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Effect of Cold Exposure on Serum Levels of Corticosteroid-Binding Globulin in Male and Virgin Female Rats

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EFFECT OF COLD EXPOSURE ON SERUM LEVELS OF CORTICOSTEROID-BINDING GLOBULIN IN MALE AND VIRGIN FEMALE RATS

by

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TABLE OF CONTENTS

CHAPTER		PAGE
I	INTRODUCTION	1
II	MATERIALS AND METHODS	9
	Animal Experimental Groups	9
	Measurement of Serum Protein Concentration	11
	Measurement of Serum Corticosterone Concentration	11
	Measurement of Corticosterone Binding Globulin	13
	Multiple equilibrium dialysis measurement of CBG	13
	Absorption measurement of CBG	15
III	RESULTS	17
	Quantification of Analysis of CBG	17
	Effect of Cold Exposure on Male Rat CBG	17
	Effect of Cold Exposure on Female Rats CBG	18
IV	DISCUSSION	24
	Methods of Determining CBG	25
	Effects of Cold Exposure on DBG	26
	Measurement of Adrenocortical Responses to Cold	27
	Comparison of Male and Female Responses	27
V	LITERATURE CITED	29

INTRODUCTION

Protein binding of serum corticoids has been demonstrated in a number of vertebrate species (Seal and Doe, 1966). This function is performed by a glycoprotein α 2 globulin which appears to be produced by the liver (Gala and Westphal, 1965B; Gala and Westphal, 1966C). This protein, corticosterone-binding globulin (CBG), serves as the primary vascular depot and as a transport vehicle for corticosteroids (Bennhold, 1963). A species specificity for CBG molecules exists such that a given CBG is more specific for the major corticoid of that species (Murphy, 1967). For example, rat CBG is more specific for corticosterone than for cortisol whereas rabbit CBG is more specific for its endogenous corticosteroid, cortisol. Although CBG has some affinities for other steroids, such as progesterone, its affinity for corticosteroids is much greater (Westphal et al., 1961).

Physiochemical evaluations of CBG through amino acid composition and carbohydrate analysis prove a distinct molecule is present in each of the different species that have been examined. Rat CBG has 1.1 binding sites/mole at 37°C (Rosner and Hochberg, 1972) and appears to have a greater concentration of binding sites in its serum than several other species including man, rabbit and guinea pig (Westphal, 1967). Its affinity for corticosterone is maximal at pH 8 with decreasing affinity with either increased or decreased pH. Elevation of

temperature also decreases affinity with complete dissociation at 45°C (Westphal, 1966; Westphal, 1967). Normally 90% of the total blood corticoids will be bound to CBG, serum albumins also bind corticosteroids, but not to the extent that CBG does. Even with the high blood concentrations of albumins, only approximately 8% of the total serum corticoids are bound to albumin (Perrin and Forest, 1975).

Steroids when bound to serum proteins are generally thought to be in an inactive form (Yates and Urquhart, 1962; Milkovic and Domac, 1973). Some evidence suggests that this is not completely true in the corticosteroid-CBG relationship. Corticosterone will block the histamine induced increase in ACTH, but will not when corticosterone is bound to CBG (Kawai and Yates, 1966). In addition, corticoid binding does not stop inducement of hepatic alanine amino transferase by corticosterone (Keller et al., 1969). This could be due to variations in capillary permeability present in the two organ systems.

In the developing mammal there is a progressive increase in CBG concentration from low levels at birth to an asymptotic in adulthood. Sex differences vary among species with female rats having twice the concentration of serum CBG as male rats (Gala and Westphal, 1965B). There are no sex differences in CBG content in humans.

Changes in the physiological state of the animal will also effect the CBG levels. These include hormonal applications and/or experimental manipulations (surgery). Involved in these

manipulations is the hypothalamic-pituitary axis which has been shown to play an important role in the regulation of serum CBG. Hypophysectomy, thyroidectomy, ACTH administration and glucocorticoids decrease serum CBG while TSH administration and adrenalectomy cause an increase (Gala and Westphal, 1966A; Gala and Westphal, 1966C; Labrie et al., 1968).

The decrease induced by hypophysectomy is due to the indirect decrease in thyroxine following loss of TSH. TSH administration increases CBG in both intact and hypophysectomized animals, but does not in thyroidectomized animals (Gala and Westphal, 1966C). Throxine (T-4), which is controlled by TSH, is the thyroidal hormone necessary for maintaining or increasing CBG levels in the blood (Labrie et al., 1968).

ACTH potentiates the decrease of CBG through its action upon the adrenal gland. ACTH administration increases corticosteroidogenesis in the adrenal gland resulting in increased serum corticoids. It is these serum corticoids which cause the decrease in CBG concentration and ACTH administration does not effect the levels of CBG after this procedure (Gala and Westphal, 1966A).

The thyroid-adrenal relationship appears to be associated with the control of serum CBG concentration. Corticosterone has an inverse relationship with CBG in that when peripheral corticosterone concentrations increase there will be a concomittant decrease in CBG and visa versa (Gala and Westphal, 1966B). Thyroxine has the opposite effect in as much as increases in T-4 cause a further increase in CBG (Labrie et al., 1968). Interrelated

with these controls is the observation that a balance between T-4 and corticosteroids exists. Increases in T-4 result in increased serum corticoid concentrations and, in contradistinction, increased corticoids in the blood will induce a dimunition in T-4 release (Bondy and Hagewood, 1952; Fortier et al., 1970). Final evidence for TSH and T-4 as controllers of CBG concentration is that following hypophysectomy the expected increases resulting from adrenalectomy, estrogen treatment in males, and progesterone treatment in females do not occur (Gala and Westphal, 1965B; Gala and Westphal, 1966C). A final postulation of thyroid-adrenal control may be a combined feedback response on the liver in as much as both glucocorticoids and thyroid hormones are known to have binding sites associated with this organ (Suyemitsa and Terayama, 1975; Torresani and Degroot, 1975). Either hormone could then have the potential of regulating CBG synthesis.

Daily fluctuation in serum corticosterone levels are a normal occurrence. Circadian rhythms control corticotropin releasing factor (CRF), ACTH, and corticosterone secretion in rats (Krieger and Krieger, 1967). Pituitary ACTH will reach peak serum concentrations 4 hours (males) and 5 hours (females) prior to the resulting corticosterone peaks (Cheiftz, 1971). Increases in corticosterone are also diurnal in occurrence with peak levels occurring just prior to awakening and the onset of activity (Perkoff et al., 1950; Guillemin et al., 1959). Serum concentrations will then decrease back to baseline before rising again. During times of stress deviations from this normal circadian pattern in corticosterone will occur.

The term "stress" usually relates to a number of conditions sufficiently removed from normal which result in a physiological and/or pathological change within the organism. Conditions such as heat, cold, crowding, handling, restraint, ether exposure, electrical shock, and other noxious stimuli are often classified as stressors (Cook et al., 1963; Friedman et al., 1967). In general they all initiate activity within the hypothalamic-pituitary-adrenal axis resulting in an increase in serum corticoids (Christian and Davis, 1964). This occurs through stimulation of pituitary ACTH secretion which in turn stimulates the fasiculata-reticularis zone of the adrenal cortex to increase glucocorticoid production (Stachenko and Giroud, 1959). It is this increase in serum corticoids that is responsible for many of the adaptive effects of stress which include gluconeogenesis and increased fat mobilization.

Physiological and pathological responses to elevated serum corticosteroids include leukopenia, gastrointestinal lesions, and psychic changes (Metcoff et al., 1952; Southwick et al., 1955; Good et al., 1957). However, in pregnancy more deleterious effects can be seen. Corticosteroids have exhibited fetotoxicity and/or teratogenicity in rabbits, hamsters, rats, and mice. Included in these effects are retarded development, cleft palates, embryonic mortality, and vascular lesions (Courrier and Colonge, 1951; Decosta and Abelmann, 1952; Hensleigh and Johnson, 1971; Chaudhry and Shah, 1973). Behavioral changes in offspring have also been noted (Southwick, 1955; Christian, 1956). Beyond this, failure of implantation, abortion, and fetal reabsorbtion are also common occurrences (Shah, 1956; Pasqualini, 1971). These conditions appear

to be the result of a stress response of the mother rather than the fetus. Perhaps due to free corticoid movement across the placenta (Migeon et al., 1956; K. Milkovic et al., 1973; S. Milkovic, 1973) any stress upon the mother that increases serum corticoids could therefore effect the fetus. This free movement of corticoids across the placenta increases the possibility of deleterious results.

Placental transfer of corticosterone has been demonstrated in rats. Adrenalectomy in pregnant rats will result in adrenal hypertrophy in the fetus and maintenance of serum corticosterone in the mother (K. Milkovic et al., 1973). Blockage of corticosterone synthesis in the fetuses of adrenalectomized rats resulted in dimunition of maternal serum corticoids (Milkovic et al., 1975). This shows the important fetal involvement in adrenal responses. These responses appear to be independent of maternal ACTH with maximum response by the fetus occurring after day 18 of gestation in the rat (S. Milkovic et al., 1973). Therefore, the 18th day of gestation is the proposed time of initiation of the pituitary-adrenal axis in the fetus. Prior to this the fetal adrenal is largely inactive. The fetal adrenal response to conditions of low maternal corticoids does not appear to be independent of maternal control, because after parturition there is a decline in fetal adrenal weight (Josimovich et al., 1954). In addition ACTH administration to the new born induces only a slight corticoid response at birth; the adult response does not occur until day 15 (Allen and Kendall, 1967). Full circadian periodicity is not attained in the young rat till the 30th day postpartum (Allen and Kendall, 1967).

These low responses after birth further confirm some additional prepuberal maternal control.

In the mother corticosteroids will increase considerably from day 12 of pregnancy till gestation in the rat (Solem, 1966). This increase in corticosterone coincides with a subsequent increase in CBG (Gala and Westphal, 1965A). This alleviates any increase of corticoids reaching the fetus under normal conditions. Further increases of corticosterone through short term, low level stress have little effect on developing fetuses (Migeon et al., 1956). It is only during long term exposure and high concentrations of corticosterone does the aforementioned fetal abberations occur (MacFarlane et al., 1957; Pennycuik, 1966; Burton and Jeyes, 1968). It is also known that conditioning an animal to a stressor will decrease fetal abberations when the animal is reexposed during pregnancy (MacFarlane et al., 1957; Pennycuik, 1966). How the conditioning can prevent any abberations is still unanswered.

Recently, this increase in CBG in pregnancy was studied when pregnant animals were exposed to different stressors (heat, restraint, and cold) (Hussain, 1974). This study concluded that following stress there was an increase in CBG. Questions about the validity of this work have risen which is partially the basis for the present work. Cold was reported to be the strongest elicitor of an increase in CBG (40%) with heat and restraint also increasing CBG (Hussain, 1974).

The purpose of this investigation was to determine whether there is an alteration in serum CBG content in response to exposure to cold and whether this alteration occurs in both sexes? Cold is

known to be active in the inducement of the pituitary-thyroid axis (Woods and Carlson, 1956; van Beugen and van der Werff ten Bosch, 1961). So it can be postulated that a cold induced increase in CBG is possible through this increase in serum thyroxine. TSH levels rise within the first ten minutes of exposure to cold followed by increases in serum triiodothyronine (T-3) and T-4 levels within two hours (Hefco et al., 1975). This is probably mediated through the hypothalamus since local cooling of the 'heat loss center' (anterior median emminance) in the hypothalamus causes an increase in TSH secretion rates (Andersson et al., 1963). This pituitary-thyroid relationship that is active in cold has also been shown to be a major influence in CBG regulation (Labrie et al., 1968; Westphal, 1971). The effect of cold exposure of 2 and 5 hours will be studied in male and virgin female rats with CBG determination utilizing two different methods of analysis. Known methods of increasing and decreasing serum CBG levels will also be utilized to confirm the adequacy of the assays to measure physiological changes in CBG concentration.

MATERIALS AND METHODS

Animal Experimental Groups

All experiments utilized adult male and female rats (Upjohn Co.) weighing 200-300 grams. They were housed four to a cage for a minimum of one week prior to experimentation. Lighting was maintained on a twelve hour light:dark schedule (700 - 1900 hrs) with a mean temperature of 24°C. Water and Purina Rat Chow were provided ad libitum.

Six to ten animals were placed in each of 6 separate groups for each sex. These groups consisted of rats exposed to cold for 2 or 5 hours. In addition separate control groups for each cold group, a positive control and a negative control.

The cold exposure regimen consisted of completely wetting an animal and placing it in an individual cage in a dark environmental chamber for either 2 or 5 hours on each of 5 consecutive days. A temperature of $4 \pm 1^{\circ}C$ was maintained throughout each period. Exposure to cold was started at 1200 hrs and was completed at either 1400 or 1700 hrs. These times correspond to basal levels (1400 hrs) and peak levels (1700 hrs) of serum corticosterone in the rat (Dunn et al., 1972). Stressors in general have shown the ability to increase corticosterone at both times.

In order to correlate the CBG binding data positive and negative controls were utilized for each sex. Males treated with estradiol or thyroidectomized served as the positive and negative controls, respectively (Gala and Westphal, 1965; Labrie et al., 1968). In

females treatment with progesterone and testosterone accomplished the same respective goals (Gala and Westphal, 1965). All hormones, which were individually dissolved in peanut oil, were injected subcutaneously on each of ten consecutive days. The animals were killed on the eleventh day. The following hormone dosages were administed in a volume of 0.2 ml; estradiol:10 ug, progesterone:10 mg and testosterone: 5 mg (Sigma) (Gala and Westphal, 1965).

Thyroidectomy was accomplished with the aid of a binocular microscope utilizing a mid-ventral incision approximately 1.5 cm in length from a point just superior of the hyoid bone to the sternum. Glands were removed intact and visually inspected for damage. 30,000 U procaine penicillin G (Pfizer) was administered 12 hours prior to surgery. 50 ug of atropine sulfate (Lilly) was given preceeding surgery to aid respiration. Equithesin (Jensen-Salsbery Laboratories), 2.2 ml/kg, proved to be an effective anesthetic. Rats with less than a 5% weight gain after two months were considered hypothyroid (Evans et al., 1960). The tracheal region was grossly inspected for residual thyroid tissue at termination. Trunk blood was collected immediately at the conclusion of cold exposure on day five. The serum portion was separated by centrifugation at high speed (IEC centrifuge) for 15 minutes and then frozen. Control animals were sacrificed at times corresponding to the stressor regimens (1400 and 1900 hours) following the same procedure. In order to avoid increasing serum corticosterone concentrations through handling, all controls were sacrificed within 30 seconds of handling (Barret and Stockham, 1963; Ramaley, 1972). The same procedure was applied to positive and

negative control animals. In addition to serum samples, adrenal and thymus glands were also removed, cleaned and weighed for further quantification of a stress response (Selye, 1936).

Measurement of Serum Protein Concentration

Total serum protein concentration was measured using the Biuret Method (Gornall et al., 1949). In this procedure 8.0 ml of biuret reagent was added to 0.1 ml serum and 2.0 ml normal saline. This solution was incubated at 37°C for 30 minutes. Spectrophotometric absorbancy of this solution at 550 nm was compared to known protein concentrations. Absorbancy due to serum pigmentation was corrected by mixing 0.1 ml serum, 2.0 ml normal saline and 8.0 ml alkaline tartrate in biuret diluent. This value was subtracted from the original absorbance.

Biuret reagent was prepared by dissolving one biuret reagent tablet (Cambridge Chemical Products) in 70 ml hot distilled water with the subsequent addition of 30 ml 10% sodium hydroxide followed by filtration. The biuret diluent solution was 0.5% potassium iodide (w/v) in 0.25 N sodium hydroxide (carbon dioxide free). To this solution 0.9% (w/v) sodium potassium tartrate (Rochelle salt) was added for the pigmentation correction solution.

Measurement of Serum Corticosterone Concentration

Serum corticosterone was measured utilizing a modification of Murphy's competitive protein-binding radioassay (Murphy, 1967). In this method serum corticosterone competes with titiated corticosterone in a dilute solution of corticosteroid binding globulin.

Corticosterone not bound to CBG can be absorbed and thereby separated by the addition of florisil. The number of displaced molecules absorbed to florisil is proportional to the amount of added non-radioactive corticosterone.

Serum was thawed and 0.15 ml of each sample was extracted with 3 X 2 ml of petroleum ether utilizing 5 seconds of vigorous agitation. Separation of the aqueous and organic phases was achieved by freezing the aqueous phase in a dry ice-acetone bath. The upper organic phase (containing the less polar steroids) was then discarded. Duplicate 50 ul aliquots were then mixed with 2.0 ml absolute ethanol in order to extract corticosterone, vortexed for 2 seconds, and then centrifuged. A 0.2 ml fraction of the ethanol extract was dried at 45°C with dry, filtered air. Following addition of one ml of fresh CBG reagent the mixture was incubated for 30 minutes at 45°C and promptly cooled to 0°C for a minimum of ten minutes. Thirty five mg of purified and activated florisil (60 - 100 mesh, Sigma) was added to each tube, vortexed for exactly 30 seconds and placed in a 0°C water bath for an additional 30 seconds. A 0.5 ml fraction of the supernatant was removed and mixed with 10 ml scintillation fluid (850 ml toluene, 150 ml BBS-3, 6 gm PPO and 150 mg POPOP) and placed in a scintillation vial. Radioactivity was measured in a packard tricarb scintillation vial with CPM from each sample compared with a range of corticosterone standards (0.5 - 10.0 ng) and expressed as ug/100 ml serum.

The CBG reagent was prepared by adding 0.336 ng/ml tritiated corticosterone (specific activity 60 uc/mMol, New England Nuclear)

to an aqueous solution of 0.8% normal male rat serum. This solution was incubated for 30 minutes at 45°C and then cooled at 4°C until used.

Measurement of Corticosterone Binding Globulin

Two different approaches were used in this study to quantify corticosterone binding globulin. In the first, multiple equilibrium dialysis (Gala and Westphal, 1966) measures the serum equilibrium binding as it occurs in physiological conditions. In the second, absorption to dextran coated florisil was used to show the total corticosterone binding capacity in serum (Trapp and West, 1969).

Multiple equilibrium dialysis measurement of CBG

In the multiple equilibrium dialysis method described by Westphal, tritiated corticosterone was used to determine CBG activity (C-value). After determining serum protein concentration, with the biuret method, each sample was diluted to 5 mg/ml with 0.05 M phosphate buffer (pH 7.40). Dialysis tubing (12,000 MW exclusion, 5/8" diameter, Arthur Thomas) was soaked for a minimum of one hour in phosphate buffer and then rinsed twice more with the buffer. A 2.5 ml aliquot of the diluted serum was placed in the tubing, baged, rinsed with phosphate buffer and placed in a beaker. Six to eight bags were dialysized against twice the total bag volume in phosphate buffer containing tritiated corticosterone (2.52 ng/ml outside solution). Five hundred U/ml penicillin (Sigma) and 20 ug/ml streptomycin sulfate (Upjohn) were added to prevent bacterial growth and were found not to interfere with assay results. A pooled sample was

included in each system to check for variations in testing. The beakers were covered with parafilm to prevent evaporation and shaken (100 - 150 times per minute) for forty eight hours to assure equilibration. Bags were then removed, rinsed twice with distilled water and a 0.2 ml aliquot was removed from each dialysis bag and the outside solution. These were placed into individual scintillation vials and ten ml of scintillation fluid added to them. Radioactivity was measured in Packard Tricarb Scintillation Counter. C-value was determined with the following formula:

$$C = \frac{S\ Bound}{S\ Unbound\ (P)}$$

C = C-value in 1/gm

S Bound = DPM in dialysis bags

S Unbound = DPM in outside solution

P = Serum protein concentration in gm/l

Bound and unbound levels of corticosterone can be determined by utilizing the data from this formula and the known endogenous corticosterone concentration in the blood. In this calculation S bound equals 100 - X and S unbound equals X.

$$C = \frac{100 - X}{X\ (P)}$$

Solving the formula for X gives the percentage of unbound corticosterone (Westphal, 1971). This value multiplied with the total corticosterone gives the amount of unbound corticosterone in ug/100 ml. The difference between these was the amount of bound corticosterone in the blood.

Absorption measurement of CBG

Absorption of corticosterone to dextran coated florisil (DCF) was used to determine corticosterone binding capacity (CBC). This gives a more quantitative measurement of total serum binding capacity of corticosterone by CBG. CBG has a greatly increased association constant at 4°C (versus 37°C) (Westphal, 1967) and by having in the system excess corticosterone the total binding capacity can be measured.

In order to prepare DCF, 50 gm of dextran (clinical grade, 60,000 - 90,000 MW, ICN Pharmaceuticals) was dissolved in 300 ml distilled water and 50 gm florisil (purified and activated, 60 - 100 mesh, Sigma) added. The mixture was stirred for 12 hours, decanted and filtered on a sintered glass funnel without washing. Final drying was achieved in a vacuum desicator at room temperature. Phosphate buffer (0.1 M, pH 7.4) was freshly prepared containing 0.1 M potassium chloride 2 ng/ml tritiated corticosterone (specific activity 60 ug/mMol, New England Nuclear) and 200 ng/ml exogenous corticosterone (Sigma). A 0.1 ml serum aliquot was mixed with 0.5 ml phosphate buffer, vortexed for 2 seconds and placed in a refrigerator at 4°C for 16 hours (for equilibration). Samples were then placed in an ice bath and 155 mg DCF was added. These were shaken (100 - 150 times per minute) for one hour at 0°C. DCF was allowed to settle and 0.1 ml aliquots of the supernatant are removed and placed in individual scintillation vials.

Ten ml of scintillation fluid was added and the samples counted on a Packard Tricarb Scintillation Counter. In order to account for

non-specific binding to serum albumin separate serum samples were heated at 60°C for 25 minutes to destroy binding by the serum CBG fraction. Corticosterone bound by CBG in 0.1 ml serum was calculated from the following formula:

$$CBC = Ft \frac{\text{CPM in 0.6 ml supernatant}}{\text{Total CPM in tube}}$$

Ft in ng/ml being the sum of added steroid and endogenous corticosterone present in the serum. The difference between the heated and non-heated samples gives the final CBC in ug/100 ml. Westphal's procedure of pooling serum to eliminate individual hormonal variations and effects was utilized for all the assays (Gala and Westphal, 1965; Westphal, 1971). This procedure allows for truer group means since each sample was exposed to the same variations. For this procedure the serum is thawed and equal volumes of serum from each individual within a group is pooled together and refrozen until needed. All tests are then preformed on this pooled aliquot. The Students-t-test was used for statistical treatment of data with a 95% confidence interval (two tailed) as the lower limit of acceptability.

RESULTS

Quantification of Analysis of CBG

Corticosteroid-binding globulin was measured utilizing two different methods of determination, absorption and multiple equilibrium dialysis. In males the ability of these assays to measure serum binding of corticosterone was confirmed in as much as estrogen treatment significantly increased CBG while thyroidectomy significantly decreased CBG (Table 2).

Progesterone and testosterone treatments of females were utilized to accomplish the same purpose. Progesterone significantly increased CBG over the values in testosterone treated rats and the rats exposed to cold for two hours (Table 4). Testosterone decreased serum CBG content as compared with all other groups except the 1700 hour control (Table 4).

Effect of Cold Exposure on Male Rat CBG

Five hours of exposure to cold significantly increased CBG when measured by the absorption or multiple equilibrium dialysis methods. CBC concentration was 50.15 ± 0.76 ug/100 ml of serum as compared to 44.30 ± 1.72 ug/100 ml in the 1700 hour control (Table 2). The corresponding C-value was 0.516 ± 0.01 1/gm for the rats exposed to cold and 0.435 ± 0.01 1/gm for the control rats (Table 2). Adrenal weights were increased and thymus weights decreased by 5 hours of cold exposure (Table 1). As would be

expected cold exposure elevated serum corticosterone. In this case 7.63 ug/100 ml serum (Table 1).

The male rats exposed to cold for 2 hours on 5 consecutive days also exhibited the adrenocortical responses to stress. Adrenal weights were increased and thymus weights decreased as compared with the 1400 hour control group (Table 1). In addition, the serum corticosterone concentration in the 2 hour cold group was also significantly increased over the 1400 hour control by 26.45 ug/100 ml of serum (Table 1). CBG, however, was increased in only one of the methods of evaluation. CBG as determined by the absorption method was significantly increased by 7.18 ug/100 ml serum over the 1400 hour control (Table 2). There was no difference in C-value in this group as compared to control rats.

Effect of Cold Exposure on the CBG of Female Rats

Exposure of female rats to 2 hours of cold for 5 consecutive days had the effect of significantly lowering the CBG levels. This was seen in both methods of evaluation. The absorption method showed a 23.82 ug/100 ml decrease in serum binding of corticosterone and the dialysis method showed a 0.092 1/gm decrease in C-value (Table 4). There was no change in either the adrenal weights nor thymus weights in these treated animals, however, serum corticosterone was increased by 24.43 ug/100 ml of serum (Table 3).

A cold exposure of 5 hours on 5 consecutive days gave rise to the recognized stress responses. Adrenal weights increased, thymus weights decreased, and serum corticosterone was increased as compared

to the 1700 hour control (Table 3). There was no difference between the 5 hour cold exposure and the 1700 hour control group in terms of serum CBG content as measured by either absorption or multiple equilibrium dialysis (Table 4).

Table 1

Average Corticosterone Concentration, Adrenal and Thymus Weights in Male Rats

Experimental Group	No. of Animals	Adrenal Weight (mg/100 gm B.W.)	Thymus Weight (mg/100 gm B.W.)	Total Corticosterone (ug/100 ml)
Estrogen Treated	10	17.49±1.9	100.91±26.8	19.23±2.6
Thyroidectomized	10	10.20±1.5	55.61±16.4	19.35±2.2
1400 Hr. Control	7	15.82±2.6	319.93±44.0	2.72±3.0
2 Hr. Cold Stress	8	19.77±2.1*	237.30±37.6	29.17±1.4*
1700 Hr. Control	8	14.70±0.7	322.56±44.4	12.40±4.5
5 Hr. Cold Stress	8	19.85±1.9**	230.46±35.8**	20.17±0.9@

All values expressed as mean ± standard deviation

* Significantly different ($P < 0.01$ by the Student-t-test) from 1400 Hr. control male

** Significantly different ($P < 0.001$ by the Student-t-test) from 1700 Hr. control male

@ Significantly different ($P < 0.05$ by the Student-t-test) from 1700 Hr. control male

Table 2

Average Corticosterone Binding Capacity (CBC) and Corticosterone Binding Globulin Activity (C-Value) in Male Rats

Experimental Group	No. of Animals	CBC (ug/100 ml)	C-Value (1/gm)	Bound Corticosterone (ug/100 ml)	Unbound Corticosterone (ug/100 ml)
Estrogen Treated	10	80.9 ± 0.5*	0.625 ± 0.01*a	18.80	0.43
Thyroidectomized	10	37.3 ± 0.8**	0.359 ± 0.02**	18.72	0.63
1400 Hr. Control	7	51.2 ± 0.6	0.476 ± 0.01	2.65	0.07
2 Hr. Cold Stress	8	58.4 ± 1.4@	0.477 ± 0.01	28.41	0.75
1700 Hr. Control	8	44.3 ± 1.7	0.435 ± 0.01	12.02	0.38
5 Hr. Cold Stress	8	50.1 ± 0.7*a	0.516 ± 0.01*@	19.58	0.59

CBC and C-Value expressed as mean $\pm$ standard deviation

* Significantly different ($P < 0.002$ by the Students-t-test) from all male groups

*a Significantly different ($P < 0.05$ by the Students-t-test) from all male groups

** Significantly different ($P < 0.05$ by the Students-t-test) from all male groups

@ Significantly different ($P < 0.05$ by the Students-t-test) from 1400 Hr. control male

*@ Significantly different ($P < 0.05$ by the Students-t-test) from 1700 Hr. control male

Table 3

Average Corticosterone Concentration, Adrenal and Thymus Weights in Virgin Female Rats

Experimental Group	No. of Animals	Adrenal Weight (mg/100 gm B.W.)	Thymus Weight (mg/100 gm B.W.)	Total Corticosterone (ug/100 ml)
Progesterone Treated	10	19.77 ± 2.1	92.57 ± 20.9	37.55 ± 1.4
Testosterone Treated	10	19.14 ± 2.0	47.33 ± 14.2	42.20 ± 2.7
1400 Hr. Control	7	28.66 ± 1.9	171.58 ± 31.6	28.18 ± 2.3
2 Hr. Cold Stress	6	30.12 ± 4.1	183.87 ± 25.1	52.61 ± 3.5*
1700 Hr. Control	6	28.25 ± 2.5	192.24 ± 16.6	43.95 ± 2.1
5 Hr. Cold Stress	8	35.32 ± 3.4**	151.51 ± 21.0**	81.34 ± 5.0**

All values expressed as mean $\pm$ standard deviation

* Significantly different ($P < 0.005$ by the Students-t-test) from 1400 Hr. control female

** Significantly different ($P < 0.002$ by the Students-t-test) from 1700 Hr. control female

Table 4

Average Corticosterone Binding Capacity (CBC) and Corticosterone Binding Globulin Activity (C-Value) in Virgin Female Rats

Experimental Group	No. of Animals	CBC (ug/100 ml)	C-Value (1/gm)	Bound Corticosterone (ug/100 ml)	Unbound Corticosterone (ug/100 ml)
Progesterone Treated	10	123.1 ± 4.3*	0.707 ± 0.01*	36.89	0.66
Testosterone Treated	10	105.7 ± 0.1*a	0.584 ± 0.01**	46.27	0.93
1400 Hr. Control	7	132.1 ± 1.6	0.721 ± 0.01	27.67	0.51
2 Hr. Cold Stress	6	108.2 ± 0.4@	0.627 ± 0.01*@	51.45	1.16
1700 Hr. Control	6	116.7 ± 3.8	0.715 ± 0.02	43.13	0.82
5 Hr. Cold Stress	8	111.9 ± 2.6	0.733 ± 0.02	79.69	1.65

CBC and C-Value expressed as mean $\pm$ standard deviation

* Significantly different ($P < 0.05$ by Students-t-test) from Testosterone and 2 Hr. cold stressed females

*a Significantly different ($P < 0.05$ by Students-t-test) from all female groups except 1700 Hr. control

** Significantly different ($P < 0.05$ by Students-t-test) from all female groups

@ Significantly different ($P < 0.005$ by Students-t-test) from 1400 Hr. control female

*@ Significantly different ($P < 0.05$ by Students-t-test) from 1400 Hr. control female

DISCUSSION

This investigation was undertaken to determine whether the exposure to cold and the associated physiological adaptations would induce an increase in CBG in rats. Among these adaptations is an elevation of serum thyroxine. It is well established that exposure to temperatures of 1-6°C will stimulate the hypothalamo-pituitary-thyroid system in the rat. Serum TSH levels rise within ten minutes followed by a subsequent increase in serum levels of T3 and T4 which remain elevated throughout the duration of cold exposure (Hefco et al., 1975). Among other things, thyroid hormones also increase serum levels of CBG (Labrie et al., 1968; Westphal, 1971), probably through a direct effect upon liver synthesis of this serum protein. This function is independent of sexual differences, although estrogens will potentiate the thyroidal effects on CBG synthesis (D'Angelo, 1968; Gala and Westphal, 1966c).

A second hormonal effect of cold exposure on rats is the increased synthesis and release of corticosterone. Increased corticoids have the opposite effect of thyroxine upon CBG serum concentration; as peripheral corticosteroid levels rise there will be a subsequent decrease in CBG and visa versa (Gala and Westphal, 1966b). This cold induced increase in corticosterone is mediated through the hypophyseal hormone ACTH. While short term "stressors" will result in maximal ACTH release, long term stresses result in a lessor release of ACTH, and consequently, relatively lower corticosterone levels (Stark et al., 1963). The experimental design of this study using five consecutive days of cold

exposure is aimed at optimizing both situations; long term thyroid release and decreasing adrenal responsiveness.

Evaluation of Determining CBG

Numerous methods for the determination of CBG are available. While each method has its advantages, there is no method that provides unequivocal accuracy. For this reason two different methods were used in this study. The first, multiple equilibrium dialysis, allows determination of the amount of corticosteroids bound by all serum proteins under physiological conditions. This procedure provides identical equilibrium conditions to be established with all sera measured in the same dialysis system. As a consequence any hormonal, ionic or pH variations existing between serum samples are minimized. In order to determine total corticoid binding by CBG specifically, an absorption method was utilized. This method provides the total binding capacity of serum CBG with the addition of excess corticosterone at $4^{o}C$. However, this method also allows correction for nonspecific binding due to proteins other than CBG. Through the use of these two methods the results obtained can be compared and a more accurate physiological interpretation be reached. In order to guarantee further reliability in the study, known physiological methods of increasing and decreasing CBG were utilized.

Estrogen treatment increased and thyroidectomy decreased serum CBG in male rats as measured by absorption and multiple equilibrium dialysis. Estrogen potentiates thyroid hormone activity in the synthesis of CBG (D'Angelo, 1968; Gala and Westphal, 1966c).

Thyroidectomy, through the loss of thyroid hormones and decreased metabolic activity results in decreased CBG (Labrie et al., 1968). In as much as both assay methods demonstrated the expected alterations in CBG concentration, it is likely that they would show any significant change in CBG concentration that resulted from exposure to cold.

On the other hand, progesterone treatment in females did not produce the expected increase in CBG as compared to control animals. Testosterone, however, did have the effect of lowering CBG as measured by the assay systems used in this study. The lack of response of progesterone may have been due to some artifactual reason and was probably not due to an inability of the animals to respond to the hormones in this way (Gala and Westphal, 1966c). The results of these measurements of CBG in females treated in these ways, while not as clear as in the male, do demonstrate the ability of the assays used to measure alterations in serum binding of corticoids due to CBG in female rats.

Effects of Cold Exposure on CBG

Exposure of male rats to cold from either 2 hours/day or 5 hours/day on each of five consecutive days increased serum CBG levels. In the five hour cold group this increase could be demonstrated by each method of determination. The two hour cold group only exhibited a significant elevation as measured by the absorption method.

Virgin female rats on the same regimen of cold exposure as the males did not have increased serum concentrations of CBG. Five hours

of cold for 5 consecutive days had no effect on serum CBG levels. In addition, the female rats exposed to cold for 2 hours/day for 5 days exhibited decreased serum CBG as measured by absorption and multiple equilibrium dialysis.

Adrenocortical Responses to Cold

The male rats exposed to 4°C for 2 or 5 hours/day for five consecutive days exhibited many of the classical stress responses including increases in adrenal weights and serum corticosterone (Selye, 1936; Hussain, 1974). These result from ACTH stimulation of corticosteroidogenesis in the adrenal gland. The associated decrease in thymus weights result from the well documented thymolytic activity of serum corticoids (Selye, 1936; Dorfman, 1962).

Exposure of virgin female rats to cold for 5 hours/day for 5 consecutive days resulted in the same stress response as measured in the male rat. Adrenal weights were increased along with serum corticoids while decreased thymus weights were recorded. The female rats exposed to cold for 2 hours/day for 5 consecutive days did not exhibit all of these effects. No significant difference was seen in either the adrenal or thymus glandular weights. Serum corticoids when measured just after the last period of cold exposure did show a significant increase over the appropriate controls.

Comparison of Male and Female Responses

A differential response between male and virgin female rats is clearly indicated by the data presented. While both groups show some

degree of stress response as measured by the parameters in this study there is a difference in the degree to which CBG concentration was altered. The main parameter measured which could account for this would be serum corticosterone. Control female rats had resting corticosterone levels that were significantly increased over the stress levels of corticosterone in the males. Even the stressed male rats did not reach the high levels of corticoids recorded in virgin females. Because of this the males are less likely to exhibit the degree of inhibitory effects that corticosteroids can have on thyroid hormone release. With less inhibition of thyroid hormones by corticosterone in the males there is a greater possibility for thyroid stimulation of CBG production.

It is possible that the observed decrease of CBG in the female group exposed to 2 hours of cold was due to the increased corticoids decreasing CBG synthesis with reduced thyroid hormone release due to a low cold exposure stress. Coupled with this is the corticosterone effect on the thyroid with increases in iodine clearance and thyroxine metabolism in the cold exposed rat (Bondy and Hagewood, 1952; Kassenaar et al., 1959). Increased corticoids will decrease serum levels of CBG (Gala and Westphal, 1966b). While the data presented here gives information about serum concentrations of CBG and corticosterone, information regarding serum thyroid hormone levels and the rate of endogenous synthesis and degradation of CBG would be necessary to confirm this hypothesis.

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