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## Effect of "Stressors" on Corticosteroid-Binding Globulin Activity in the Rat during Pregnancy

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EFFECT OF "STRESSORS" ON  
CORTICOSTEROID-BINDING GLOBULIN ACTIVITY  
IN THE RAT DURING PREGNANCY

by

Muhammad Nasir Hussain

A Thesis  
Submitted to the  
Faculty of The Graduate College  
in partial fulfillment  
of the  
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Muhammad Nasir Hussain

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EFFECT OF "STRESSORS" ON CORTICOSTEROID-BINDING  
GLOBULIN ACTIVITY IN THE RAT DURING PREGNANCY.

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Biology

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## INTRODUCTION

"Stressors" affect the hypothalamo-pituitary axis resulting in adrenocorticotrophin (ACTH) secretion, which stimulates the adrenal cortex to secrete corticosteroids. These steroids, in turn exert negative feedback control on the rate of ACTH release from the pituitary gland.

Corticosteroids in blood are largely bound by corticosteroid binding-globulin (CBG) which serves as a vascular depot and as a transport vehicle for corticosteroids (Bennhold, 1963). There are variations in the concentration of blood corticosteroids and their binding to protein in the rat during pregnancy (Solem, 1966). Most are bound to CBG which increases markedly during pregnancy. The free, unbound corticosteroids, to which all biological activities are attributed, decrease initially and then increase several fold from the 12th day of pregnancy (Solem, 1966).

There have been a number of prominent fetal developmental aberration associated with maternal stress exposure in a wide variety of species. These alterations in normal development include failure of implantation, abnormal fetal development, placental vascular alterations, increased reabsorption of embryos and poor neonatal viability (Pasqualini, 1971). These effects seem to be due to the maternal stress response and not a specific manifestation of the stressor agent. This hypothesis is supported by several important observations including: (1) Treatment of pregnant animals with epinephrine, adrenocorticotrophic hormone (ACTH) or corticosteroids (Fernadex, 1958;



Pennycuik, 1966) in large doses produces similar fetal alterations (Defree et al., 1967; Robinson and Sharaf, 1952). (2) Removal of the material adrenal prior to stress exposure has been shown to prevent fetal changes under certain conditions (Fernadex, 1958, Yang and Yang, 1969; Hensleigh and Johnson, 1971). (3) Animals conditioned to stressor agents show decreased fetal alterations when reexposed to the same agent during pregnancy (Macfarlane et al., 1957; Pennycuick, 1966).

The adrenal therefore may play an important role in mediating maternal stress response. Corticosterone which is the predominant adrenal corticoid in the rats has been shown to increase in concentration three and four fold from resting levels in conjunction with the application of stressor agents (Cook, et al., 1963).

Developing fetuses are relatively resistant to elevated levels of corticosteroids in the blood due to a typical stress response even though the placenta is permeable to corticosteroids (Migeon et al., 1956). However, when relatively high levels of corticosterone are administered to pregnant rats, a number of the aforementioned fetal developmental aberrations can be observed (Pennycuik, 1966; Macfarlane et al., 1957). This leads to hypothesis that the developing fetus and/or the pregnant female in some way protects the fetus from low intensity stress elicited increases in blood corticosterone concentrations.

It has been demonstrated that stress alters other endocrine functions as well. For example FSH and LH are found to be decreased and increased in stressed animals, respectively (Selye, 1936;

Eleftheriov and Church, 1967). Stress also results in an increase in serum prolactin and growth hormone in rats (Grosvenor, 1965; Charles et al., 1960; Roth et al., 1963; Roth et al. 1963).

Possible mechanisms that prevent the deleterious effects of high corticosterone levels in the fetus during stressful events that the pregnant rat experiences include:

1. An increase in maternal or fetal corticosteroid-binding globulin activity which will result in a relative decrease in the amount of biologically active, placentally-transferable corticosteroids.
2. An increase in maternal or fetal catabolism of corticosteroids into biologically inactive molecules.
3. A decrease in placental transport of corticosteroids.
4. Disposal of maternal corticosteroids by the fetus via urine.
5. Return of corticosteroids and their metabolites to the maternal circulation through the placenta.

The intent of this study is to examine whether CBG activity (C-value) becomes elevated when mid-pregnant animals are exposed to either cold, heat or restraint. If both CBG activity and corticosterone concentration are increased it is possible that this is one mechanism by which elevated corticoids are prevented from achieving high concentrations in the fetus when maternal stress occurs.

## LITERATURE REVIEW

There are a variety of prominent fetal developmental aberrations associated with maternal stress exposure. These alterations in normal development include failure of implantation, abnormal fetal development, placental vascular alteration, increased resorption of embryos, and poor neonatal viability (Pasqualini, 1971). Alterations in fetal development appear to be a result of the maternal stress response, rather than a specific manifestation of the stressor agent. These may be directly related to increased production of maternal adrenal corticoids because of the following observations: (1) Treatment of pregnant animals with epinephrine, ACTH or corticosteroids (Fernandez, 1958; Pennycuick, 1966) in large doses produces similar fetal alterations (Robinson and Sharaf, 1952; Defrees et al, 1967). (2) Removal of the maternal adrenals prior to exposure to stressors has been shown to prevent fetal changes under certain conditions (Hensleigh et al., 1971; Fernandez 1958; Yang et al., 1969). (3) Animals conditioned to stressor agents show decreased fetal alterations when exposed to the same agent during pregnancy (Pennycuick 1966; Macfarlane et al., 1957).

The hypothesis that fetal alterations appear to result from the mother's response to a stressor, rather than a direct effect of the stressor on the fetus is supported by experiments examining retardation of fetal growth following maternal heat stress. Hensleigh and Johnson (1971) demonstrated that heat stress causes retardation of fetal rat growth.

Shah (1956) while he was working on transplantation of early rabbit embryos, noticed that embryonic degeneration induced by high body temperature was due to the effect on the mother rather than upon the embryos. He showed that when the temperature of a donor rabbit was raised the embryos were normal when transplanted to rabbits with normal body temperature. However, when the temperature of the recipient was raised, degeneration of embryos obtained from a donor with normal body temperature occurred.

#### Corticoid Responses to Stress

"Stress" results in the arousal of the brain-pituitary adrenal axis (Christian and Davis, 1964). Elevated levels of ACTH from the pituitary gland stimulates the adrenal glands to synthesize and release increased amounts of corticosteroids to the blood. Corticosterone, which is the predominant adrenal corticoid in rats, has been shown to increase in concentration three and four fold from resting levels in conjunction with the application of stressor agents (Cook et al., 1963). Activation of this endocrine axis can be produced by intense light, heat, cold, restraint, noises, handling and other stimuli which represent alterations in the animals environment.

The effects of various forms of stress on the adrenal cortex has been extensively studied; however, few studies have been performed in pregnant animals. On the other hand, the effect of pregnancy on adrenocortical function has been investigated (Anderson and Turner, 1962). Blood and pituitary ACTH content, as well as plasma and adrenal corti-

costerone concentration have been found to be markedly elevated (Voogt et al., 1969) in pregnant rats on the day of delivery (139% higher than the 20th day of pregnancy). Studies seeking to determine the adrenocortical response of pregnant rats to stressors such as repeated handling, have reported a more attenuated and transient reaction to stress in pregnant than in nonpregnant animals (Grotta and Ader, 1970). This attenuated response to stress in the pregnant animals may result from a decrease in the responsiveness of the hypothalamo-hypophyseal-adrenocortical axis, which is known to occur when basal "non stress" levels of adrenal corticosteroids are elevated (Dunn and Critchlow, 1969) as they are in pregnancy.

#### Placental Passage of Corticosteroids

Passage of corticosterone through the placenta from fetus to mother has been demonstrated in the rat by Milkovic et al., (1973). On the 21st day of gestation, carbon-14-labeled corticosterone was injected subcutaneously into the fetuses through the uterine wall after maternal laparotomy. Three fetuses of each pregnant female were injected with a mixture of 100 ug unlabeled corticosterone plus 0.33 uCi labeled corticosterone per fetus. Fifteen minutes later the animals were killed and the radioactivity in the blood, placenta and liver immediately analyzed. The blood, liver and placenta of injected fetuses showed high degree of radioactivity in the form of corticosterone as determined by thin layer chromatography. At the same time the radioactivity per gram of the maternal liver was higher than that

of fetal livers. The blood, liver and placenta of non-injected fetuses showed significantly lower activities than those found in injected fetuses and their mother (Milkovic et al., 1973). When the radioactive label was injected into the maternal blood, considerable corticosterone radioactivity was found in fetal liver thirty minutes later. Therefore, reciprocal transplacental passage of corticosterone occurs. Similar studies have been performed by Migeon et al. (1956). He injected radioactive cortisol into pregnant woman 20-75 minutes prior to abortion and found cortisol specific radioactivity in the free fraction of fetal plasma, ranging from 1.36 to 2.67% of the administered dose per liter of plasma. These concentrations were approximately one-third those of the maternal plasma radioactivity; the fetal concentration of cortisol was proportional to amount present in the maternal circulation (Migeon et al., 1956). Supporting the concept of fetus to mother passage of steroid are the findings of several other workers, who demonstrated in several species that pregnant adrenalectomized animals survive longer than nonpregnant adrenalectomized animals (Collings, 1941).

#### Teratogenic Effects of Corticoids

Although the passage of corticosteroids across the placenta has been demonstrated in human, rats, mice, and sheep (Migeon et al., 1956; Hanngren et al, 1964; Burton and Jeyes, 1968; Milkovic et al., 1973), in most cases the fetuses are relatively resistant to teratogenic effects of corticosteroids administered to the mother. In the

mouse, teratogenic effects of corticosteroids in some strains are caused only by relatively high doses (Burton and Jeyes, 1968). Cleft palate formation has been reported in offspring of selected corticosteroid administration during pregnancy (Levine et al., 1968). The incidences of this malformation was shown to be dependent upon drug dosage and strain of the mice involved. The effects of corticosteroids upon palate formation in mice has been demonstrated by Fraser and Fainstat (1951). One group of females received the teratogenic regimen of four single daily intramuscular injections of (2.50 mg) cortisone acetate given on the 11th to 14th days of gestation. Another group of females received four single, daily intramuscular injections of sterile physiological solution given at the same time cortisone was administered to the first group. All animals were killed on the 18th day of pregnancy. Palatine shelves were found to be relatively heavier in the offsprings from cortisone-treated mothers than in control of offspring (Jacob, 1964). The effect of corticosteroid administration during pregnancy in rats has been demonstrated by Gunberg (1956). He showed that daily administration of cortisone (2.5 mg) to rats in the first trimester of pregnancy resulted abortion in the majority of cases. Davis and Plotz (1954) noticed that cortisone administered to pregnant rats in the second and third trimester did not interrupt the pregnancy, but the fetuses were considerably smaller.

Inter-relationship Between Maternal and Fetal Hypophyseal-Adrenal System

The observation of Ingle and Fisher (1938) and Walaas and Walaas (1944) have shown that adrenalectomy of pregnant rats results in hypertrophy of the fetal adrenal glands. This hypertrophy can be prevented by the administration of deoxycorticosterone. This observation raised certain questions concerning the inter-relationship of the fetal and maternal hypophyseal adrenal-cortical axis and the functional integrity of the fetal pituitary-adrenal system. Specifically, is the fetal adrenal hypertrophy observed following adrenalectomy of the mother engendered by an increased secretion of ACTH from the fetal pituitary gland, or both?

A considerable amount of evidence (Jost, 1953) indicates that the fetal pituitary-adrenal axis is functional by the 18th day of pregnancy in rats and exhibits many of the inter-relationships described in adult animals. The suggestion has been further made (Jones et al., 1953; Jost, 1953) that maternal ACTH probably does not cross the placenta under physiological circumstances. The fetal adrenal gland may be, at least in part, under the control of the maternal hypophysis as has been implied by the study of Josimovich et al. (1954) which demonstrated that there was a decline in fetal adrenal weight immediately after parturition.

#### Effect of Stress on Fetal Behavior

"Stress" during pregnancy appears to alter normal fetal behavior in that fighting is usually increased among offspring (Southwick, 1955; Christian, 1956). Some work has been focused on the relationship between prenatal and adult behavior of offspring (King, 1956;



King, 1957), which shows that stress during gestation period alters neonatal behavior.

Thompson (1957) discovered that mid pregnant rats subjected to social stress (crowding) produce offspring that showed less overall activity than pups from unstressed mothers. The offspring of the stressed mothers also showed longer latencies for leaving the home cage and reaching food after 24 hours of food deprivation. Similar results were obtained by Keeley (1962) using pregnant albino mice subjected to social stress by crowding. He found that 100 day old litters from crowded mothers were less active, slower to respond to unfamiliar stimuli and defecated less in an unfamiliar environment than control groups born to uncrowded mothers. These differences were found whether the mice were raised by a crowded or by an uncrowded foster mother. This also occurred at age 30 days in litters born to crowded mothers but raised by uncrowded foster females. He showed that nursing and other postnatal modifications did not abolish behavior trait acquired in utero. One explanation may be that aberrant endocrine activity in the crowded, pregnant female impairs the development of fetal response system.

There is direct evidence that animals pass through "critical periods" during their development (Scott, 1962). An alteration of the chemical environment in the fetus during this period may result in permanent changes in physiological and behavior pattern (Levine and Mullin, 1966). Inasmuch as "stress" during pregnancy results in behavioral alteration in offspring (Lieberman, 1963) and "stress" also

causes increased corticosteroid secretion (Cook et al, 1963), it is possible that the effect of "stress" on behavior is, in part, mediated through this endocrine axis. Greater than normal amounts of corticoid in the maternal blood during "critical period" of fetal-brain development could permanently change physiological and behavioral patterns.

#### Effect of "Stress" on Follicle Stimulating and Lutienizing Hormones

In addition to the pituitary adrenal response to stress, there are many reports showing a concomitant decrease in ovarian function in response to stress. Selye (1936) noted that stress in nonpregnant animals resulted in regression of gonads. Charter et al. (1969) found that following surgical stress, women showed slight and transient decrease in plasma follicle stimulating hormone.

It has been demonstrated that there is elevation of plasma LH in response to stressor agents. Eletherio and Church (1967) found that mice which were stressed by exposure to aggression and defeat showed increased luteinizing hormone (LH) production. In this study C57B1/6s strain mice were exposed to trained fighter mice of the same strain twice a day for 1, 2, 4, 8, or 16 days. Twenty minutes after the last exposure to fighting, plasma and pituitary glands were collected. Assay of plasma LH showed increases, within four days after exposure to the fighting regimen to values of 2.95 ng/ml as compared to concentration of 0.78 ng/ml in control mice not exposed to fighting. Pituitary LH exhibited minor nonsignificant fluctuations. It is assumed that exposure to defeat produces a stimulation, similar to

other forms of stress that possibly may act through the hypothalamus to release the neurohormone affecting LH release.

#### Effect of "Stress" on Prolactin

Stressed rats were shown to release increased prolactin (Grosvenor, 1965), resulting initiation of lactation (Charles et al, 1960). In this study, virgin females, previously primed with estrogen injection, were stressed by cold, heat, restraint, starvation or formalin injections. The stressed groups all showed variable degree of lactation after treatment. It was concluded that nonspecific stresses can promote the secretion of prolactin from the anterior pituitary in amounts adequate to induce lactation in estrogen primed rats.

#### Effect of "Stress" on Growth Hormone

Roth et al. (1963 ab), demonstrated both the marked lability of growth hormone release and its dynamic relationship to certain altered metabolic and physical conditions of human subjects. Insulin-induced hypoglycemia, exercise, and surgery were all found to stimulate GH release, much in the same manner as they were shown to stimulate ACTH release. Thus it has become evident that GH, like ACTH was in all probability a "stress" hormone. Gross (1966) has demonstrated that plasma GH levels were consistently elevated in monkeys immediately after capturing and removing them from their cages. Brown et al. (1967) has also shown that the stress of ether anesthetization produces a marked rise in plasma GH levels in the squirrel monkey.

### Corticosteroid-Binding Globulin

Corticosteroids in blood are bound to an  $\alpha_2$  globulin fraction of plasma known as corticosteroid-binding globulin (CBG) (Gala and Westphal, 1965), which serves as a vascular depot and as a transport vehicle for corticosteroids (Bennhold, 1963). CBG increases in the blood of developing mammal from a low concentration in the young to adult levels which are similar in man and woman, but twice as high in the female rat than the male. The affinity of the CBG for corticosteroids is maximal at approximately pH 8, decreasing at either higher or lower pH's, and decreases with increasing temperature. Study of the relative binding strength for different steroids has clearly indicated a species specificity of the CBG molecules. This was confirmed by the isolation of CBG from serum of man, rat and rabbit by a variety of chromatographic procedures. The pure glycoprotein CBG's which are homogenous by physicochemical criteria, proved to be distinct molecules on the basis of their amino acid, and carbohydrate composition and other properties (Westphal, 1970). They possess one principal steroid binding site per molecule. Thermodynamic data indicate a very tight fit of corticosteroid with the interacting CBG. Removal of about 90% of corticosterone from CBG of rat by gel filtration at 23°C results in polymerization of the glycoprotein molecule to dimeric, tetrameric and octameric forms. Recombination of the polymeric mixture with corticosterone reforms the monomer. This reversible polymerization of binding steroid hormone may have a possible biological significance which at this time is unknown.

The presence in the serum of a macromolecular component that binds corticosteroid hormones with high affinity is common to all vertebrate species examined to date. On the basis of their extensive studies, Seal and Doe (1966) concluded that the fundamental mechanism of serum protein binding of corticosteroids appeared early in the history of the vertebrates mediated by a specific protein of unique conformational structure.

Corticosteroids bound by serum CBG are not available for metabolic alteration in the liver, the major site involved in steroid inactivation and removal from the vascular system. Recent studies have shown that increased concentration of CBG occurs during pregnancy in rats (Westphal, 1971), which results in an increase in total maternal plasma corticosteroids and a decrease in the rate of degradation of the steroids. Such an increased retention of corticosteroids in maternal circulation may provide a protective effect upon the fetuses against possible corticosteroid-mediated teratogenic defect.

Westphal (1971) showed that gonadectomy, either before or after puberty, resulted in an increased CBG change in the female; the increased values in the castrated male, however, did not reach the female level. Rats castrated prepuberally had corticosteroid-binding activity rather close to those observed in sera from intact females.

#### The Role Of Pituitary In Corticosteroid-Binding Globulin Activity

The pituitary gland plays an important role in CBG activity (corticosteroid binding capacity of protein). Other factors such as

adrenalectomy, estrogen treatment in male rats, and progesterone administration to female rats, which elevate CBG binding activity, become ineffective in hypophysectomized rats irrespective of sex (Gala and Westphal, 1966). This could be due to loss of a specific pituitary factor which may or may not be one of the known pituitary hormones exerting a previously unrecognized action. On the other hand, it may be due to the loss of a combination of pituitary hormones.

The concentration of corticoids is inversely related to the corticosteroid-binding globulin activity; that is, increased peripheral corticosteroids concentration results in a decrease in CBG activity and vice versa. Administration of corticosterone decreases CBG activity (Gala and Westphal, 1966). An increase in the ACTH level by either unilateral adrenalectomy or by injection of ACTH into hypophysectomized animals does not affect the CBG activity (Gala and Westphal, 1966). This work shows that ACTH is not the pituitary factor responsible for the increase (Gala and Westphal, 1966).

Thyroid-stimulating hormone (TSH) has been shown to exert a controlling influence on CBG activity although a certain level of CBG appears to be independent of TSH-thyroid control (Westphal, 1971). Corticosteroid administration will decrease CBG activity in intact rats, prevent its rise in adrenalectomized rats, and reduce the high CBG level established following adrenalectomy. The mechanism of action is thought to be through inhibition of TSH production at the pituitary level, or possibly by direct action at the site of CBG biosynthesis which is assumed to be the liver (Westphal, 1971).

The Role of CBG In The Regulation of ACTH Secretion and Corticoid  
Distribution

In the rats treated chronically with corticotropin (ACTH), some stressors produce no rise in the plasma corticosterone level 24 hours after the last injection (Acs and Stark, 1967; Stark et al., 1968). Since the plasma CBG level is low in ACTH-treated animals (Acs et al., 1967), it seemed possible that the feedback action of the extremely high levels of plasma free corticosterone, induced by the last ACTH injection, continues to operate at least 24 hours. This possibility has been tested by an experiment (Acs and Stark., 1973a), in which male rats were given an intramuscular injection of either 3 I.U. of corticotrophin (ACTH) or 0.05 ml 0.9% saline daily for 14 days. Thirty minutes after both the 13th and the 14th injection, the treated and the control rats received an intravenous injection of either CBG or physiological saline. Animals were subjected to stress by the injection of 1% formalin, 24 hours after the CBG injection. Plasma corticosterone concentration was determined 1 hour after formalin treatments. In ACTH-treated animals formalin raised the plasma corticosterone level only if the animals had been given CBG on the last two days of ACTH treatment. In control animals resting plasma corticosterone was not affected by CBG injections.

A possible interpretation of these results is that administration of CBG to ACTH-treated animals decreased the feedback activity of the extremely high plasma corticosterone induced by ACTH injection, and as a consequence the stress reaction (corticosterone elevation) to

formalin was not inhibited 24 hours later. These data lend support to the hypothesis of Keller et al. (1969) that CBG plays an important role in ensuring sensitive regulation of the hypothalamo-hypophyseal adrenal system.

The role of CBG in the distribution of corticosterone in the rat has been demonstrated by Acs et al. (1967); Acs et al. (1969; Acs et al., 1973b), in this study, one group of rats was given CBG and another group of rats received physiological saline following chronic treatment with ACTH. Twenty four hours after the last injection, a suitable number of the CBG treated and saline treated control rats received tritiated corticosterone with albumin. Blood samples were collected from the carotid artery at different time intervals including 5, 10, 15, 20, 30, 40, 60 and 80 minutes after corticosterone administration. Radioactivity in the serum was measured and related to the time elapsed following administration. The distribution volume of corticosterone was found to be significantly lower in CBG treated than in control animals. From this result it could be concluded that CBG affects the distribution of corticosterone probably by inhibiting its movement from the vascular system to the extracellular space.

On the basis of the known functional properties of CBG, it is possible that alteration of CBG activity in stressed pregnant rats may be one of the mechanisms that attenuate the potential effects of the maternal response to stress upon fetal development. Therefore, this study will attempt to show whether an alteration of CBG activity occurs in response to several stressors, (including heat, cold and restraint) applied during pregnancy.



## MATERIALS AND METHODS

Virgin, 200-250 g, female rats were obtained from the Upjohn Company and placed in breeding cages (3 females with 1 male). Females with microscopical evidence of vaginal sperm were designated as in day 1 of pregnancy and placed in individual cages. All animals were maintained ad libitum on Purina Laboratory Chow and tap water.

The pregnant rats were divided among four groups of fifteen rats each; one group of which contained untreated controls. In addition, there was one group of virgin control females. The animals in the remaining three groups were subjected to either cold, heat or restraint stress for four hours on the 13th through 17th days of pregnancy. Cold-stressed animals were soaked in water and placed in individual cages in a refrigerator maintained at 5-8°C for four hours per day prior to termination. Heat-stressed animals were placed in an incubator (three per cage) at 41°C. The rectal temperature of heat-stressed rats was elevated on the average from a control value of 37.8 to 39.8°C at the fourth hour of stress each day, while the body temperature of cold-stressed animals remained unchanged. Restraint-stressed animals were anesthetized with ether and placed on a 3 by 10 inch wooden board and a trough-shaped piece of quarter inch mesh screen was placed over each animal and tightened by means of hooks located on the opposite side of the wooden board so that no locomotory activity was possible.

### Positive Controls

In order to determine whether the multiple equilibrium dialysis method has the sensitivity to measure changes in CBG activity, (C-value), the following types of physiologically-altered rats were prepared. In order to show the effect of hypo and hyperthyroidism on metabolic rats and CBG activity, eight male hypothyroid rats were prepared by providing 0.02% solution of thiouracil (Nutritional Biochemical Corporation) as drinking water for two weeks. Eight male hyperthyroid rats were prepared by providing a 1.5% mixture of iodinated casein (Nutritional Biochemical Corporation) with ground rat chow for one week. Six euthyroid male control rats were also used. In order to show an elevated CBG activity, five female rats were bilaterally adrenalectomized and ovariectomized through dorso-lumber incision following anesthesia by administration of 0.3 ml Equithesin (Jensen Salsbery Laboratories) per 100 gram body weight and maintained on saline water for two weeks. In addition five male rats (400 g) were given 10 ug estradiol dissolved in sesame oil by subcutaneous injection each day for twelve days.

### Animal Termination Procedures

Immediately after the last stress treatment or at the designated time in the positive controls each rat was killed by withdrawal of blood by heart puncture following light ether anesthetization. The blood was allowed to clot and the serum resulting from centrifugation at 2500 rpm for 10 minutes was frozen and stored until used for

measurement of serum protein concentration, corticosteroid-binding globulin activity and serum corticosterone concentration. The thymus, adrenal glands and fetuses were removed from pregnant animals and weighed. In addition, the stomach and duodenum of each animal was inspected for ulceration.

#### Determination of Serum Protein Concentration

In this investigation serum protein concentration was determined by the biuret method (Reinhold, 1953). Absorbency due to pigmented serum was corrected by one of the following methods: (1) In the alkaline tartrate method, 0.1 of serum was placed in a test tube and 2.0 ml of saline was added. Alkaline tartrate was added to pigmented serum and saline mixture to make a total volume of 10.1 ml. The blank was prepared from 2.1 of saline and 8.0 ml of alkaline tartarte. Absorbances were read at 550 nm and subtracted from the biuret color readings. These corrected readings were used to calculate protein concentration. (2) In the cyanide method, pigment and turbidity errors were corrected by adding 0.25 ml of saturated KCN to each cuvette after obtaining biuret color absorbencys. KCN removes the Cu from the red-violet, copper-protein complex by forming non-ionized Cu (NH)<sub>2</sub>, thus clearing the tube of any biuret color. Absorbancy of the cyanide-treated solution representing absorbancy of pigments and turbidity, was subtracted from biuret color readings which provided the correct concentration of serum protein.

### Determination of Corticosteroid-Binding Globulin Activity

Corticosteroid-binding globulin-activity C-values (CBG activity) was measured by multiple equilibrium dialysis using tritiated corticosterone (Gala and Westphal, 1966). Following determination of serum protein concentration of each sample, the serum was diluted with 0.05M phosphate buffer (pH 7.4) to give 5 mg protein per ml; 2.5 ml each of the diluted sera was placed into a dialysis bag made from 5/8 inch diameter dialysis tubing (12,000 MW exclusion). The contents of up to as many as six bags were then simultaneously dialysed against twice their total volume of buffer containing the radioactive corticosteroid. Tritiated corticosterone (New England Nuclear, 40 C/mM) was added to the dialysis system at the concentration of 3.9 ng/ml of outside solution. Streptomycin and penicillin were added to the system at levels of 20 ug and 500 units/ml, respectively, which have been found not to interfere with the CBG binding activity. Multiple equilibrium dialysis was performed in duplicate at 37°C for 48 hours in a water bath with shaking at 100 - 120 rpm. The dialysis bags were then removed from the buffer solution and washed with 0.05 M phosphate buffer. An 0.5 ml aliquot of serum was removed from each of the dialysis bag and from the outside labeled buffer solution and placed into scintillation vials. Ten ml of scintillation fluid (750 ml toluene, 250 ml BBS-3, 6g PPO and 150 mg POPOP) was added to each vial. The radioactivity was measured in Packard Tricarb scintillation counter. The resulting counts per minute (cpm) were corrected for background radiation. Corticosteroid-binding globulin activity (C value) of each serum sample

was calculated by using the following formula:

$$C = \frac{S \text{ bound}}{S \text{ unbound (P)}}$$

- S bound = cpm from dialysis bag solution.  
 S unbound = cpm from outside solution.  
 P = Total serum protein concentration in grams/liter  
 C = CBG activity in liters/gram

#### Determination of Serum Corticosterone Concentration

In this investigation corticosterone concentration was determined by a competitive protein-binding radioassay (Murphy, 1967). This method depends upon displacement of similar radioactive steroid molecules from a binding protein. The number of displaced molecules is proportional to the concentration of added nonradioactive steroid molecules. Displaced radioactivity by means of standards can be related to plasma concentration by use of a standard curve.

#### Preparation of Standards

Duplicate 12 X 75 mm glass tubes containing 0.0 (0.25 ml ethanol), 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 10.0, 15.0, and 20 ng of corticosterone (Sigma) were prepared in 100% ethanol and evaporated to dryness in a 45°C water bath with dry filtered air.

#### Preparation of Serum

Many steroids less polar than corticosterone were removed by extracting duplicate 0.1 ml of serum contained in a 12 X 75 mm test tube

with 2 X 1 ml of petroleum ether (B.P. 35.5 - 49.7°C). The upper organic phase was separated from serum by freezing the test tube contents in dry ice and decanting the liquid organic portion of the tube contents. Duplicate fifty ul aliquot of the extracted serum were placed in 12 X 75 mm test tubes. Corticosterone was extracted by adding 2.0 ml of 100% ethanol. Following centrifugation, 0.2 ml of each ethanol extract was placed in 12 X 75 mm test tubes. The ethanol extract was evaporated at 45°C with dry filtered air.

#### Radioassay

One ml of corticosteroid-binding globulin solution (0.5% solution of 10 day estradiol-treated male rat serum in Cutter's sterile, pyrogen-free water and 0.027 uCi of tritiated corticosterone/ml) was added to each of the 12 X 75 mm test tubes containing dried ethanol extracts of serum. The mixture was vortexed for two seconds. The tubes were incubated in a water bath at 45°C for ten minutes and then vortexed for three seconds. The tubes were then incubated in an ice water bath at 5°C for ten minutes or until completion of the assay. Eighty mg of purified and activated 60-100 mesh Florisil (Sigma) was added, and vortexed for exactly thirty seconds. The tubes were placed at an angle in the test tube rack for thirty seconds to allow the Florisil to settle. Then 0.5 ml of supernatant was withdrawn and placed in a scintillation vial containing 10 ml scintillation fluid.

Counts per minute obtained from the corticosterone standards were graphed against ng of corticosterone and unknown values were obtained

from this standard curve. Serum corticosterone concentration was expressed in ug/100 ml serum. A blank value obtained from the serum of adrenalectomized male rats was subtracted from the value obtained from experimental animals.

Determination of Metabolic Rate in Euthyroid, Hypothyroid And Hyper-Thyroid Rat

In order to determine the effect of the drugs on metabolic activity, the animals were placed in a chamber containing soda lime which was then sealed. The consumption of 5 ml of oxygen was measured using a manometer to indicate equalized pressure. Five replications of the procedure were performed on each rat. Surface area (square centimeters) was calculated from body weight by using this formula:

$$K = A(\text{Sq. CM})/W^2/3(\text{gm}), \text{ assuming } K = 10$$

Observed gas volume was corrected to the volume that would have been observed under conditions of standard temperature and pressure. These values were then related to heat production by multiplying the volume of oxygen consumed under standard conditions by 4.8 which is the gram calories of heat produced when a ml of oxygen is consumed. Finally total metabolism was divided by surface area giving metabolic rate in gram calories/hour/cm<sup>2</sup> surface area.

## RESULTS

This investigation was designed to determine whether an elevation of corticosteroid binding-globulin activity occurs in response to cold, heat, and restraint stress applied during late pregnancy in the rat.

### Effect of Pregnancy on CBG Activity, Serum Corticosterone Concentration

An elevation of corticosteroid-binding globulin activity and a decrease in serum corticosterone concentration were observed in pregnant animals as compared to virgin control animals. Pregnant rats exhibited significantly different corticosteroid-binding globulin activity ( $1.06 \pm 0.08$ ) than control animals ( $0.96 \pm 0.03$ ) (Table 1). The average serum corticosterone concentration of  $20.02 \pm 0.88$  ug/100 ml in pregnant rats was significantly different than the  $26.93 \pm 3.18$  ug/100 ml value observed in virgin control rats (Table 2). An increase in thymus weight and a decrease in adrenal weight were noticed, which correlates with a decrease in corticosterone concentration levels (Table 1, 2). Virgin control animals averaged  $24.55 \pm 3.18$  ug/100 ml and  $2.38 \pm 1.00$  ug/100 ml of bound and unbound corticosterone, respectively (Table 1) whereas the pregnant rats had an average of  $18.38 \pm 3.30$  ug/100 ml bound and  $1.64 \pm 0.19$  ug/100 ml unbound corticosterone.

### Effect of Cold, Heat, and Restraint Stress on CBG Activity and Corticosterone Concentration

Corticosteroid-binding globulin activity and corticosterone concentration, and bound and unbound corticosterone were significantly



elevated in pregnant stressed rats as compared to virgin and pregnant control animals (Table 1). Maximum elevation of CBG activity ( $1.40 \pm 0.08$  liters/gram) occurred in cold stressed rats and minimum CBG activity ( $1.18 \pm 0.01$ ) occurred in restraint stressed rats (Table 1). The highest corticosterone levels were noted in heat stressed animals ( $103.75 \pm 15.72$  ug/100 ml) and lowest levels ( $41.33 \pm 4.93$  ug/100 ml) in cold stressed rats (Table 1).

As indicators of chronic stress, adrenal weight was increased and thymus weight significantly decreased in all stressed rats as compared to virgin and pregnant control animals (Table 2). These stresses produced no other obvious deleterious effects on fetuses other than a decrease in average fetal weight in stressed groups (Table 2).

#### Endocrine Effects on CBG Activity

In order to determine whether the multiple equilibrium dialysis method had the sensitivity to measure changes in CBG activity, three experiments were performed in which CBG elevation was expected. These included measurement of C values in hyperthyroid rats, adreno-ovariectomized virgin rats and estradiol treated male rats. In addition, they were measured in hypothyroid rats which are expected to have decreased CBG activity. Iodinated-casein treated (hyperthyroid) male rats showed significant increase in CBG activity as compared to control rats. Average values for corticosteroid-binding globulin activity were found to be  $0.77 \pm 0.01$ ,  $0.52 \pm 0.01$  and  $0.62 \pm 0.01$  in hyperthyroid, hypothyroid and control animals, respectively (Table 4).

Metabolic rates as expected were found to be increased in hyperthyroid rats and decreased in hypothyroid rats. Average metabolic rates were  $6.24 \pm 0.26$ ,  $4.59 \pm 0.12$  and  $5.11 \pm 0.10$  cal/hr/cm<sup>2</sup> in hyperthyroid, hypothyroid and control animals respectively (Table 5).

Corticosteroid-binding globulin activity in adreno-ovariectomized and control animals were found to be  $1.40 \pm 0.56$  and  $0.96 \pm 0.03$ , respectively, which was a significant elevation of CBG activity in adreno-ovariectomized rats as compared to control animals (Table 6).

Corticosteroid-binding globulin activity was found to be elevated in estradiol-treated male rats, as compared to control male animals. Average C values were  $0.71 \pm 0.12$ , and  $0.34 \pm 0.01$  in estradiol-treated and control male rats, respectively, (Table 3).

Table 1

Corticosteroid-binding globulin activity (C value) and serum corticosterone concentration in stressed (cold, heat or restraint stress) and control rats

Experimental Group	No. of Animals	C Values At 37°C	Serum Corticosterone Concentration		
			Total	Bound (ug/100 ml)	Unbound (ug/100 ml)
Virgin Control	15	0.96 <sup>±</sup> 0.03	26.93 <sup>±</sup> 3.18	24.55 <sup>±</sup> 3.19	2.38 <sup>±</sup> 1.00
Pregnant Control	15	1.06 <sup>±@</sup> 0.08	20.02 <sup>±@*</sup> 0.88	18.38 <sup>±@</sup> 3.30	1.64 <sup>±@</sup> 0.19
Cold Stressed	15	1.40 <sup>±@</sup> 0.08*	41.33 <sup>±@*</sup> 4.93	37.70 <sup>±@*</sup> 4.40	3.63 <sup>±@*</sup> 0.86
Heat Stressed	15	1.26 <sup>±@</sup> 0.12*	103.73 <sup>±@*</sup> 15.12	95.76 <sup>±@*</sup> 15.12	7.24 <sup>±@*</sup> 1.89
Restraint Stressed	15	1.18 <sup>±@</sup> 0.01*	67.73 <sup>±@*</sup> 11.68	61.83 <sup>±@</sup> 10.68*	5.90 <sup>±@*</sup> 2.05*

All values are expressed as mean <sup>±</sup> standard deviation.

@ Significantly different (P < 0.05 by the Student-t-test) from virgin control.

\* Significantly different (P < 0.05 by the Student-t-test) from pregnant control.

Table 2

Average Adrenal, Thymus and Fetal Weights of Cold, Heat or Restraint Stressed Pregnant Rats

Experimental Group	No. of Animals	Body Weight (g)	Average Fetal Weight (g)	Adrenal Weight (mg)	Thymus Weight (mg)
Virgin Control	15	267.53 <sup>±</sup> 10.98	-	72.00 <sup>±</sup> 3.58	432.00 <sup>±</sup> 11.04
Pregnant Control	15	290.00 <sup>±@</sup> 33.29	1.20 <sup>±</sup> 0.61	68.00 <sup>±@*</sup> 4.00	470.00 <sup>±@*</sup> 35.57
Cold Stressed	15	312.73 <sup>±@</sup> 21.32	0.77 <sup>±*</sup> 0.17	83.00 <sup>±@*</sup> 3.88	308.00 <sup>±@*</sup> 31.33
Heat Stressed	15	301.12 <sup>±@</sup> 17.35	0.78 <sup>±*</sup> 0.35	85.00 <sup>±@*</sup> 3.44	370.00 <sup>±@*</sup> 31.58
Restraint Stressed	15	281.86 <sup>±@</sup> 17.16	0.60 <sup>±*</sup> 0.25	86.00 <sup>±@*</sup> 4.48	359.00 <sup>±@*</sup> 31.20

All values are expressed as mean <sup>±</sup> standard deviation

@ Significantly different (P < 0.05 by the Student-t-test) from virgin control

\* Significantly different (P < 0.05 by the Student-t-test) from pregnant control

Table 3

Corticosteroid-binding Globulin Activity (C Value) and Serum Corticosterone Concentration in Control and Estradiol-Treated Male Rats

Experimental Group	No. of Animals	C Values at 37°C	Serium Corticosterone Concentration		
			Total (ug/100 ml)	Bound (ug/100 ml)	Unbound (ug/100 ml)
Control male	5	0.34 <sup>+</sup> -0.01	28.80 <sup>+</sup> -1.85	26.29 <sup>+</sup> -1.59	2.51 <sup>+</sup> -0.25
Estradiol treated male**	5	0.71 <sup>+</sup> -0.12 *	30.00 <sup>+</sup> -2.02	27.64 <sup>+</sup> -1.39	2.36 <sup>+</sup> -0.39

All values are expressed as means <sup>+</sup> - standard deviation

\* Significantly different ( $P < 0.05$  by the Student-t-test from control male.

\*\* Estradiol-treated male (injection of 10 ug estradiol/day were given for 12 days before sacrifice).

Table 4

Corticosteroid-binding globulin activity (C values) and serum corticosterone levels in control, iodinated-casein treated (hyperthyroid) and thiouracil-treated (hypothyroid) male rats

Experimental Group	No. of Animals	C values at 37°C	Serum Corticosterone Concentration		
			Total (ug/100 ml)	Bound (ug/100 ml)	Unbound (ug/100 ml)
Control male	6	0.62 <sup>±</sup> 0.01	25.50 <sup>±</sup> 1.03	23.66 <sup>±</sup> 1.63	2.50 <sup>±</sup> 0.74
Hyperthyroid	8	0.77 <sup>±</sup> 0.01*	25.25 <sup>±</sup> 0.99	23.10 <sup>±</sup> 3.77	2.15 <sup>±</sup> 0.70
Hypothyroid	8	0.52 <sup>±</sup> 0.01*	26.16 <sup>±</sup> 0.83	23.12 <sup>±</sup> 3.27	2.37 <sup>±</sup> 1.04

All values are expressed as means <sup>±</sup> standard deviation

\* Significantly different (P<0.05 by the Student-t-test) from control male.

Table 5

Average adrenal, thymus, thyroid weight and the metabolic rate of iodinated casein-treated (hyperthyroid), thiouracil-treated (hypothyroid) and control male rats.

Experimental Group	No. of Animals	Body Weight (g)	Adrenal Weight (mg)	Thymus Weight (mg)	Thyroid Weight (mg)	Metabolic Rate cal/hr/cm <sup>2</sup>
Control male	6	240.16 <sup>±</sup> 12.92	44.66 <sup>±</sup> 3.32	399.66 <sup>±</sup> 6.97	21.50 <sup>±</sup> 0.83	5.11 <sup>±</sup> 0.10
Hyperthyroid male	8	218.25 <sup>+*</sup> 13.86	43.00 <sup>+</sup> 4.59	330.87 <sup>±</sup> 9.42	20.87 <sup>+</sup> 0.99	6.24 <sup>+*</sup> 0.26
Hypothyroid male	8	239.75 <sup>+</sup> 9.91	47.25 <sup>+*</sup> 4.43	339.66 <sup>+</sup> 7.80	24.25 <sup>+</sup> 1.03	4.59 <sup>+*</sup> 0.12

All values are expressed as means<sup>+</sup> standard deviation

\* Significantly different (P < 0.05 by the Student-t-test from control males.)

Table 6

Corticosteroid-binding globulin activity (C value) and thymus weights of adrenalectomized-variectomized virgin female rats

Experimental Group	No. of Animals	Body Weight (g)	Thymus Weight (mg)	C Values at 37°C	Corticosterone (ug/100 ml serum)
Control female	15	267.53 <sup>+</sup> -10.98	432.00 <sup>+</sup> -11.05	0.96±0.03	26.93 <sup>+</sup> -3.19
Adrenalectomized and ovariectomized females	5	215.00 <sup>+</sup> -11.18*	412.20 <sup>+</sup> -5.63*	1.40 <sup>+</sup> -0.56*	00.00 <sup>+</sup> -00.00

All values are expressed as means <sup>+</sup> - standard deviation

\* Significantly different (P<0.05 by the Student-t-test) from control female.



## DISCUSSION

This investigation was designed to correlate the differences in plasma corticosterone levels with corticosteroid-binding globulin activity in response to stress during pregnancy in rats. The results obtained in this investigation indicate that corticosteroid-binding globulin activity and corticosteroid levels elevate in stressed pregnant rats as compared to virgin and pregnant control animals.

It has been well established that when an animal is exposed to physical stress of severe intensity it responds to this treatment with a number of pathological changes. Classically, there are three physical measurements which can be used to indicate the extent of an animal's response to a physical stress. They are adrenal weights, thymus weight, and plasma corticosterone concentration. During intense physical stress, adrenal weights increase due to increased corticosterone biosynthesis. Thymus weights decrease due to increased plasma corticosterone levels which decrease thymus cellular activity and cause thymocytolysis (Dorfman, 1962). It is easily seen by reference to Table 1 and 2 that in all cases, animals subjected to stress responded appropriately in terms of these three indicators. Adrenal weights were significantly higher in all stress groups and thymus weights significantly decreased. Blood plasma corticosterone concentrations were elevated significantly in all three stress groups in late pregnancy.

Effect of Pregnancy on CBG Activity and Peripheral Corticosterone Concentration

The results show that there is a decrease in corticosterone concentration and an increase in CBG activity in pregnant rats which are in agreement with Westphal's (1971) findings. This probably is due to a suppressive effect of progesterone on corticosterone concentration, which elevates during pregnancy (Westphal, 1971).

It has been demonstrated that administration of progesterone to female rats increases CBG activity (Westphal, 1971; Vogt, 1955). A possible explanation for the decrease in corticosterone concentration is that an increased progesterone levels in pregnancy may displace corticosterone from the binding sites on CBG. The initial increase of unbound corticosterone would lower the adrenal corticoid secretion by feedback regulation and lead to increased CBG levels. Since a large number of the corticosteroid-binding sites are occupied by the progesterone, the equilibrium between bound and unbound corticosterone is such that the level of peripheral corticosterone is lower than prior to pregnancy and the coincidental decrease of peripheral corticosterone levels in the female rat resembled the response after adrenalectomy when the corticosteroid-binding globulin activity rises following depletion of the circulating corticosteroids (Westphal, 1971). The sequelae of progesterone administration may represent a second example of a general mechanism by which a lowering of the corticosterone level is accompanied by increased CBG activity. This mode of influence on binding appears to be fundamentally different from that observed after

estradiol injection into male rat or after testosterone administration to the female rat. In these, the elevation and decrease of CBG activity caused by the estrogenic and androgenic steroid hormones, respectively, are accompanied by parallel changes in the corticosterone level (Westphal, 1971).

#### Effect of Pregnancy on Thymus and Adrenal Gland Weight

The results show that there is an increase in thymus weight during pregnancy. Thymus weight is correlated inversely with the amount of free biologically active corticosterone. It has been reported that certain steroid hormones, such as estradiol, testosterone and progesterone potentiate the effect of corticosteroids on thymus involution (Dofman, 1962). It appears that during pregnancy a decrease in corticosterone concentration counter-balances the potentiating effect of progesterone on thymus involution, resulting an increase in thymus weight. The result shows that there is a decrease in adrenal gland weight during pregnancy in rats. During pregnancy, the equilibrium between bound and unbound corticosterone results in increased negative feedback on ACTH secretion which leads to a decrease in adrenal gland weight. In short during pregnancy corticosteroid-binding globulin activity increases and serum corticosterone concentration decreases. As a consequence, indicators of corticosterone levels such as thymus weight and adrenal weight show an increase and decrease, respectively.

#### Effect of Stress on CBG Activity

When pregnant rats were stressed, CBG activity was found to be

elevated. This finding can possibly be explained on the basis of endogenous factors. In cold-stressed pregnant rats TSH acting upon the thyroid gland, is one of the endogenous factors which elevates CBG activity (Westphal, 1971). Either TSH or thyroxin has controlling influence on CBG biosynthesis. In addition to TSH or thyroxin, there are other pituitary hormones, such as growth hormone, follicle stimulating hormone, and prolactin, which are found to be elevated in response to stressor agents (Charles et al. 1960; Roth et al., 1963 and 1963b; Grosvenor, 1965; Elekerio and Church, 1967).

These hormones may have some influence on CBG biosynthesis. However, with the exception of thyroxin, none have yet been tested. While TSH-induced elevation of CBG activity in the cold stressed animals is a probable explanation of this observation, no mechanism can be firmly proposed for the elevation seen following heat and restraint stress.

#### Relationship Between Serum Corticosterone Levels and CBG Activity

It is well established that in rats that ACTH or corticosterone injection will result in increased plasma corticosterone levels; this in turn is related to CBG activity. CBG activity and peripheral corticosterone levels have inverse relationship as noted by Gala and Westphal (1966) and Westphal (1971). In this investigation we find an elevation of CBG activity as well as peripheral corticosterone concentration. This finding may possibly be explained in the case of pregnant control rats by the presence of increased concentrations of progesterone which nullifies the effect of corticosterone on CBG activity.

In this investigation there is four fold more corticosterone concentration in heat stressed-pregnant rats than pregnant controls. The difference between the corticosterone concentration found in stressed-pregnant rats and controls is about 70 ug/100 ml but there are minor differences between the value of unbound biologically active corticosterone in stressed pregnant as compared to control rats, a difference of about 5 ug/100 ml. The result shows that most of the increased corticosterone concentration in response to stress is bound by CBG molecules in pregnant stressed rats. From these observations it is fair to state that CBG plays a major role in the regulation of corticosteroid homeostasis in the fetal and maternal circulation.

#### Comparative Effect of Different Stressors on Pregnant Rats

The results obtained in this study show that there is a increase in corticosterone concentration in stressed pregnant rats. Among stressed groups, serum corticosterone concentration varies from one group to another group. The highest corticosterone concentration was found in heat-stressed animals (Table 1). Similarly, corticosteroid-binding globulin activity in one stressed group differs from another group. Maximum corticosteroid-binding globulin activity was found in cold-stressed animals and minimum elevation in restraint-stressed animals. It is possible that the maximum increase in corticosterone concentration can be explained on the basis that heat stress greatly increases ACTH release. As a consequence, heat results in a greatly ele-

vated corticosterone concentration and a lesser increase in CBG activity because of the inverse relationship between corticosterone concentration and CBG activity. On the other hand, the CBG response in cold-stressed rats may be due to activation of control mechanisms that are not affected by heat; that is TSH release and consequent stimulation of the thyroid. It seems likely that stressor effects were mediated in different ways, or that the stress regimens used in this experiment, varied in their intensity upon the CBG elevating mechanism. From this study it is obvious that CBG elevation and corticosterone elevation in response to stress are independently controlled. An explanation of the mechanisms controlling CBG activity alteration must await further work.

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