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Radioimmunoassay (RIA) of Serum Corticosterone in Rats[1,2] (41391)

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Abstract. A radioimmunoassay for corticosterone was developed and characterized using corticosterone antiserum (377, Niswender) and a simple ethanol extraction procedure. The antiserum appeared to be highly specific for corticosterone. Intraassay variability was 3.67 ± 0.75% (mean ± SE) at 50 pg of corticosterone; interassay variability with a mean value of 51.15 pg was 6.98 ± 1.01%. Assay sensitivity was 9.48 ± 0.60 pg. Utilizing this assay, serum obtained from adrenalectomized and adrenalectomized–ovariectomized rats yielded lower corticosterone values than serum from intact or ovariectomized rats. Intact females had lower corticosterone values than intact males. Rats exposed to elevated temperature (32.5°) displayed significantly ($P < 0.001$) elevated plasma corticosterone levels (48.48 ± 4.37 μg/100 ml) compared to control (24.5°) animals (21.31 ± 2.02 μg/100 ml). The high specificity, sensitivity, precision, recovery level, and ease of this technique make it useful for the study of either serum or plasma corticosterone.

Animals exposed to conditions for which they are unaccustomed or nonadapted exhibit responses similar to those resulting from ACTH stimulation (1) including increased levels of glucocorticoids and mineralocorticoids. Corticosteroids, in particular, have been shown to be sensitive and reliable indicators of an animal's internal environment (2). Thus, it is common practice (3, 4) to use fluctuations in corticosteroid levels as indicators of physiological stress.

Fluorometric (5, 6) or competitive protein-binding (7) techniques do not accurately measure levels of corticosterone in serum. Moreover, the development of a radioimmunoassay to measure corticosterone levels in laboratory animals has been difficult because of antibody cross-reaction (8, 9), large sample requirements, and the need for chromatographic purification of samples. Recent availability of an antiserum containing a high affinity for and specificity to corticosterone has enabled the development of a reliable radioimmunoassay for measuring corticosterone in rat serum. This assay and its validation are described here.

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Materials and Methods. *Animals.* Male and female Sprague–Dawley rats [Caw: CFE (SD)] were housed in metabolic cages (Acme, Model 4-641-000) and floor space was equal to the recommended value (10). Food (Purina Laboratory Chow) and water were available *ad libitum*.

Surgical procedures. Five female rats were ovariectomized (O); five females were adrenalectomized–ovariectomized (A/O); and, six males were adrenalectomized (A). All surgical procedures were performed though the bilateral dorsal approach using ether anesthesia. Following adrenalectomy, animals were maintained on a 0.9% NaCl drinking solution, available *ad libitum*. Completeness of adrenalectomy was verified at necropsy and by serum corticosterone levels (11). A group of five intact females (I_f) and a group of seven intact males (I_m) were used as controls. Rats were decapitated between 0900 and 1100 hr; blood was collected in culture tubes and serum was separated and frozen.

Experimental conditions. RIA of serum or plasma corticosterone was performed on rats from two different experimental conditions:

1. A, O, A/O, and I groups. Control temperature of 25.0 ± 1.0°, 50 ± 5% relative humidity, and a 12L:12D photoperiod (L = 0600 to 1800 hr) were maintained until

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decapitation, 10 days after surgery for all animals except those in the A group. The A animals were individually caged in a controlled environment room (Forma Scientific, Model C7-88) for 4 weeks following surgery. The O, A/O, and I animals were caged in pairs.

2. *Heat-stressed group.* The 12 male rats used in this experiment were caged individually in a controlled environmental room. Relative humidity of 50 ± 2% and a 12L:12D photoperiod (L = 0900 to 2100 hr) were maintained for all experiments. Continuous monitoring of the ambient temperature in the environmental room was accomplished through thermistors (YSI, Model 43TD) interfaced with a data acquisition system (Esterline Angus, Model PD2064). The dry-bulb temperature was maintained at either 24.5° (control) or 32.5° (experimental). The animals were at 24.5° for 10 days followed by acute (1–2 days) exposure to heat (32.5°). Blood samples were collected by heart puncture between 0900 and 1100 hr into heparinized tubes within 40 sec of opening the animal's cage. It previously has been reported (12) that plasma corticosterone is not elevated within 4 min of cage opening. The blood was centrifuged and the plasma was frozen.

Reagents. Radioactive corticosterone [1,2,6,7-^{3}H(N)] with a specific activity of 82.1 Ci/mmol (New England Nuclear, NET-399) and corticosterone standard (Sigma Chemical) were used. Charcoal (Sigma Chemical), dextran T-70 (Pharmacia), gelatin (Sigma Chemical), and ethanol (U.S. Industrial Chemicals) are required reagents. Sephadex LH-20 (Sigma Chemical), benzene (Baker Chemical), and methanol (Burdick and Jackson) were used to compare alcohol extraction procedures.

Solutions. Phosphate-buffered saline with gelatin (PBS-g), 1.0 *M*, pH 7.2, containing 0.1% gelatin and 0.85% NaCl, was used in the assay procedure. The charcoal suspension, for separating free from bound steroid, was composed of 0.625 g charcoal and 0.0625 g dextran in 100 ml of PBS-g buffer. The scintillation fluid consisted of a 2:1 aqueous counting scintillant (ACS, Amersham):toluene (Mallinckrodt) solution. The anticorticosterone–BSA-3 antiserum 377 was produced in rabbits.[3] The affinity constant for this antiserum was calculated from a Scatchard plot (13).

Assay procedure. A previously reported assay procedure (14) was modified as follows: The serum was extracted with 200 μl of ethanol from a freshly opened bottle. For rats, 10 μl of serum was sufficient. The assay tubes containing serum were agitated with a vortex mixer and allowed to stay at room temperature for 10 min. Serum proteins were pelleted by centrifugation (Beckman, Model TJ-6) at 2500 rpm for 10 min; 50 μl of the supernatant was used for assay.

The solvent was evaporated by passing a stream of dry air over the samples, which were heated in a water bath at 60°. The samples were resuspended in 350 μl of PBS-g buffer, vortexed gently, and allowed to stand at room temperature for 20 min. One hundred microliters of a 1:2000 dilution of antiserum was added to each tube and vortexed gently. The antiserum dilution of 1:2000 was sufficient to bind 40% of the [^{3}H]corticosterone added. Twenty minutes later, 0.05 ml of radioactive corticosterone (10,000 cpm) in PBS-g buffer was added and each tube vortexed.

After incubation overnight at 4°, the tubes were placed in a cold room (4°) and 200 μl of charcoal suspension was added while stirring. After 10 min of incubation, the tubes were centrifuged at 2500 rpm for 10 min. Using a dilutor (Labindustries, Model 81001), 300 μl of supernatant was transferred to a scintillation counting vial with 3 ml of ACS–toluene scintillation fluid and vortexed. The scintillation vials were allowed to settle in a cool dark place (4°) for 15 min prior to counting (Packard, Model 3320). All vials were counted for 1 min.

Pooled plasma and serum samples from rats in each of four groups (A, I_m, heat stressed, and control) were assayed after ethanol extraction alone, methanol extrac-

[3] The authors gratefully acknowledge the donation of antiserum by Dr. Gordon D. Niswender, Department of Physiology and Biophysics, Colorado State University.

tion alone, and after column chromatography purification. Methanol extraction involved the same procedure described above for ethanol extraction. After ethanol extraction, the dried samples were subjected to Sephadex LH-20 column chromatography using benzene:methanol (85:15) as eluant (15). After collection, fraction samples (250 μl) were dried and residues were analyzed for corticosterone by radioimmunoassay.

Standard curve. A standard curve was prepared by assaying varying volumes of corticosterone standard (10 pg/μl) between 10 and 1000 pg. The standard curve (Fig. 1) was plotted according to the logit transformation (16). Calculations were made with a programmable calculator (Texas Instruments, Model TI-59) interfaced with a printer (Texas Instruments, PC-100A). Results were expressed as micrograms corticosterone/100 ml serum or plasma.

Recovery error. Using the same extraction procedure, corticosterone was recovered from a representative subset of samples equalling about 10% of the total number of unknown samples.

Various amounts of corticosterone (0.5 to 50 ng) were added to 200 μl of serum from each of six group A rats. The samples were vortexed, extracted, and assayed for corticosterone levels.

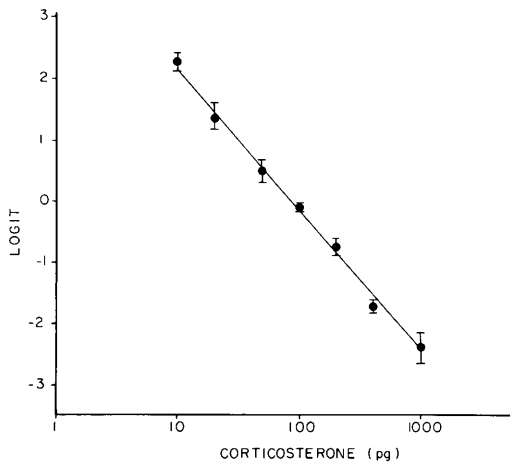

FIG. 1. Standard curve for corticosterone. Each point represents the mean value and the vertical bars the standard errors.

Cross-reactions. The percentage cross-reactivity was calculated as $C/X \times 100$ where C is the amount of corticosterone, and X is the amount of cross-reacting steroid at which binding of labeled corticosterone is reduced by 50% (13).

Statistical analysis. Comparisons between groups were determined by analysis of variance. The paired comparison t test was utilized to confirm parallelism of the slopes of the dilution curves of the A and I_m groups with the standard curve for corticosterone. Significance was assumed when $P < 0.05$.

Results. *Antiserum specificity.* Data on the cross-reaction of antiserum with corticosterone and other steroids are presented in Table I. The antiserum proved to be highly specific for corticosterone. The affinity constant for this antiserum was 1.91×10^{10} liters/mole.

Recovery of added corticosterone. Recoveries averaged 69.96 ± 0.42% (mean ± SE) and all samples were corrected for this error. The relationship between the amount of corticosterone assayed compared to the amount added to the A sera is described in Fig. 2.

TABLE I. THE CROSS-REACTION OF ANTI-CORTICOSTERONE-BSA-3 ANTISERUM TO CORTICOSTERONE AND A VARIETY OF STEROIDS

Compound	Percentage cross-reaction
Corticosterone	100.000
Deoxycorticosterone	7.080
11-β-Hydroxyprogesterone	7.000
Progesterone	0.952
11-α-Hydroxyprogesterone	0.321
11-Deoxycortisol	0.200
21-Deoxycortisol	0.160
Cortisol	0.114
20-β-Hydroxyprogesterone	0.053
Cortisone	0.033
Testosterone	0.007
20-α-Hydroxyprogesterone	0.006
17-Hydroxyprogesterone	0.005
Dehydroepiandrosterone	0.004
17-β-Estradiol	0.003
Aldosterone	0.002
Estriol	0.001
Estrone	<0.001
Cholesterol	<0.001

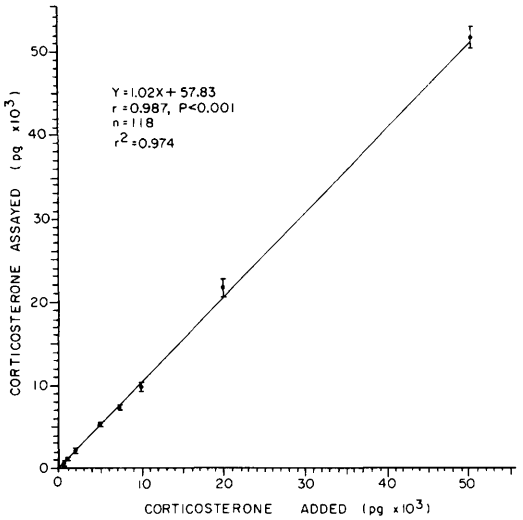

FIG. 2. Values for recovered amounts of corticosterone added to adrenalectomized rat sera. Each point represents the mean value and the vertical bars the standard errors.

Precision. Sera unknowns from A and I_m rats, assayed in serial dilution (10, 20, 40, 60, 80 μl), paralleled the standard curve (Table II). Intraassay variability expressed as a coefficient of variation was 3.67 ± 0.75% at 50 pg of corticosterone. Interassay variability with a mean corticosterone value of 51.15 pg was 6.98 ± 1.01%. Duplicates of 17 assays were used in these calculations (16).

Sensitivity. This is defined as the smallest amount of corticosterone that is significantly different from that in the zero standard. The sensitivity of this assay was 9.48 ± 0.60 pg/assay tube.

Physiological responses. Serum obtained from the A group (0.64 ± 0.04 μg/100 ml; n = 33) and the A/O group (3.59 ± 0.40; n = 40) of rats yielded significantly ($P < 0.001$) lower corticosterone values than either I_f (15.26 ± 2.58; n = 15), I_m (19.08 ± 0.68; n = 47), or O (24.12 ± 4.43; n = 22) animals. The I_f values are significantly lower than both I_m ($P < 0.001$) and O ($P < 0.05$) groups; and, the I_m values are significantly lower than the O ($P < 0.01$) group. Corticosterone concentrations in A sera was significantly ($P < 0.001$) decreased below that of the A/O sera.

The heat-stressed (32.5°) rats (48.48 ± 4.21 μg/100 ml; n = 14) displayed a significantly ($P < 0.001$) elevated plasma corticosterone levels compared to control rats at 24.5° (21.31 ± 2.02; n = 24). These control plasma values are not significantly different from those obtained from I and O sera but are increased ($P < 0.05$) over the I_f values.

There were no significant differences between corticosterone values obtained from ethanol extraction alone compared to methanol extraction alone and column chromatography purification (Table III).

Discussion. In many of the referenced experiments (2, 3, 5–7, 12, 17–20) corticosterone was measured utilizing a fluorometric technique. The wide range of reported control values for male rats, from about 13 μg/100 ml (19) to 25 μg/100 ml (20), is probably due to this technique. The radioimmunoassay reported here for control male rats is less variable.

Serum corticosterone levels of I, O, A/O, and A rats are similar to those previously reported (3, 11, 12, 18, 19). The specificity of corticosterone antiserum was very high

TABLE II. CHARACTERISTICS OF REGRESSION EQUATIONS OBTAINED FROM SERUM SAMPLES FOR ADRENALECTOMIZED (A) AND INTACT MALE (I_m) RATS ASSAYED IN SERIAL DILUTION[a]

Group	Serum corticosterone (μg/100 ml)	Slope (m)	Correlation coefficient (r)	Coefficient of determination (r^2)
Corticosterone standard	—	−2.37	−0.998	0.996
I_m	19.08 ± 0.68	−2.46	−0.967	0.935
A	0.64 ± 0.04	−2.26	−0.988	0.976

[a] Serial dilutions: 10, 20, 40, 60, 80 μl. For each dilution, triplicate serum samples were assayed for each rat in the group. There were no significant differences between any of the compared slopes.

and interference from other steroids was variable (Table I). Because no significant differences in corticosterone levels were observed between chromatographic purification and ethanol or methanol extraction (Table III), the observed cross-reactions for deoxycorticosterone (7.08%) and 11-β-hydroxyprogesterone (7.00%) do not appear to affect the usefulness of this RIA as a sensitive and precise technique for studying either serum or plasma corticosterone.

The large affinity of corticosterone antiserum for corticosterone allowed for greater sensitivity (10 pg) and made small sample (10–100 μl) use possible. Because chromatographic purification of the extract was unnecessary (Table III), the assay was easily performed. The intra- and interassay variability and recovery level of 70% contribute to the high reproducibility of this assay. As this RIA also is more specific than the competitive protein-binding (7), fluorometric (5, 6), and other RIA techniques (8, 9), it should be the preferred method for measuring serum or plasma corticosterone. The ease of this technique allows handling of large numbers of samples; one person can assay 400 samples per week.

It has been reported that plasma-free corticosteroids reach 0% of control levels in 4 hr (19) or that they are maximally reduced in 4–6 hr (18) postadrenalectomy in rats. It also has been shown (11) that there are no differences in corticosterone levels between plasma samples taken at 12 hr and 28 days following adrenalectomy. In the present study, ovariectomy and adrenalectomy were performed 10 days prior to decapitation in the O and A/O groups.

Plasma corticosterone levels increased significantly ($P < 0.001$) on exposure to heat. Similar observations have been reported (3) in rats exposed to temperatures of 29.2 and 34.0°. Increased adrenal activity is associated with the onset of stress and results from the stressor-stimulated release of ACTH, which helps the animal to adjust physiologically (1). The experimental temperature (32.5°) used in this experiment is therefore considered stressful to these animals. The results further support the applicability of this radioimmunoassay for measuring corticosterone in physiological studies using rats.

The control values in the heat experiment are similar to some previously reported (3) but are higher than others (19). This variation may be accounted for by the technique used for corticosterone measurement or alterations in biorhythms due to time of blood sampling. Influences of daily biorhythms do not appear to be responsible for this variation (20). Also, in the present study, the effect of biorhythms was minimized by collecting blood samples at the same time of day from all groups.

Corticosterone levels of male rats (intact 19.08 ± 0.68 μg/100 ml; control 21.31 ± 2.02) were significantly ($P < 0.001$) elevated above the values of the females (15.26 ± 2.58). These results indicate the existence of sex differences in peripheral corticosterone levels of the rat as previously reported (17). Other reports (21, 22) suggest that changes in serum corticosterone are associated with reproductive function and status of the rat. The gonadal hormones mediate corticosterone secretion at several levels, including the hypothalamus, pitu-

TABLE III. COMPARISON OF CORTICOSTERONE VALUES OBTAINED FROM ETHANOL EXTRACTION ALONE, METHANOL EXTRACTION ALONE, AND FROM CHROMATOGRAPHIC PURIFICATION

Group	Ethanol extraction (μg/100 ml)	Methanol extraction (μg/100 ml)	Chromatographic purification (μg/100 ml)
Intact males (I_m)	19.04 ± 1.24[a]	19.16 ± 1.19	18.98 ± 0.80
Adrenalectomized (A)	0.84 ± 0.14	0.80 ± 0.09	0.87 ± 0.08
Control (24.5°)	20.63 ± 2.20	21.29 ± 2.68	21.13 ± 1.42
Heat stressed (32.5°)	39.02 ± 1.72	38.07 ± 2.22	40.34 ± 2.44

[a] Mean ± SE; values are based on triplicate determinations for each sample.

itary, liver, and the adrenal cortex (17, 21). The elevated corticosterone levels (24.12 ± 4.43) found in ovariectomized rats may result from altered adrenal metabolism (23).

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