32 ± 0.003 nM; $t_8 = 10.70$, $P < 0.0001$).

DISCUSSION

Several species of *Microtus* have high basal concentrations of corticosterone (McDonald and Taitt, 1982; Boonstra and Boag, 1992; DeVries *et al.*, 1995) in the absence of apparent glucocorticoid-induced pathology. We have conducted the first systematic study of glucocorticoid resistance in *Microtus*. Prairie voles (*M. ochrogaster*) had significantly greater adrenal-to-body weight ratios, and significantly higher basal plasma corticosterone and ACTH concentrations, than both montane voles (*M. montanus*) and rats. These parameters were not significantly different between montane voles and rats, indicating that hyperactivity of the HPA axis is not characteristic of all New World *Microtus* species.

Our studies showed that basal plasma levels of total corticosterone in male prairie voles were approximately 10-fold higher than those of male rats. It is unlikely that this relative adrenal hyperactivity was

due to chronic stress. First, prairie voles exhibit no apparent negative consequences of glucocorticoid excess, such as immunosuppression (Klein *et al.*, 1996). Second, following chronic stress, corticosterone levels in rats do not exceed 300 ng/ml (Vernikos *et al.*, 1982; Kiss and Aguilera, 1993), less than half of the basal level of corticosterone in male prairie voles. However, some glucocorticoid resistant New World nonhuman primate species (Chrousos *et al.*, 1982a), and Townsend's voles (*Microtus townsendii*; McDonald and Taitt, 1982), have basal plasma corticosterone levels similar to those of prairie voles.

Our measurements of basal CBG and estimated free corticosterone concentrations for prairie voles are similar to those of both Townsend's and meadow voles (*M. pennsylvanicus*) (McDonald and Taitt, 1982; Boonstra and Boag, 1992). There was a large difference between the estimated and directly measured concentrations of free corticosterone, which is probably due to the presence of the albumin-bound fraction of corticosterone in the latter, decreasing the amount of the free fraction in the ultrafiltrate, as previously reported (Pugeat *et al.*, 1984; Chrousos *et al.*, 1982a). Because albumin does not cross the filter, this method does not measure albumin-bound steroid, which is biologically active. However, using either method of measurement, free corticosterone levels were significantly greater in prairie voles than in rats.

Despite indices of HPA axis hyperactivity, corticosterone levels of prairie voles remained responsive to a stressor, and retained a blunted circadian rhythm. Therefore, the 10-fold difference in basal corticosterone levels between male prairie voles and rats was not an artifact of opposing circadian rhythms. Prairie voles, like rats (Persengiev *et al.*, 1991), displayed a morning trough of corticosterone levels. Although the variation was not statistically significant for male prairie voles, corticosterone levels were lowest in the early morning and rose throughout the day. The pattern of corticosterone secretion in prairie voles corresponds to their activity rhythm (Dewsbury, 1980; Moffatt *et al.*, 1993), and the low amplitude of corticosterone changes from peak to trough may be explained by their greater diurnal activity relative to other nocturnal rodents (Dewsbury, 1980), or by hippocampal-hypothalamic-pituitary resistance to glucocorticoids. Exposure to continuous light resulted in constantly high levels of

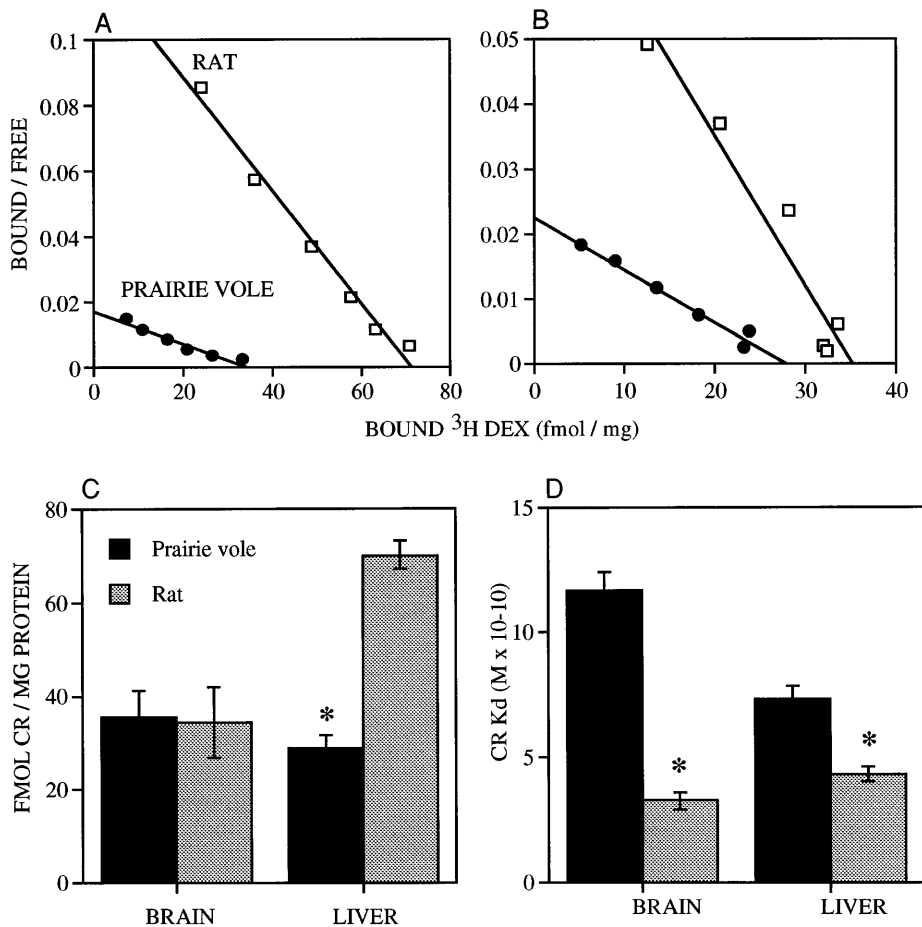


FIG. 7. Functional assay of GRs and CRs in male prairie voles vs male rats. Representative Scatchard plots of (A) liver cytosol GR binding and (B) whole brain cytosol CR binding in one male prairie vole, and one male rat. (C) CR/GR concentration (B_{max}) in whole brain and liver cytosol. Bars represent mean \pm SEM from five male prairie voles and five male rats. *Prairie vole liver cytosol has significantly less GR than rat liver cytosol ($P < 0.0001$). (D) The dissociation constant (K_d) of CR/GR for DEX in whole brain and liver cytosol. Bars represent mean \pm SEM from five male prairie voles and five male rats. *The affinity ($K_a = -1/K_d$) of CR/GR for dexamethasone is significantly lower in both prairie vole whole brain and liver cytosol than in the same tissues in rats.

corticosterone in female prairie voles (at least 1000 ng/ml), suggesting that the corticosterone rhythm was strongly influenced by light cues. Prolonged, high-corticosterone surges also occur in rats exposed to constant light (Persengiev *et al.*, 1991).

The HPA axis of prairie voles was responsive to light cues and to swim stress, but was strongly resistant to suppression by dexamethasone, using both DEX injection dosage and plasma DEX concentration as the criteria. The significant differences in plasma DEX concentrations, after administration of the same DEX dose to prairie voles and rats, may have been due to species differences in absorption or metabolism of

DEX. Such data suggest that the plasma concentration of DEX necessary for corticosterone suppression should be determined for valid interspecies comparisons of DEX sensitivity.

Dexamethasone resistance in prairie voles suggested that their HPA axis has a relatively high set-point for regulation, in order to compensate for decreased target tissue sensitivity to glucocorticoids. The results of the GR binding assay were consistent with this hypothesis. The binding assay did not distinguish between type I (classical mineralocorticoid receptors) and type II (GR) receptors. In rats and primates, type II receptors in the brain are approximately 10-fold more abundant

than type I, and type II receptors have a 3-fold higher affinity for DEX than type I (Johnson *et al.*, 1996b; Spencer *et al.*, 1990), suggesting that in these species the contribution of type I to dexamethasone binding is minor. Presumably, the contribution of type I receptor binding is also negligible in prairie voles; however, the relative distribution and affinities of type I and type II receptors have not been determined in prairie voles as yet. Therefore, when referring to DEX binding in brain cytosol we have used the broader term of corticosteroid receptors.

In prairie voles, as in glucocorticoid resistant squirrel monkeys (Chrousos *et al.*, 1982a) and guinea pigs (Kraft *et al.*, 1979), there was a significant decrease in the affinity of liver GRs for ligand. Whole brain cytosol from prairie voles also showed decreased affinity for DEX, relative to rat whole brain cytosol. Our findings do not preclude the possibility that specific reductions in the concentration of hippocampal, hypothalamic, or anterior pituitary corticosteroid receptors might also contribute to HPA axis hyperactivity in prairie voles.

Decreased corticosteroid receptor concentration, which also has been associated with familial glucocorticoid resistance (Karl *et al.*, 1993), was found in prairie vole liver, but not whole brain, cytosol. This may reflect greater proteolysis in prairie vole liver than rat liver, or, it may be functionally significant. Reduction in both the affinity and the concentration of peripheral GRs of prairie voles might account for the absence of adverse glucocorticoid-induced effects, despite their corticosterone excess.

The mechanism(s) responsible for glucocorticoid hypersecretion and resistance in prairie voles appears to differ from those characterized in guinea pigs. Glucocorticoid resistance in guinea pigs appears to be part of a larger alternative strategy for the maintenance of glucose homeostasis (Keightley and Fuller, 1996). In guinea pigs, nucleotide alterations in the gene encoding proopiomelanocortin (POMC), the ACTH precursor, lead to production of a "superagonist" ACTH which circulates at "normal" levels (Smith *et al.*, 1987; Keightley *et al.*, 1991), causing hypercortisolism. However, the presence of elevated plasma levels of ACTH in prairie voles makes the existence of "superagonist" POMC/ACTH alterations unlikely in this species.

The mechanism(s) responsible for glucocorticoid resistance of prairie voles appears to differ from that of

New World nonhuman primates. In the latter, glucocorticoid resistance appears to be part of a generalized syndrome of steroid hormone resistance ("pan-steroid" resistance), possibly mediated through the actions of a protein that interferes with steroid-receptor binding, or with the actual steroid hormone transcription machinery (Lipsett *et al.*, 1985; Brandon *et al.*, 1989; Gacad and Adams, 1991; Onate *et al.*, 1995). Prairie voles are probably not pan-steroid resistant, as estrogen and testosterone levels are similar in prairie voles and rats (Cohen-Parsons and Carter, 1987; Nelson *et al.*, 1989).

The decreased abundance of GR in prairie vole liver cytosol and decreased affinity of prairie vole corticosteroid receptors in liver and whole brain cytosol (2- and 3-fold, respectively) might contribute to glucocorticoid resistance in this species. New World (corticoreistant) monkeys show a similar 2.5-fold decrease in GR affinity relative to Old World (corticosenitive) monkeys (Chrousos *et al.*, 1982). Other defects found in New World monkey glucocorticoid signal transduction suggest that the 2.5-fold decrease in affinity of the GR is not solely responsible for glucocorticoid resistance. Similarly, the combined actions of several inhibitory mechanisms at various stages of glucocorticoid signal transduction may result in glucocorticoid resistance in prairie voles.

We conclude that prairie voles are a novel rodent model for generalized glucocorticoid resistance, associated with alterations in target tissue corticosteroid receptors. Thus, glucocorticoid resistance is found in two widely divergent rodent families (prairie voles, Arvicolidae; and guinea pigs, Caviidae) and some nonhuman primates, all with New World origins. This suggests that evolutionary pressures unique to the New World may have favored the development of glucocorticoid resistance (Keightley and Fuller, 1996). Nonpathological glucocorticoid resistance probably arose on three separate occasions. The range of endocrine axis anomalies in guinea pigs suggests that their lineage may have branched off before the divergence among other rodents, and among primates (Li *et al.*, 1992; Keightley and Fuller, 1996). Glucocorticoid resistance arose again after the New and Old World monkeys diverged, ~30–50 million years ago (Cronin and Sarich, 1978; Chrousos *et al.*, 1982a). Our data suggest that montane voles are not glucocorticoid

resistant. Therefore, glucocorticoid resistance in prairie voles presumably arose for the third time, within the last 0.7 million years, when the lineages of prairie voles and montane voles diverged (Chaline and Graf, 1988).

The possible selective advantages conferred by non-pathological target tissue resistance to glucocorticoids remain unclear. Some evidence suggests that glucocorticoid resistance may be advantageous to prairie voles, through direct or indirect interactions between their corticosterone excess and social behaviors, such as those associated with monogamy. The HPA axis of prairie voles both responds to and influences social behaviors. For example, prairie voles exhibit declines in plasma corticosterone levels within minutes of the onset of pair-bonding and reproductive activation (DeVries *et al.*, 1995), and in turn, corticosterone and vasopressin modulate pair-bonding (Winslow *et al.*, 1993; DeVries *et al.*, 1995; Carter *et al.*, 1995) and biparental care (Wang *et al.*, 1994).

In contrast to glucocorticoid resistance in humans, which is a pathological condition, this state has arisen in guinea pigs, some New World primates, and prairie voles, as a nonpathological, adaptive response to as yet unknown selective pressures unique to the New World. Because voles, including corticosterone-resistant prairie voles and corticosterone-sensitive montane voles, can be studied both in nature and in the laboratory, these rodents offer excellent models for the analysis of both the physiological and behavioral consequences of glucocorticoid resistance.

APPENDIX

Barsano and Baumann equation:

$$H_f = 0.5[H_t - \text{MBC} - 1/K_a \pm [(MBC - H_t + K_a)^2 + 4(H_t/K_a)]],$$

where H_f = free hormone (M), H_t = total hormone, (M), $\text{MBC} = \text{MCBC} (M)$, and K_a = affinity of binding protein for hormone (M^{-1}).

Tait-Burstein equation:

$$K_{\text{CBG}}N[i]^2 + \left\{ N + K_{\text{CBG}} \left[\left(\sum_{\text{PCBG}} \right) - \left(\sum_i \right) \right] \right\} \left[[i] + \left[\sum_i \right] \right] = 0,$$

where K_{CBG} = affinity of binding protein for hormone

(M^{-1}), $\sum P_{\text{CBG}} = \text{MCBC} (\mu\text{g}/\text{dl})$, $N = 1 + K_{\text{ALB}} [\sum P_{\text{ALB}}]$ (where K_{ALB} = affinity of albumin for hormone in M^{-1} , and $\sum P_{\text{ALB}}$ = concentration of albumin binding sites in $\mu\text{g}/\text{dl}$), $\sum i$ = total corticosterone ($\mu\text{g}/\text{dl}$), and $[i]$ = free corticosterone ($\mu\text{g}/\text{dl}$).

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