EFFECTS OF CHRONIC ACTH STIMULATION, GONADAL HORMONES, THYROIDAL HORMONE TREATMENT AND AGE ON ADRENOCORTICAL FUNCTION OF THE RAT

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ABSTRACT

EFFECTS OF CHRONIC ACTH STIMULATION, GONADAL HORMONES, THYROIDAL HORMONE TREATMENT AND AGE ON ADRENOCORTICAL FUNCTION OF THE RAT

By

Gerald Dale Hess

The primary objective of this study was to gather physiological data related to endocrine function in aged rats with particular emphasis on the adrenal cortex. The effect of chronic adrenocortical stimulation was studied in young (5 month) and old (26 month) male rats. These rats were exposed to daily injections of a long-acting (depo) ACTH preparation for 6 weeks. Adrenocortical response (corticosterone concentration, μg/100 ml plasma) to ether stress was depressed (50% of control) in young male rats but was not altered in old male rats during the 6 weeks of treatment.

Adrenocortical response to acute ACTH stimulation (saline based exogenous ACTH preparation) was elevated in both young and old male rats following depo-ACTH treatment. Adrenocortical response to ether stress was also depressed in young (3 month) but not old (25 month) female rats following chronic ACTH treatment, while adrenocortical response to ACTH was elevated in both age groups. These results are

indicative of a marked alteration of the adrenocortical feedback control mechanism sensitivity (presumably to circulating corticosteroids) with increasing age in the rat.

Ovariectomy of young (6 month) and old (24 month) female rats did not affect adrenocortical response of either age group to ether stress or exogenous ACTH. Gonadal hormone replacement therapy with progesterone (800 μg daily) or estradiol (30 μg daily) for 1 week did not alter adrenocortical responsiveness of ovariectomized rats. Adrenocortical responsiveness to exogenous ACTH was reduced in young female rats that had been ovariectomized at 30 days of age (response determined 6 weeks later). Castration of young (5 month) and old (24 month) male rats did not reduce adrenocortical responsiveness to ACTH. These experiments indicated that gonadal hormone deficiencies are of limited importance with respect to age-related changes which exist in adrenocortical function of the rat. They also suggested that the principal influence of estrogens on adrenocortical function in the female rat occurs by the onset of puberty with little additional influence of physiological dosages of estrogens on adrenal steriodogenesis during the adult lifespan of the female rat.

Adrenocortical responses of male and female rats to ether stress were found to change between 23 and 200 days of age. The fact that adrenocortical stress response changes between 23 and 200 days of age in the rat should be considered when animals are chosen for studies of adreno-cortical functional parameters.

Young and old male and female rats were exposed to thyroid hormone treatment (Protamone, .02%, .04% and .08% of diet) for a period of three weeks. Adrenocortical response (plasma corticosterone levels) was higher in young (3 month) male rats fed the .04% (81 μg%) and the .08% Protamone diets (106 μg%) than in controls (59 μg%). Adrenocortical responses of old (23 month) male rats were not significantly different from those of non-treated control rats after 3 weeks of Protamone treatment. Adrenocortical response of young (4 month) female rats treated with .08% Protamone was significantly higher (161 μg%) that of controls (110 μg%), but adrenocortical response was not altered by any level of Protamone treatment in old (25 month) female rats. These findings suggested that the pituitary-adrenal axis of aged rats is less sensitive to thyroid hormone stimulation than the pituitary-adrenal axis of young adult rats of both sexes. This conclusion is in agreement with the previous hypothesis concerning age-differences in corticosteroid feedback influences.

Biological half-life, distribution volume and production rate of corticosterone were estimated in young (5 month) and old (27 month) female rats by calculating disappearance curves (single compartment model) for plasma radioactivity following rapid injection of H^3-cortisosterone.

The estimated biological half-life of corticosterone was longer in old female rats (10.6 minutes) than in young female rats (9.3 minutes), but the calculated corticosterone production rate was significantly higher in young (11.4 μg/min) than in old (7.8 μg/min) female rats. These findings suggested that adrenocortical secretory dynamics change with age in the rat. A similar study in young female rats suggested that chronic ACTH stimulation of the pituitary-adrenal axis does not alter the biological half-life and distribution volume of corticosterone. The H^3-corticosterone distribution technique was also used to study the influence of Protamone treatment (.08% for 3 weeks) on corticosterone distribution volume in the male rat. Corticosterone distribution volumes were significantly lower (36% of body weight) in treated than in control rats (48% of body weight). These data support the hypothesis that corticosterone distribution volume is altered by thyroid hormone treatment. Resting adrenocortical responses determined in these rats gave evidence of no significant influence of thyroid hormone treatment on this parameter of adrenocortical function.

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INTRODUCTION

Although progress in experimental gerontology has been less rapid than that in many other fields of biology, a number of significant studies dealing with the biology of aging have been reported in recent years. A comprehensive review of progress in experimental gerontology was presented by Comfort (1970). A detailed search for individual error processes, presumably at the cellular level, has constituted an important facet of recent gerontological investigation. Despite the recent emphasis upon studies of aging at the cellular and molecular level, the importance of studies at the systemic level is also recognized.

Sacher (1968) criticized the molecular approach in experimental gerontology because of its persistent lack of consideration for certain systemic aspects of aging and death. The molecular approach implies that the aging process can be explained purely on the basis of physical phenomena. The fact that a mouse is senile at three years while a man is senile at 90 years of age, although both are almost identical at the molecular level, illustrates the problem with this assumption. Sacher (1968) concluded that a greater emphasis must be placed upon the systemic aspects of aging if experimental gerontology is to develop as a complete discipline. Studies of aging at the systemic level are required to produce such an emphasis.

Frolkis (1968), Comfort (1970) and others regard the aging process as a gradual loss of homeostasis in highly complex organisms. Many reasons can be given to explain this homeostatic decrement. Curtis (1964) proposed that spontaneous mutations in the somatic cells are involved in the aging process. The organs of the body become full of defective cells and the resulting loss of efficiency constitutes the deterioration known as aging. According to Bjorksten (1968) the progressive cross-linkage of protein and nucleic acid molecules is responsible for the aging of biological organisms. The resulting molecular alteration leads to a progressive deterioration of physiological performance and the eventual death of the organism. Age-related changes in the control mechanisms of the central nervous and endocrine systems were examined by Frolkis (1968) and co-workers. They obtained evidence that endocrine functional activity diminished with increasing age. The sensitivity of tissue to hormones, determined by finding the lowest dose of hormone capable of producing metabolic and functional changes, was found to increase with age. Increased tissue sensitivity to hormones was thought to represent an adaptative element to compensate for decreased functional activity of the aging endocrines. Included in this adaptation was an increased sensitivity of endocrine glands to hormones produced by other endocrine glands. On the other hand, the same group (Frolkis, 1968) found that tissue reactivity to hormones, i.e., the potential range of metabolic and functional changes taking place in response to large doses of hormones, decreases with increasing age.

Endocrine homeostasis can be maintained in old age because of these "allometric" changes which occur in the endocrine control systems. However, the onset of pathological or other extreme conditions in aged individuals may upset the adapted regulatory mechanisms and cause the collapse of homeostasis.

Studies of the biological aging process have been limited despite the recent surge in basic biological research. Many theories have been proposed to account for the aging process, but these have suffered from a lack of the experimental evidence needed to substantiate them. The loss of homeostatic capacity is a well-known characteristic of biological aging. Because of its importance in maintaining body homeostasis as well as its control by the nervous system, the endocrine system is a logical prospect for involvement in the aging process, whether this involvement be cause or effect of the aging phenomenon.

The primary objective of this study was to gather physiological data related to endocrine function in aged rats with particular emphasis on the adrenal cortex. To this author's knowledge, few if any studies dealing with functional measurements of the pituitary-adrenal axis in aged rats have been reported heretofore other than those from our laboratory. In several studies undertaken for this dissertation, experiments in aged animals were prefaced by considerable investigation of the systems involved in young adult rats.

LITERATURE REVIEW

Control of the Pituitary-Adrenal Axis

Steroid-Sensitive Feedback Control

The existence of a steroid sensitive feedback mechanism for controlling pituitary ACTH release and synthesis is well documented. MacKay and MacKay (1926) demonstrated that unilateral adrenalectomy induces compensatory hypertrophy of the remaining adrenal cortex. De Groot and Harris (1950) and Hume and Wittenstein (1950) working independently, showed that the hypothalamus influences ACTH secretion. Studies by Saffran *et al*. (1955) and Guillemin and Rosenberg (1955) suggested the existence of a corticotrophin-releasing factor (CRF) of hypothalamic origin.

Other investigators also showed that the hypothalamus is involved in the control of pituitary ACTH release. Bogdanove and Halmi (1953) and McCann (1953) found that lesions of the medial basal hypothalamus (MBH) may cause adrenal atrophy. Lesions of the MBH blocked the compensatory hypertrophy which normally follows unilateral adrenalectomy in several studies (Ganong & Hume, 1954; Endröczi & Mess, 1955; Fulford & McCann, 1955).

Implantation of corticosteroids in the MBH has been used in a number of more recent studies to show that the brain is involved in control of the pituitary-adrenal axis. Endröczi _et al_. (1961) inhibited both resting adrenal secretion and stress induced increases in adrenal secretion with cortisone implants into the MBH of normal cats and rats. Smelik and Sawyer (1962) confirmed these results in rabbits bearing median eminence implants of cortisol. Corbin _et al_. (1965) found that plasma and adrenal corticosterone levels and adrenal weight could be reduced significantly by median eminence implants of Dexamethasone. They also showed that a similar but less extensive effect could be produced by implants of cortisol. Dexamethasone implants into the pituitary or cerebral cortex did not affect the parameters studied. Median eminence implants of either cortisol or corticosterone blocked adrenocortical stress responses in studies by Davidson _et al_. (1965). These implantation studies have shown rather clearly that the MBH is involved in the control of the pituitary-adrenal axis, but have failed to determine whether the MBH merely conveys signals from other brain structures to the anterior pituitary or exerts a controlling influence itself.

Demonstration of changes in the CRF content of the median eminence in response to pituitary-adrenal activation has provided additional evidence for central nervous system involvement in the control of adrenocortical function.

Vernikos-Danellis (1965) demonstrated that median eminence CRF activity closely parallels or precedes stress induced changes in blood and pituitary ACTH concentrations. Exposure of female rats to ether stress, sham adrenalectomy or true adrenalectomy increased the CRF content of the median eminence while the median eminence CRF content was reduced and the stress induced rise of median eminence CRF was completely blocked by cortisol pretreatment. This study was important in validating the concept that CRF's are physiologically involved in the synthesis and release of ACTH.

The medial basal hypothalamus (MBH), also called the hypophysiotropic area, appears to be the source of the neural factors which control anterior pituitary function. Halasz _et al_. (1962) showed that the normal function and structure of the anterior pituitary was maintained following transplantation to the MBH region. Transplantation of pituitaries to other hypothalamic or extrahypothalamic areas resulted in a loss of normal structure and function, hence the name hypophysiotropic area. The significance of the hypophysiotropic area in adrenocortical control was studied further following neural isolation of this region. Using an ingenious knife assembly mounted in a stereotaxic apparatus, Halasz and Pupp (1965) were able to completely sever the hypophysiotropic area from the rest of the brain without breaking its contact with the pituitary. Although vascular regeneration occurred subsequent to this procedure, neural isolation endured for the course of the experiments.

Halasz et al. (1967) found that complete deafferentation of the hypophysiotropic area does not alter compensatory hypertrophy of the remaining adrenal following unilateral adrenalectomy. Similarly, Palka et al. (1968) showed that neural isolation of this region did not prevent dexamethasone suppression of non-stress plasma corticosterone levels. Even more surprising is the finding by several groups (Matsuda et al., 1964; Halasz et al., 1967; Palka et al., 1968) that a normal adrenocortical response to ether stress can still be produced following neural isolation of the hypophysiotropic region. Voloschin et al. (1968) also found that the pituitary-adrenal axis was capable of responding to ether stress after neural isolation of the MBH, but the observed response was at a submaximal level. Matsuda et al. (1964) found no response to traumatic stress in MBH isolated rats despite a normal response to ether stress in these animals.

Upon reviewing studies of adrenocortical function following isolation of the hypophysiotropic area conducted in several laboratories including his own, Halasz (1969) concluded that adrenocortical responses to ether stress, immobilization and corticosteroid feedback are mediated through the MBH. Studies of adrenocortical function based on neural isolation of the hypophysiotropic area do not preclude the possibility that the MBH maintains normal anterior

pituitary structure and function, with the stress response and corticoid feedback being mediated at least partially by the anterior pituitary. Since MBH neural isolation blocks some stress responses while leaving others intact, Halasz (1969) concluded that several factors activate ACTH release by different mechanisms. The release of vasopressin following ether (Turner, 1966) could represent one such mechanism.

Experimental evidence now available suggests that steroid sensitive receptors active in controlling pituitary-adrenal function exist outside of the hypothalamus. Endröczi _et al_. (1961) and Corbin _et al_. (1965) found that corticosteroid implants into the midbrain region depressed pituitary-adrenal function in rats. Davidson and Feldman (1963) reported that midbrain implants of cortisol were partially effective in blocking adrenal compensatory hypertrophy after unilateral adrenalectomy. Slusher (1966) inhibited diurnal changes in adrenal and plasma corticosterone levels of rats with cortisol implants into the hippocampus or midbrain. Kendall _et al_. (1969) found that systemically ineffective amounts of corticoids could reduce ACTH secretion when placed in certain areas of the ventricular system. They concluded that previous reports of extrahypothalamic feedback receptor sites in the rat forebrain might be explained by transport of corticoids from their implantation site to a single feedback site located in the basal hypothalamus or the anterior pituitary via the ventricular system.

Evidence from other studies suggests that the pituitary gland may contain receptor sites for the adrenal steroid feedback mechanism. De Wied (1964) found that dexamethasone pretreatment prevented pituitary ACTH release by hypothalamic extracts containing CRF while Chowers et al. (1967) showed that dexamethasone implants into the median eminence or pituitary reduced pituitary ACTH content. Only median eminence implants reduced CRF content (Chowers et al., 1967). Arimura et al. (1969) found that dexamethasone suppresses the action of CRF at the pituitary level, although this blockade does not develop immediately. Dexamethasone injected bilaterally into the anterior pituitary by Russel et al. (1969) prevented pituitary ACTH release in response to both exogenous and endogenous CRF. Similar results were obtained when dexamethasone was injected into the median eminence or into the septal region of the brain. Using a fluorescent dye, Russel et al. (1969) found that materials injected into the median eminence or septal region spread very rapidly to the pituitary. They concluded that previous evidence for hypothalamic feedback sites for pituitary-adrenal control resulted from corticoids which had traveled to the pituitary and exerted an effect there. The fact that other investigators did not demonstrate a local inhibitory effect of dexamethasone at the pituitary was attributed to inadequate exposure of the pituitary to dexamethasone. A recently reported study of ACTH release in dogs (Gonzalez-Luque et al., 1970) also suggested that adrenal steroid feedback control resides in the pituitary. Although this group as well as others have inhibited the release of ACTH with dexamethasone at the pituitary, this effect has not been shown for physiological levels of natural corticoids.

Short Feedback Control

The pituitary gland is regulated by two different types of feedback mechanisms. The peripheral target gland hormones serve as feedback signals for the classic control system while the pituitary hormones themselves are involved in a short feedback control system. Short feedback control systems have been demonstrated for all the anterior and intermediate lobe pituitary hormones, including prolactin, growth hormone and melanocyte stimulating hormone which have no peripheral target glands and hence are not regulated by the classic feedback system (Motta et al., 1969).

The short feedback receptors are located primarily in the brain (hypothalamus) (Mess & Martini, 1968; Motta et al., 1969). The pituitary hormones may reach the brain by either the general circulation or by transport up the pituitary stalk. Török (1964) demonstrated a vascular system passing from the posterior surface of the anterior pituitary to the capillary complex. Several pituitary hormones have been isolated where this vascular system ends in the basal hypothalamus (Guillemin et al., 1962; Schally et al., 1962; Johnson & Nelson, 1966).

Early evidence that ACTH influences its own secretion by feedback on CNS structures was reported by Hodges and Vernikos (1958, 1959). They found that stressful stimuli induced a greater secretion of ACTH in adrenalectomized rats with low initial plasma ACTH levels than in those with high initial levels. Apparently, high levels of circulating ACTH reduced the reactivity of the hypothalamic-pituitary-adrenal axis. Kitay _et al_. (1959) increased pituitary ACTH stores in proportion to the dose of exogenous ACTH administered in adrenalectomized rats. Similarly, Kitay _et al_. (1959) blocked the stress induced fall of pituitary ACTH in adrenalectomized rats with exogenous ACTH. Further support for short feedback control of ACTH is provided by studies of Vernikos-Danellis and Trigg (1967) utilizing ACTH secreting pituitary tumors. In the presence of such tumors, stress induced increases of plasma ACTH in adrenalectomized rats were lower than usual and the elevation of plasma and pituitary ACTH normally seen after adrenalectomy was absent.

Dallman and Yates (1968) considered the effects of chronic ACTH pretreatment on stress induced ACTH secretion in intact rats. Response to stresses such as noise and ether applied 24 hours after the last ACTH injection were partially inhibited but response to other stresses (e.g., scald, laparotomy) was not inhibited. They concluded that ACTH can block stress induced rises in ACTH secretion, although the inhibition appears to depend on the type of stimulus employed. In response to the argument that an accumulation of endogenous corticoids might be producing the blocking effects, the inhibitory effects of ACTH pretreatment were studied in intact animals whose classic feedback receptors had been saturated by dexamethasone administration. Histamine and laparotomy caused ACTH release even after high doses of dexamethasone, but this response was blocked when animals received both dexamethasone and ACTH simultaneously.

Vernikos-Danellis (1965) and Motta et al. (1968) reported increases in the hypothalamic CRF concentration following adrenalectomy. A further increase in hypothalamic CRF content above adrenalectomy levels occurred when animals were both adrenalectomized and hypophysectomized (Motta et al., 1968). The fact that CRF has been found in the circulation after hypophysectomy (Brodish and Long, 1962), suggests that eliminating the ACTH feedback signal activates both synthesis and release of CRF. Treatment of adrenalectomized-hypophysectomized animals with exogenous ACTH (Motta et al., 1968) reduced CRF stores to the level found in animals adrenalectomized only, but would not lower them to pre-adrenalectomy levels.

Interaction of Stress and Resting Responses

Experimental evidence substantiates the fact that the pituitary-adrenal axis is controlled by negative feedback mechanisms which are sensitive to either ACTH or corticosteroids. This control mechanism is thought to regulate adrenocortical function under "resting" conditions. The role of negative feedback control during stress activation of the pituitary-adrenal axis is not completely understood.

The concepts of stress and stress activation of the pituitary-adrenal axis were popularized by the General Adaptation Syndrome of Selye (1950). Ganong (1963) has reviewed the role of the many different non-specific stimuli termed stresses in activation of the pituitary-adrenal axis. Some authors have attempted to classify stressors according to the different sites and modes of action involved in stimulation of the pituitary-adrenal axis. Mangili et al. (1966) proposed that the stimulating power of stressors might be a more valid basis for classification than their modalities of action.

Diurnal fluctuations of "resting" plasma corticosterone levels have been observed in many different species including humans and the rat (Liddle et al., 1962; Critchlow et al., 1963; Ganong, 1963). Sayers and Sayers (1947) and Yates et al. (1961) proposed that pituitary ACTH secretion is closely regulated by a mechanism sensitive to circulating plasma corticosterone levels. According to their hypothesis, stress stimulation of the pituitary-adrenal axis resulted in a "resetting" of the control mechanism so that higher plasma corticosteroid levels were attained, but precise control by the negative feedback mechanisms was still maintained.

Subsequent evidence from other investigators suggested the existence of a more complicated control mechanism. The failure of large corticosteroid doses to block ACTH release following acute stress suggested a lack of precise negative feedback control under certain conditions (Hodges & Jones, 1963; Smelik, 1963). Zimmerman and Critchlow (1967) proposed that mechanisms regulating acute pituitary-adrenal response to stress and those regulating the diurnal rhythms of the non-stress response are independent of one another. Slusher (1964) had previously reported a dissociation of stress and non-stress mechanisms following studies on male rats bearing hypothalamic lesions.

In more recent studies, Zimmerman and Critchlow (1969a, 1969b) have shown that relatively low doses of dexamethasone injected subcutaneously or implanted intracerebrally selectively suppress non-stress levels of corticosterone without blocking acute plasma corticosteroid responses to stress. In a third study (Zimmerman & Critchlow, 1969c), intravenous corticosteroid injections which produced elevated (but physiological) plasma corticosteroid levels blocked non-stress levels without affecting acute stress responses. A double bleeding technique was used in these studies. The plasma corticosterone level of the initial plasma sample, obtained within three minutes of handling of the rat, was considered a resting response, while the plasma corticosterone level of a second sample, obtained 15 minutes after ether anesthesia, was considered to be a stress response.

Although Zimmerman and Critchlow's definition of a "resting" response can be criticized, these findings suggest that control of stress and non-stress responses of the pituitary-adrenal axis is under separate neural mechanisms.

In contrast to the earlier hypotheses of Sayers and Sayers (1947) and Yates _et al_. (1961), the findings of Zimmerman and Critchlow (1969a, 1969b, 1969c) indicated that adequate stress stimuli are capable of overriding the control mechanism which operates under resting conditions. These findings were in conflict with those of other investigators (Hedner & Rerup, 1962; Mangili _et al_., 1965; Dallman & Yates, 1968; Davidson _et al_., 1968) in which pretreatment with dexamethasone blocked acute adrenocortical response to ether in the rat. Several reasons were given by Zimmerman and Critchlow to account for the discrepancies in blocking of the stress response. Differences in the degree of stress may have been responsible. According to this argument, the stress used by investigators reporting an apparent block of acute stress response was inadequate to overcome the blocking effect of dexamethasone pretreatment. Complications of Nembutal anesthesia and differences in the time interval between stress and blood sampling are given as additional explanations for the results of Zimmerman and Critchlow as opposed to those of the conflicting studies.

Results of experiments and computer simulation studies reported by Yates _et al_. (1969) indicate that the capacity of corticosteroids to inhibit stress activation of the pituitary-adrenal axis reaches a maximum; beyond which further increases in the stress intensity will activate the system despite increased corticosteroid dosage. Yates et al. (1969) found that the feedback inhibition property of the pituitary-adrenal axis has non-competitive saturation characteristics. Because of this saturation characteristic, many investigators fail to appreciate the capacity of corticosteroids to inhibit the stress response, according to Yates et al. (1969). This implies that experiments in which corticosteroids did not block the stress response involved stresses whose stimulus strength exceeded the saturation level of the feedback system. Inhibition would have been observed had a lower stimulus strength been used.

Gonadal Hormone Influences on the Pituitary-Adrenal Axis

Gonadal steroids, particularly the estrogens, exert a significant influence on adrenocortical function. The well recognized sex difference of adrenocortical function in the rat is at least in part a consequence of this gonadal steroid influence. Kitay (1961) reported that ether stress stimulation induced higher and more persistently elevated plasma corticosterone levels in female rats than in male rats. Exogenous ACTH stimulation gave similar results. This study was prompted by previous studies of Troop (1959) and Urquhart et al. (1959) which had described sex differences in the rate of corticosterone and cortisone metabolism by rat liver tissue *in vitro*.

Kitay (1961) also demonstrated that corticosteroid metabolism, both _in vivo_ and _in vitro_, occurs at a faster rate in female rats than in male rats. In a subsequent study, young adult male and female rats were treated with exogenous estrogen for two weeks (Kitay, 1963a). Following this treatment, stimulated (ether stress or exogenous ACTH) plasma corticosterone levels of intact male rats were significantly higher than levels of non-treated controls. Both _in vivo_ and _in vitro_ corticosteroid metabolism were also increased. The same level of estrogen treatment did not significantly alter stimulated plasma corticosteroid levels of intact female rats, but it decreased the rate of hepatic corticosteroid metabolism _in vivo_ and _in vitro_.

The effects of gonadectomy on adrenocortical function have also been studied by Kitay (1963b). Rats were gonadectomized at 30 days of age and adrenocortical responses were tested six weeks later. Both resting and stimulated (ether stress or exogenous ACTH) plasma corticosterone levels were reduced in ovariectomized female rats but castration did not affect these parameters in male rats. Corticosteroid metabolism was increased in castrated male rats and decreased in ovariectomized female rats. Estradiol added directly to adrenal slices from ovariectomized rats increased _in vitro_ corticosterone production, thereby suggesting that estrogenic influences upon adrenocortical function result at least in part from a direct estrogen effect on the adrenal cortex (Kitay, 1963a).

Evidence for estrogenic stimulation of adrenocortical function at the hypothalamo-hypophyseal level has been reported. Kanematsu and Sawyer (1963) and Telegdy (1964) found that estradiol implants in the hypothalamic area increased adrenocortical function. Richard (1965/66) found basal levels of plasma corticosterone were increased by implantation of estradiol-17 β into either the accurate nucleus, the anterior pituitary or the lateral mammillary area of ovariectomized rats. The exact site of estradiol stimulation of adrenocortical function at the hypothalamo-hypophyseal level is not known. Coyne and Kitay (1969) demonstrated that estradiol stimulates ACTH secretion by increasing pituitary responsiveness to CRF-like activity and by increasing pituitary ACTH synthesis. The effects of estradiol on the hypothalamo-hypophyseal system are thought to occur independently of corticosteroid feedback (Coyne and Kitay, 1969).

Estrogens are known to cause increased levels of corticosteroid binding protein in the circulation. Westphal et al. (1962) have demonstrated the presence of transcortin activity in rat plasma. Tait and Burstein (1964) obtained evidence in humans, however, that increased transcortin binding of corticoids under estrogen stimulation had little effect on levels of free circulating corticoids. Since the free plasma corticoids are thought to be the primary feedback signal for regulation of resting pituitary-adrenal function (Tait and Burstein, 1964) it is likely that estrogen stimulation of transcortin has little effect upon adrenocortical function.

It is reasonable to conclude that estrogenic stimulation of the pituitary-adrenal axis occurs at multiple sites, including the hypothalamus, the pituitary, and adrenal cortex and the liver. The relative importance of estrogenic stimulation at these sites under physiological conditions remains to be determined.

Thyroidal Influences on the Pituitary-Adrenal Axis

The thyroid gland is known to exert an influence on adrenocortical function (Sayers, 1950; Wallach & Reineke, 1949). Wallach and Reineke (1949) studied adrenal response to prolonged thyroxine stimulation in rats. After receiving daily injections of thyroxine ranging from 5 to 80 μg thyroxine (T_4)/100 g for 28 days, adrenal weight and ascorbic acid content of treated rats were increased above those in non-treated controls at all dose levels above the 5 μg treatment. In a similar study, dosages of Protamone, a thyroactive iodinated protein, ranging from 0.01% to 0.16% of the diet produced no significant change in adrenal response after 4 weeks of treatment. In a separate experiment Wallach and Reineke (1949) were able to prevent the thyroxine induced increase in adrenal weight and ascorbic acid content by concurrent administration of adrenocortical extracts. This was taken as evidence that the adrenals are in a state of hypersecretion in a hyperthyroid animal. The enlarged adrenal glands of hyperthyroid animals were interpreted as evidence for an increase in ACTH secretion.

Steinetz and Beach (1963) found increased adrenal gland size in rats made hyperthyroid by feeding a diet containing 0.15% USP thyroid for eight days. Thyroidectomized rats maintained on the same diet showed similar results. Thyroid treatment was not effective in increasing adrenal weights in hypophysectomized or prednisolone pretreated rats. D'Angelo and Grodin (1964) confirmed the lack of adrenocortical response to thyroid hormone treatment in hypophysectomized rats. D'Angelo and Grodin also found that administration of tri-iodothyronine (T_3) increased adrenal weight of treated rats although adrenal ascorbic acid concentrations were consistently reduced at higher T_3 dose levels. Decreased adrenal ascorbic acid concentration was offset by adrenal hypertrophy, so that vitamin C content of the enlarged male adrenals was significantly increased. The increased ascorbic acid content of the enlarged adrenals in hyperthyroid animals confirmed the earlier work of Wallach and Reineke (1949).

Kawai (1962) also reported a significant increase in adrenal weight of hyperthyroid rats. By way of contrast, "resting" plasma corticosterone levels (blood from rapid decapitation) were similar in hypothyroid, euthyroid and hyperthyroid rats, suggesting that if increased ACTH secretion rates actually exist under basal steady state conditions they must be balanced by changes in other parameters which determine plasma corticosterone levels. Pituitary ACTH depletion 15 minutes after stress was greater in hyperthyroid than in euthyroid rats (Kawai, 1962) although pre-stress pituitary ACTH concentrations did not differ between these groups.

Steinetz and Beach (1963) measured plasma corticosterone levels one hour after injection of ACTH into intact rats. Plasma corticosterone levels were elevated in thyroid-treated rats in comparison to non-treated controls. Similar results were observed in thyroidectomized rats following treatment with thyroid hormone. Thyroid prefeeding did not alter plasma corticosterone levels after ACTH injections in hypophysectomized rats. D'Angelo and Grodin (1964) also reported increased plasma cortocosterone levels in rats following thyroid treatment. Interpretation of their results is complicated by possible inadequate standardization of the stress involved in obtaining blood samples. Bohus _et al_. (1965/66) studied the effects of thyroxine implantation into the brain on adrenocortical response. Bilateral implantation of thyroxine led to an enhancement of adrenal function when implants were located in the mid-posterior part of the arcuate nucleus. A weaker activation of the adrenals was evident when implants were placed into the anterior median eminence. When thyroxine implants were placed in other areas of the hypothalamus or in the anterior pituitary there was no change in the corticosterone output of the adrenals. The results of this study suggested that hypothalamic CRF release was stimulated by the thyroxine implants if properly placed in the median eminence.

Steinetz and Beach (1963) demonstrated a 50% reduction in the apparent distribution volume of corticosterone in hyperthyroid rats. Distribution volume was determined following intravenous injection of unlabelled corticosterone. Additional experimental evidence showed that plasma volume, red cell binding of corticosterone and "capillary permeability" were not sufficiently altered to account for the reduction in distribution volume. In vitro experiments in which adrenal corticosterone production was not markedly influenced by thyroid treatment also suggested that distribution volume was reduced in hyperthyroid rats. In hypophysectomized rats, in vivo plasma corticosterone levels one hour after ACTH were higher in animals treated with both thyroid and repository ACTH than in animals treated only with respository ACTH. These results were attributed to the 50% reduction in corticosterone distribution volume of hyperthyroid rats.

In spite of increased adrenal size in hyperthyroid animals, Steinetz and Beach (1963) found no marked difference between normal and hyperthyroid rats when in vitro adrenal steroid production was compared. However, stimulation of adrenals from both groups by equal amounts of ACTH in vitro could reflect inadequate ACTH for maximal stimulation of hyperthyroid adrenals. Thyroxine administration to rats *in vivo* caused increased activity of enzymes associated with several adrenocortical biochemical pathways including glycolysis and NADPH production and resulted in increased adrenal size (Freedland & Murad, 1969). They suggested that increased activities of the NADPH producing enzymes may be related to the increase in corticoid production following thyroid treatment that has been reported by other investigators.

Yates *et al*. (1958) reported a close correlation between adrenal size and the *in vitro* capacity of the liver to inactivate steroid hormones in hyperthyroidism. Total hepatic capacity for *in vitro* reduction of ring A of cortisone was increased by 38% in male and female rats following thyroid treatment. Increases of total steroid hydrogenases and NADPH availability in the liver were thought to account for this increase. McGuire and Tomkins (1964) found that the increased rate of steroid reduction observed in rats treated for three days with thyroxine resulted from increased levels of reduced NADPH in liver homogenates.

Studies of Endocrine Function in Aging Individuals

Aging and the Pituitary-Adrenal Axis

The concept of age-related changes in adrenocortical function originated with studies by Jackson (1919) on postnatal development of the rat adrenal gland. There was evidence of a general increase in the adrenal parenchymal cell size from one to ten weeks of age with little change thereafter. Blumenthal (1945) reported a gradual diminution in the number of mitoses occurring in the guinea pig adrenal gland with increasing age. Other studies (Dribben & Wolfe, 1947; Jayne, 1953) provided evidence of changes in connective tissue structure of the adrenal glands of aging rats. Studies on histochemical and degenerative changes in the adrenal cortex of the aging rat by Jayne (1957) indicated that cellular degeneration may substantially decrease the steroid producing ability of adrenocortical parenchymal cells. Friedman *et al*. (1965) showed that administration of adrenal and neurophypophyseal hormones significantly prolonged the life span of 24 month old rats and visibly improved general body condition. The results of this study suggested that hormonal inadequacies may facilitate the aging process.

Early studies of adrenocortical steroid output indicated that 17-ketosteroid excretion decreases with increasing age (Hamilton & Hamilton, 1948; Pincus, 1955). These compounds are primarily androgen rather than glucocorticoid metabolites. Borth *et al*. (1957) showed that there are definite correlations between urinary excretion of both 17-ketosteroids and 17-hydroxycorticosteroids and age. They concluded that 17-ketosteroid excretion was more dependent upon age than was 17-hydroxycorticosteroid excretion. Their findings suggested that glucocorticoid production by the adrenal cortex does change with advancing age.

On the contrary, Romanoff _et al_. (1957) reported that increasing age produced no change in the urinary excretion of the more characteristic corticosteroids (e.g., tetrahydrocortisone, tetrahydrocortisol, cortisone and cortisol). Romanoff _et al_. (1957) reported a 10% decrease in the number of detectable 17-ketosteroids in the urine of elderly subjects. This became a 20% decrease, as compared to young subjects, following ACTH administration.

In a subsequent study Romanoff _et al_. (1958) determined excretory levels of tetrahydrocortisol, 3α-allotetrahydrocortisol and tetrahydrocortisone in the urine of young and old men and women. The mean quantitative excretory levels of the three metabolites were significantly lower in young and old women and in old men than in young men. They found that the differences in quantitative excretion of these metabolites disappeared when urinary excretion was expressed as a function of creatinine excretion. Further investigation (Romanoff _et al_., 1959) showed that β-cortolone, a regularly occurring metabolite found in the urine of young and old subjects of both sexes, also shows an age related decline in excretory levels when expressed as mg/hr. This difference was also eliminated when cortolone excretion was expressed in terms of mg/g creatinine excretion.

In order to confirm their previous findings, Romanoff _et al_. (1961) determined the resting adrenal secretory rates of eight young and eight old men. The 24-hour secretion rate of cortisol in old men was only 75% of that in young men, regardless of the metabolite on whose excretion the determination was based. Secretion rates were the same for young and old subjects when expressed as mg/g creatinine/24 hr. This study (Romanoff et al., 1961) confirmed previous findings that apparently neither cortisol metabolism nor cortisol secretion by the adrenal differed with age when the muscle mass (based on creatinine excretion index) of the subject was considered.

Tyler et al. (1955) compared plasma 17-hydroxycorticosteroid levels of young adults and geriatric patients following a 6 hour ACTH infusion. They reported that adrenal response to ACTH and hepatic metabolism of corticosteroids did not change with increasing age although corticosteroid distribution volumes and turnover rates were decreased.

West et al. (1961) found a progressive decrease in the rate of cortisol removal from the circulation with increasing age but no difference in the distribution volume of the infused cortisol. Suggestive evidence for an age-related decrease in the cortisol production rate under resting conditions was also reported. Samuels (1956) demonstrated an increased biological half-life of cortisol in aged human subjects. The apparent distribution volume of cortisol was also lower in old than in young subjects. Because of these changes, the secretory rate required to maintain normal resting plasma cortisol levels was much lower (about 50%) in old men than in young men. This implied that the adrenals of older persons were less functional than those of younger individuals.

Moncloa *et al*. (1963) showed that 17-hydroxycorticosteroid excretion/24 hr decreased progressively with increasing age in healthy men ranging from 20-85 years of age. They concluded that decreased production and increased biological half-life of cortisol accounted for these changes. Standard tests for liver function performed on their subjects indicated no abnormalities. This implies that decreased hepatic metabolism was not the reason for increased half-life. Moncloa *et al*. (1963) also found that 17-hydroxycorticosteroid production was lower in old subjects than in young subjects in response to multiple levels of ACTH infusion.

The conclusion by Samuels (1956) and Moncloa *et al*. (1963) that adrenocortical response to ACTH decreases with increasing age was also supported by the findings of Riegle and Nellor (1967). Following ACTH infusion, plasma glucocorticoid levels in bulls showed a marked and progressive decrease with age, indicative of a decreased adrenocortical responsiveness to ACTH. Plasma ACTH levels were higher in old animals than in young ones. Histological studies revealed varying degrees of cortical degeneration with age which were positively correlated with the relative insensitivity to exogenous ACTH infusions. Apparently the increased levels of plasma ACTH were required in older animals to maintain normal plasma glucocorticoid levels. Subsequent studies in goats (Riegle *et al.*, 1968) also revealed an age-related decrease in adrenocortical responsiveness to ACTH.

Age related changes in adrenocortical function have also been reported in guinea pigs (Fajer & Vogt, 1963) monkeys (*M. mulatta*) (Bowman & Wolf, 1969), and mice (Grad, 1969). Recent studies in our laboratory have shown that adrenocortical responsiveness to ether stress or exogenous ACTH is decreased in both male and female rats with increasing age (Hess & Riegle, 1970). This age-difference in adrenocortical function in the rat did not exist in the absence of ether stress or ACTH stimulation.

Gonadal Function in the Aged

The concurrent progression of the aging syndrome and changes in gonadal function observed in humans, produced the theory that gonadal hormone changes caused aging to occur (Sobel, 1967). Considerable experimental evidence suggested that a sudden decrease in estrogen production occurred in human females at menopause, while a gradual decrease in 17-ketosteroid production occurred with advancing age (Gherondache *et al.*, 1967). The cessation or diminution of gonadal steroid production with increasing age disrupts the fine balance between hormones which is normally found in young adults. Following gonadal changes in aged humans, the pituitary gland secretes increased amounts of gonadotropins (Gherondache *et al.*, 1967). The development of endocrinology was expected to provide the necessary knowledge for reversing or at least controlling the aging process. It is now apparent that the aging phenomenon is much more complex than can be explained by hormonal changes with increasing age. This does not mean that gonadal hormone changes are strictly a consequence of the aging process, however.

Evidence now available suggests that the causative factors behind reproductive failure in the aging human female are quite different from those in aged rats. At menopause the human female suffers the loss of ovarian steroids, which is attributed to the absence of follicular cells (Engle, 1955). Krohn (1955) reported that three phenomena associated with the onset of menopause in women are a disappearance of ovarian oocytes, a decreased production of sex hormones and increased gonadotropin levels in blood and urine. Becker and Albert (1965) found that in post-menopausal women follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretions (based on urinary excretion of these gonadotropins) were increased 11 and 9 fold, respectively, compared to normal men. Apparently the menopause develops in women because the ovary is no longer capable of responding to gonadotropins (Krohn, 1955).

This is not the case in aged rats and mice, in which ovarian function can be restored by gonadotropin treatment (Krohn, 1955). Eckstein (1955) concluded that the decline in ovarian function of aged rats results from anterior pituitary failure rather than from ovarian senescence.

More recently, Ascheim (1965) showed that regular estrous cycles could be induced in aged constant estrous rats by LH injections.

The loss of reproductive function in lower animals occurs so gradually that its final stages may not be reached before the end of life. For example, there may be primordial and Graafian follicles and even corpora lutea present in aged rats, although in reduced numbers (Eckstein, 1955). It appears that ovarian function declines slowly in old rats and that pituitary failure rather than ovarian senescence alone accounts for this change. These findings imply that some gonadal hormone production could persist in rats beyond the cessation of normal reproductive capability.

Clemens et al. (1969) investigated the possibility that hypothalamic changes may account for the loss of normal reproductive function in old rats. Constant estrous, aged rats (20 month) were exposed to treatment with drugs, hormones, or direct electrical stimulation of the preoptic area of the brain. Ovulation, confirmed by laparotomy, was induced by progesterone, epinephrine and electrical stimulation of the preoptic area, suggesting that changes in brain function are at least partially responsible for the decline of ovarian function. Although this study did not rule out the possibility of ovarian senescence, the observation that old rat ovaries can be reactivated suggested that ovarian failure is caused by changes at a higher level.

Changes in brain neural activity are implicated in the loss of normal ovarian function and subsequent constant estrous of old rats. This concept is supported by the finding (Clemens, 1968) that hypothalamic LRF content is very low and FSHRF content is very high in old constant estrous rats compared with 3-month-old cycling rats during estrous.

Influences of Aging on Thyroid Function

For a number of years there has been evidence that thyroid function is decreased in aged subjects. Turner (1948) reported this phenomenon in poultry, while more recently Long *et al.* (1952) reported similar findings in cattle and Henneman *et al.* (1955) presented evidence that thyroid secretion rates are lower in old sheep than they are in young sheep. Age-related changes in thyroid function of the rat have been reported by Grad and Hoffman (1955), and Wilansky *et al.* (1957) while Flamboe and Reineke (1959) observed a progressive decline in thyroid secretion rate of aging goats.

Grad and Hoffman (1955) found that thyroid secretion rate (estimated by the goitrogen technique) was at least 25% lower in old (29 month) female rats than in young (4½ month) female rats. Wilansky *et al.* (1957) found that thyroid secretion rates (estimated by the thyroxine degradation technique) were 20% lower in old (24-25 month) rats than in young (4-5 month) rats. Wilansky *et al.* found no difference in circulating PBI levels between the two groups. Gregerman (1963) measured PBI levels in young and old female rats and found that they were lower in 12 month than in 24 month old animals. The thyroid hormone distribution space per unit body wt was greater in old rats than in young rats, however. Gregerman (1963) also found that the rate of thyroxine degradation was 50% higher in old rats than in young rats. This finding contradicted previous findings of Grad and Hoffman (1955) and Wilansky et al. (1957) in aged rats. Narang and Turner (1966) estimated thyroid secretion rate in rats at 30 day intervals between weaning and 4 months of age and found a progressive decrease in secretion rate during this time.

Studies of thyroid secretion rate with aging do not give a complete picture of the physiological effects of thyroid hormone in aging rats. For example, Gregerman (1963) pointed out that the exact contribution of triiodothyronine (T_3) to the metabolic effect of thyroid hormone in the rat is presently undefined as is the relationship between age and T_3 secretion rate. A complete understanding of thyroid function in the senescent rat is dependent upon acquisition of this information. Preliminary experiments conducted by Wilansky et al. (1957) suggested that a greater rise in oxygen consumption occurred in old rats than in young rats for the same dose of thyroxine per unit metabolic mass. Increased responsiveness to thyroxine in aged rats could be indicative of a homeostatic mechanism whereby senescent rats are able to compensate for a reduction in thyroid secretion rate. Grad (1969) also attempted to determine if target tissue requirements for thyroid hormone are changed in senescent animals. Basal metabolic rate (BMR), based on oxygen consumption, was used as an index of tissue responsiveness to thyroid hormone. Although BMR was higher in old female rats than in young female rats, thyroidectomy caused BMR to drop to the same extent in both young and old rats. Thyroxine treatment increased the BMR of both young and old rats but the effect was more pronounced in the old animals than in the young. This was interpreted as a greater responsiveness of target tissues to the same dose of thyroxine given in relation to metabolic mass in the senescent rats (Grad, 1969).

According to Frolkis (1968), tissue responsiveness to hormones is increased in senescent animals. Since this increased sensitivity of tissues to hormones occurs at a time when functional activity of the endocrines is declining, it represents an adaptive response to changes which occur in body control systems with aging. As an example of this change, Frolkis (1968) reported evidence from a study where thyroid stimulating hormone (TSH) treatment produced only insignificant changes in the thyroid glands of mature rats while a similar dose of TSH given to aged rats produced a

27% increase in oxygen consumption of thyroid tissue, a 69% increase in thyroid weight and a 66% increase in the height of thyroid epithelium.

MATERIALS AND METHODS

Experimental Animals

Experimental animals were rats of the Long-Evans strain bred and reared in the Endocrine Research Unit rat colony. They were maintained at approximately 22° C and subjected to illumination between 7:00 AM and 7:00 PM. All rats received MSU regular rat diet (Appendix I) and water *ad libitum* during the course of experimentation.

Statistical Analysis

The significance of plasma corticosterone differences between treatment groups was determined by the Students' ("pooled") t-test, except in experiments V and VI where Duncan's Multiple Range Test was used subsequent to Analysis of Variance (Steel and Torrie, 1960).

Determination of Adrenocortical Response

ACTH Stimulation

Exogenous ACTH stimulation of the adrenal cortex was conducted similar to the procedures of Moncloa, Peron and Dorfman (1959). Subcutaneous ACTH injections (1-2 units/100 g body wt) were found to produce a maximal increase in plasma corticosterone concentration 60-70 minutes after ACTH (Depo-ACTH, The Upjohn Company, Kalamazoo, Michigan) administration. Rats were lightly anesthetized with diethyl ether (Mallinckrodt Chemical Works, St. Louis, Missouri) immediately before bleeding.

Stress

Exposure to ether vapors is a recognized adrenocortical stressing agent in the rat (Zimmerman and Critchlow, 1967). Duration of ether exposure was employed as a standardizing criterion in these experiments. In order to insure uniform maximal stimulation of the pituitary-adrenal axis experimental animals were exposed to 40 minutes of periodic ether vapor exposure. (The rats were anesthetized at 40 minutes, 10 minutes and again just before blood sampling.)

Blood Collection

Peripheral blood samples (0.5 to 1.5 ml whole blood) were collected into dry heparinized beakers by orbital sinus puncture with a capillary tube in lightly anesthetized rats. The red blood cells were centrifuged out and plasma samples collected as soon after bleeding as possible. Plasma samples not subjected to corticosterone extraction following centrifugation were stored at -15° C until they could be assayed.

Resting Corticosterone Levels

Rats were rapidly anesthetized with ether so that blood samples could be obtained by orbital sinus puncture within two minutes or less from the time the animal was initially disturbed in its cage.

Fluorometric Assay of Plasma Corticosterone

Corticosterone concentration in rat plasma was measured by the fluorometric assay procedure of DeMoor and Steeno (1963). Fluorescence was measured with a Turner #110 fluorometer equipped with narrow band, high transmittancy filters (excitation wave length 470 mμ, emission wave length 530 mμ). Corticosterone was measured in duplicate plasma samples of different volumes (normally 0.1 and 0.2 ml plasma). The corticosterone concentration of each plasma sample was calculated by comparing its fluorescence intensity to that of corticosterone standards run concurrently with each group of plasma samples.

The reliability of the fluorometric technique for corticosterone quantitation in rat plasma was tested by measuring the plasma corticosterone concentration of a pooled plasma sample by both the simple fluorometric assay and the colorimetric blue tetrazolium reaction. The blue tetrazolium technique of Elliott *et al*. (1954) was used following purification and chromatographic separation of corticosterone as described by Riegle and Nellor (1967).

The validity of the fluorometric technique for measurement of plasma corticosterone in plasma from adrenocortically stimulated rats was verified by the close agreement of the results of the two methods (plasma cortociosterone concentration from the fluorometric analysis, 32.4 ± 0.6 μg/100 ml; and from the blue tetrazolium reaction, 32.0 μg/100 ml). The precision of the fluorometric technique was ascertained in the following manner. A total of 21 samples (0.2 ml each) from a pooled plasma supply were assayed fluorometrically with a variation (standard error of the mean) of 0.40 for the series (58.1 ± 0.40 μg/100 ml).

H^3-Corticosterone Disappearance from Plasma

Procedures similar to those of Glenister and Yates (1961) and Saroff and Wexler (1969) for measuring distribution volume, biological half-life and production rate of corticosterone in the rat were followed in measuring these same parameters in this study. Serial blood samples taken at regular intervals following intravenous injection of radioactive corticosterone were used to calculate a disappearance curve for plasma radioactivity with increasing time.

Corticosterone -1, 2-H^3 (New England Nuclear Corp.) (1.00 millicurie, 0.0115 milligram in 1 ml ethanol-benzene) was diluted to a total volume of 5 ml and served as the labelled hormone used in these studies.

Adequate volumes of this diluted isotopic corticosterone were added to a solution of unlabelled corticosterone (200 μg/ml) in saline (0.9%) to provide final concentrations of 1.2 or 2.0 μC/ml. The volume injected into each rat was 0.25 ml (0.3 or 0.5 μC H^3-corticosterone, 50 μg of unlabelled corticosterone).

Rats were anesthetized with Nembutal approximately 10-15 minutes before isotope injection (3.5 mg/100 body wt injected ip). The femoral vein injections (0.25 ml) were facilitated by small skin incisions over the injection region which were closed with surgical clips following injections. A 15 minute equilibration period was allowed between isotope injection and the start of blood sampling.

Blood samples of 0.5 to 0.75 ml were collected by the orbital sinus route at 15, 20, 25, 30 and 35 minutes post-injection in male rats. Because of the more rapid plasma corticosterone disappearance rate in female rats blood samples were collected by the orbital sinus route at 15, 19, 23, 27 and 31 minutes post-injection. Plasma was separated by centrifugation and 0.1/ml fresh plasma samples were prepared for methylene chloride extraction. The remaining plasma was stored at -15° C until assayed fluorometrically for corticosterone concentration. Methylene chloride extraction and alkali wash of plasma samples were conducted by the same procedure used in fluorometric corticosterone determinations. A 4 ml aliquot of the methylene chloride extract (5 ml original volume) was transferred to a scintillation vial and the contents evaporated under a constant air flow at room temperature. Following addition of 10 ml of a toluene based counting solution the radioactivity was counted in a Nuclear Chicago Mark I liquid scintillation spectrometer.

In order to confirm that plasma radioactivity was associated with corticosterone, 0.1 ml plasma samples were obtained from an adult female rat 15 minutes after injection of a large dose (15 μC in 0.25 ml) of H^3-corticosterone. Following chromatographic isolation of corticosterone according to methods described by Riegle and Nellor (1967), the percentage of total radioactivity eluted from the chromatogram associated with corticosterone was determined.

Calculations for parameters of adrenal secretory dynamics were patterned after those used by Saroff and Wexler (1969) and reviewed in detail by Tait and Burstein (1964), for the single compartment model. The single compartment model was assumed to adequately represent adrenal secretory dynamics for the purposes of the present studies.

The least squares line for (natural) log CPM (5 points/animal) with increasing time was calculated for the results from each rat. Extrapolating this curve to zero time, one obtained the volume of distribution, provided mixing had occurred instantaneously (the intercept of this line on the ordinate represented log CPM/0.1 ml plasma, from which the value of CPM/ml plasma was readily obtained).

Using dilution techniques, the appropriate volume was calculated.

$$\text{distribution volume (ml)} = \frac{\text{Total CPM injected}}{\text{CPM/ml at zero time}}$$

The slope of this least squares line (disappearance curve) was used to calculate corticosterone turnover rate (biological half-life):

$$t_{\frac{1}{2}} \text{ (min)} = \frac{.69315}{\text{slope}}$$

Metabolic Clearance Rate (M.C.R.), the volume of plasma completely cleared of corticosterone per unit time, was calculated for subsequent use in calculating corticosterone production rate.

$$\text{M.C.R. (ml plasma/min)} = \frac{\text{Total CPM's injected x slope}}{\text{CPM/ml plasma}}$$

Production Rate (P.R.) of corticosterone, the amount of corticosterone required per unit time to maintain plasma corticosterone levels (assuming steady state conditions), was calculated in the following manner:

$$\text{P.R. } (\mu\text{g/min}) = \text{M.C.R. x plasma corticosterone concentration}$$

The corticosterone concentration of each serial blood sample was determined fluorometrically. Average corticosterone concentration (μg/100 ml plasma) of individual animals during the blood sampling period was used in calculating P.R. values.

EXPERIMENTAL

Experiment I: Effects of Chronic Adrenocortical Stimulation in Young and Old Male Rats

Materials and Methods

The purpose of this experiment was to study the effects of chronic adrenocortical stimulation on the ability of rats to respond to subsequent exogenous ACTH or stress. Interest in these studies was in two areas: (1) the capacity of chronically stimulated adrenal cortices of young and old rats to respond to exogenous ACTH, subsequent to chronic stimulation and (2) the effect of continuous elevation of endogenous corticosterone on the ability of stressors to mobilize hypothalamic-pituitary ACTH release. Plasma corticosterone levels following adrenocortical stimulation were used as an index of adrenocortical responsiveness.

The experimental scheme used to test these objectives is shown in Figure 1. Five month and 26 month old male rats were grouped into treated and control groups, the former receiving daily injections of a depo-ACTH preparation while controls received daily injections of the gelatin vehicle alone. During the first 2 weeks of treatment all treated rats received 10 U ACTH/day in 15% gelatin. During the last 4 weeks of treatment the rats received 2 U ACTH/100 g B.W./day in order to compensate for differences in body weight among the experimental subjects. In most animals this dosage amounted to 10 U ACTH/day. Adrenocortical response was tested at five intervals throughout the course of the experiment: prior to the start of chronic ACTH treatment, after 2 weeks, 4 weeks and 6 weeks of chronic ACTH treatment and after 2 weeks recovery from chronic ACTH treatment (Figure 1).

Two types of adrenocortical responses were tested at each interval of the experiment: response to a standardized ether stress procedure and response to levels of exogenous ACTH adequate to maximally stimulate adrenocortical steroidogenesis. The latter parameter will be referred to as acute ACTH stimulation to distinguish it from depo-ACTH treatment. Plasma samples were obtained by orbital sinus puncture and corticosterone levels were determined fluorometrically. Individual body weights of experimental animals were recorded at each bleeding interval. Adrenocortical response differences between depo-ACTH treated and control rats of the same age, sex and mode of acute adrenocortical stimulation at each interval of the experiment were statistically analyzed by Student's t-test (Steel & Torrie, 1960).

Results

Adrenocortical responses to acute ACTH stimulation in old male rats receiving daily depo-ACTH injections are presented in Table 1 and Figure 2. Adrenocortical response is represented as the mean plasma corticosterone level $\pm$ S.E.M. Only after 6 weeks of chronic ACTH stimulation did treated old rat response differ ($p < .05$) from control group old rat response.

The effect of chronic adrenocortical stimulation on adrenocortical responses of young male rats to acute ACTH stimulation is presented in Table 1 and Figure 3. Although there was no difference between treated and control responses initially, there was a significant increase of response in treated young rats over that of control young rats after 2 weeks ($p < .05$), 4 weeks ($p < .01$) and 6 weeks ($p < .01$) of chronic ACTH treatment. Response of treated young rats did not differ from that of non-treated young controls after a 2 week recovery period. Adrenocortical responses of depo-ACTH treated young male rats to acute ACTH stimulation were significantly higher than those of depo-ACTH treated old male rats to acute ACTH stimulation after 2 weeks ($p < .01$) and 4 weeks ($p < .01$) of depo-ACTH treatment.

The ether stress response of both treated and control old male rats receiving chronic depo-ACTH injections is shown in Table 1 and Figure 4. The ether response of treated old rats did not differ from that of control old rats at any interval of the experiment. This is in contrast to the ether stress response of young rats (Table 1 and Figure 5). The ether stress response of young treated rats was significantly lower than young control group response after 2 weeks ($p < .01$), 4 weeks ($p < .05$) and 6 weeks ($p < .01$) of chronic ACTH treatment. After 2 weeks recovery, treated and control group responses were not significantly different.

In a composite representation (Figure 6), this data is presented as change of treated response from control response at each interval of the experiment. Change is represented as μg corticosterone/100 ml plasma above or below control response.

Group body weight data (mean ± S.E.M.) for each interval of the experiment is presented in Table 2. Although mean body weight of both young and old depo-ACTH treated rats dropped during the 6 weeks of treatment, these changes were not significant in either young or old rats.

Figure 1. Scheme of chronic ACTH experiment.

Young and old rats were each divided into treated and control groups. Adrenocortical response (plasma corticosterone levels) to ether stress (determined between 9:00 AM and noon) and to acute ACTH stimulation (determined between 2:00 and 4:00 PM) was determined at each interval of the experiment. These intervals were before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, 4 weeks and 6 weeks of depo-ACTH treatment and after 2 weeks recovery from depo-ACTH treatment.

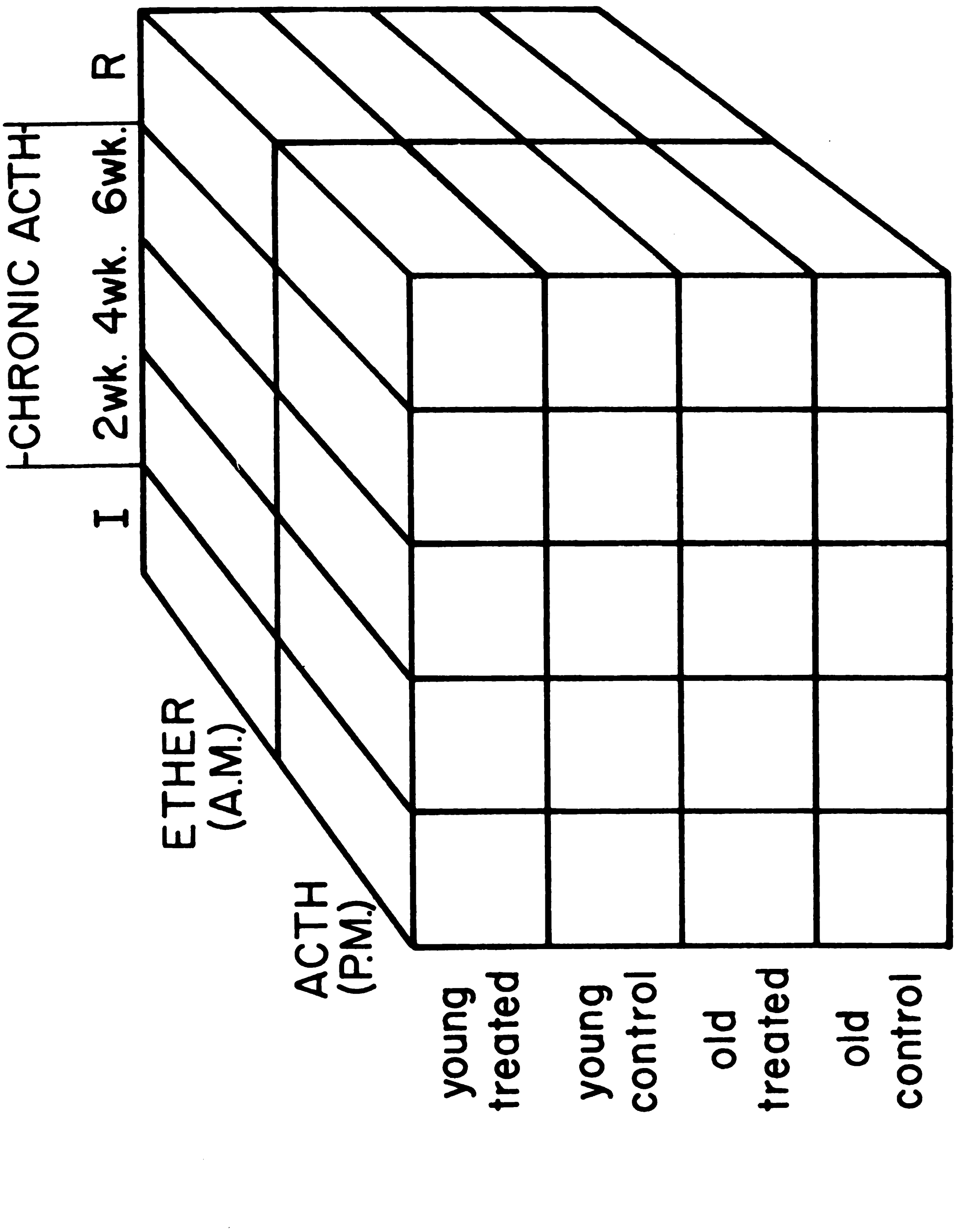

CHRONIC ACTH
I
2wk.
4wk.
6wk.
R
ETHER (A.M.)
ACTH (P.M.)
young treated
young control
old treated
old control

Figure 1

Figure 2. Adrenocortical responses of depo-ACTH treated and control old male rats to acute ACTH stimulation.

This data represents adrenocortical responses of old (26 month) male rats to acute ACTH stimulation. All rats received a saline based ACTH injection (2 U/100μg body weight) 70 minutes prior to orbital sinus blood sampling. Fluorometrically determined plasma corticosterone levels (μg/100 ml) were used as an index of adrenocortical responsiveness. Depo-ACTH treated rats received subcutaneous injections (10 U daily) of ACTH in 15% gelatin for a period of 6 weeks, during which time controls received injections of gelatin alone. Adrenocortical responses were determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, after 4 weeks and after 6 weeks of depo-ACTH treatment and after 2 weeks recovery from depo-ACTH treatment.

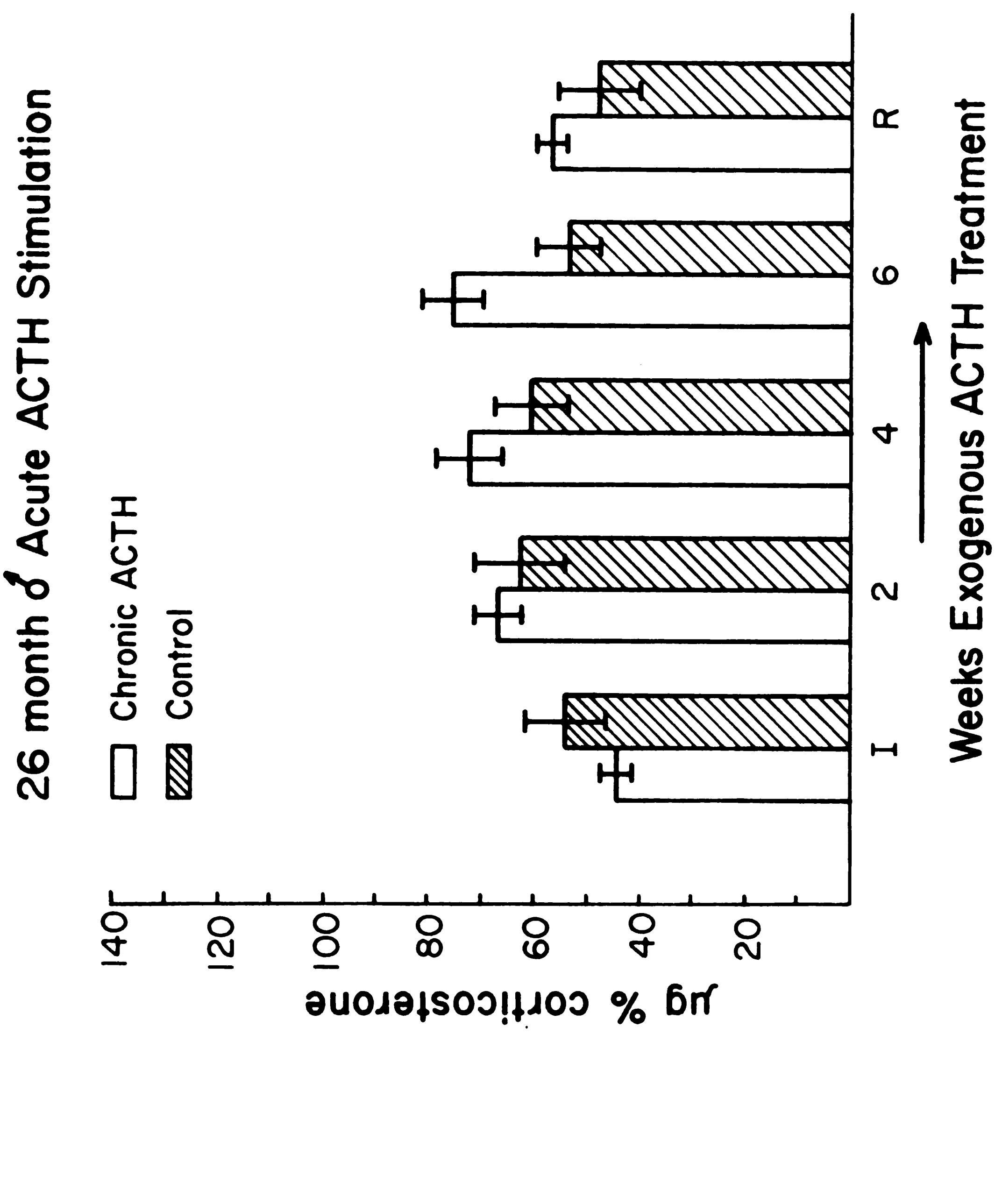

26 month ♂ Acute ACTH Stimulation
Chronic ACTH
Control
µg % corticosterone
140
120
100
80
60
40
20
I
2
4
6
R
Weeks Exogenous ACTH Treatment

Figure 2

Figure 3. Adrenocortical responses of depo-ACTH treated and control young male rats to acute ACTH stimulation.

This data represents adrenocortical responses to acute ACTH stimulation in young (5 month) male rats. All rats received a saline based ACTH injection (2 U/100 g body weight) 70 minutes prior to orbital sinus blood sampling. Fluorometrically determined plasma corticosterone levels ($\mu g/100$ ml) were used as an index of adrenocortical responsiveness. Depo-ACTH treated rats received subcutaneous injections (10 U daily) of ACTH in 15% gelatin for a period of 6 weeks, during which controls received injections of gelatin alone. Adrenocortical responses were determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, after 4 weeks and after 6 weeks of depo-ACTH treatment and after 2 weeks recovery from depo-ACTH treatment.

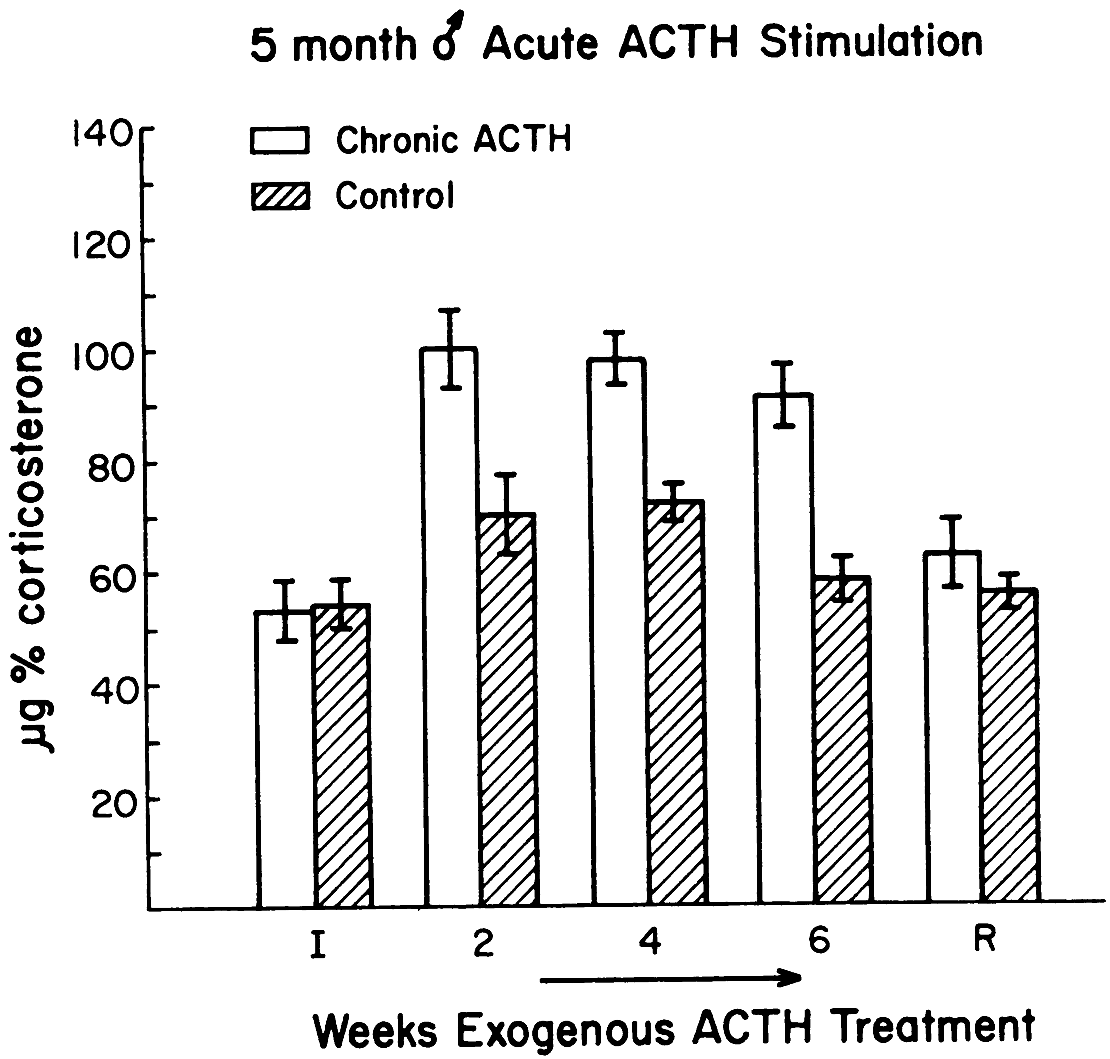

5 month ♂ Acute ACTH Stimulation
Chronic ACTH
Control
140
120
100
80
60
40
20
µg % corticosterone
I
2
4
6
R
Weeks Exogenous ACTH Treatment

Figure 3

Figure 4. Adrenocortical responses of depo-ACTH treated and control old male rats to ether stress.

This data represents adrenocortical responses to ether stress in old (26 month) male rats. Rats were exposed to ether anesthetization 40 minutes, 10 minutes and just prior to orbital sinus blood sampling. Fluorometrically determined plasma corticosterone levels (μg/100 ml) were used as an index of adrenocortical response. Depo-ACTH treated rats received subcutaneous injections (10 U daily) of ACTH in 15% gelatin for a period of 6 weeks, during which time controls received injections of gelatin alone. Adrenocortical responses were determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, after 4 weeks and after 6 weeks of depo-ACTH treatment and after 2 weeks recovery from depo-ACTH treatment.

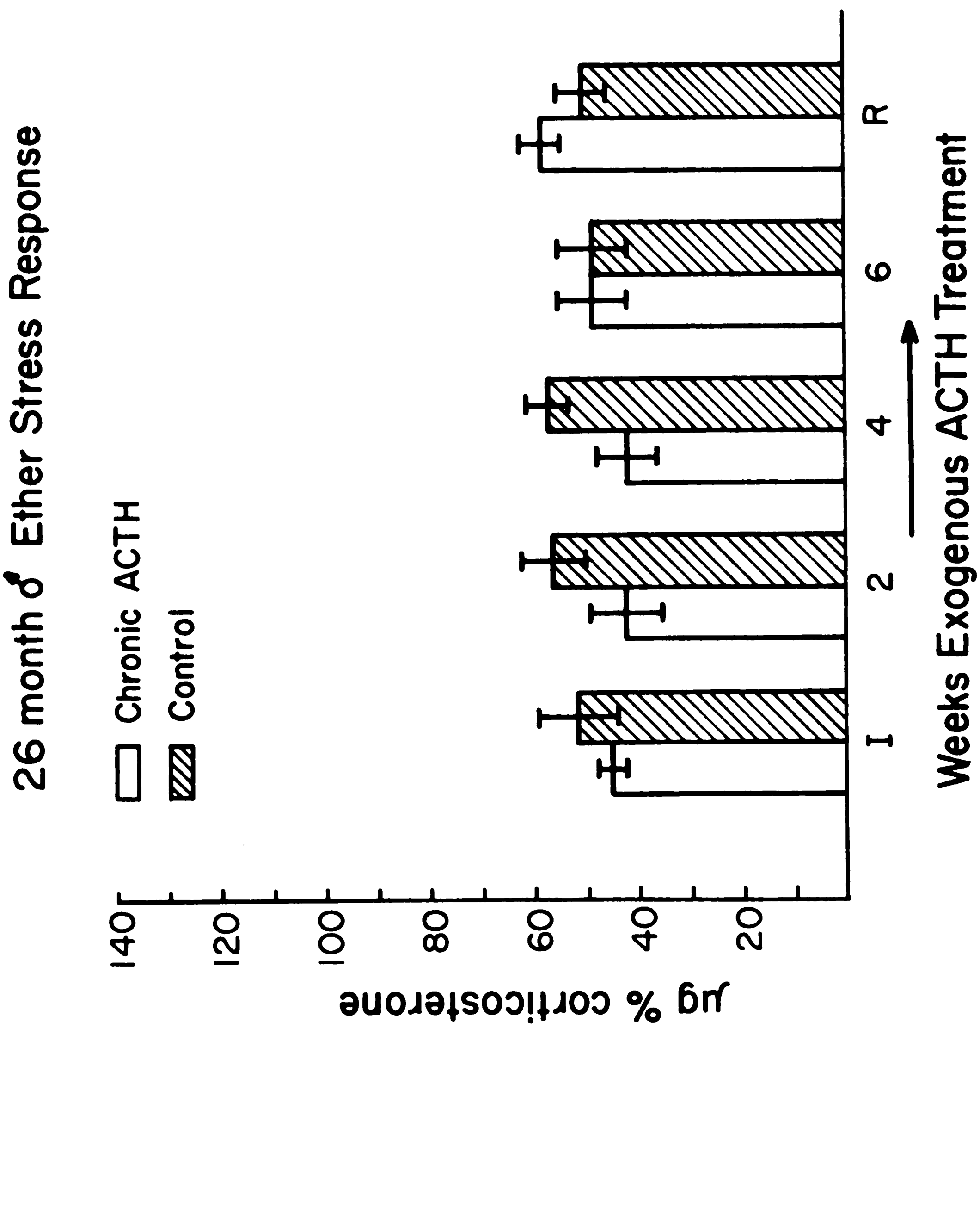

26 month ♂ Ether Stress Response
Chronic ACTH
Control
μg % corticosterone
140
120
100
80
60
40
20
I
2
4
6
R
Weeks Exogenous ACTH Treatment

Figure 4

Figure 5. Adrenocortical responses of depo-ACTH treated and control young male rats to ether stress.

This data represents adrenocortical responses to ether stress in young (5 month) male rats. Rats were exposed to ether anesthetization 40 minutes, 10 minutes and just prior to orbital sinus bleeding. Fluorometrically determined plasma corticosterone levels (μg/100 ml) were used as an index of adrenocortical response. Depo-ACTH treated rats received subcutaneous injections (10 U daily) of ACTH in 15% gelatin for a period of 6 weeks, during which controls received injections of gelatin alone. Adrenocortical response was determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, after 4 weeks and 6 weeks of depo-ACTH treatment and after a 2 week recovery from depo-ACTH treatment.

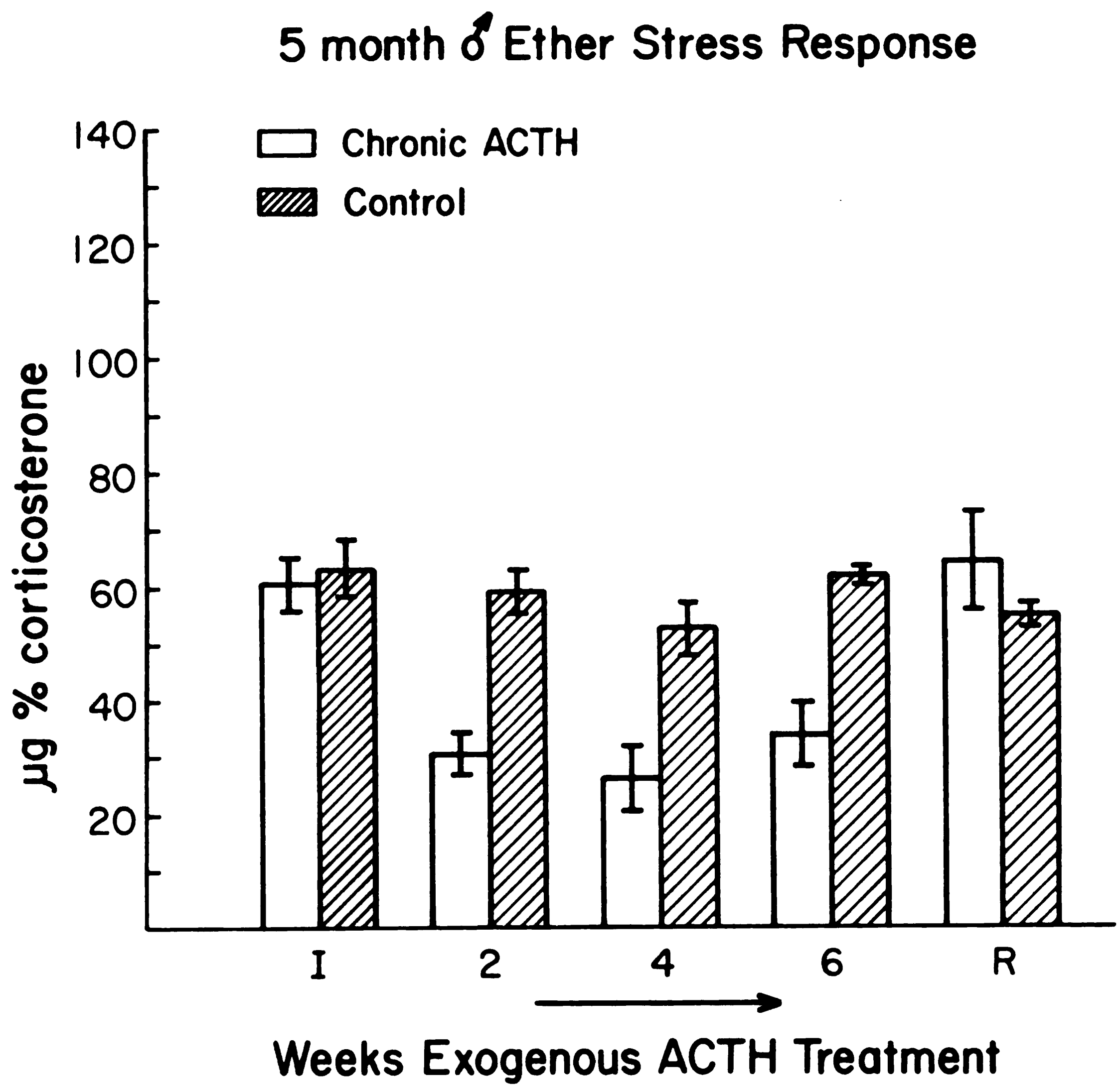

5 month ♂ Ether Stress Response
Chronic ACTH
Control
μg % corticosterone
140
120
100
80
60
40
20
I
2
4
6
R
Weeks Exogenous ACTH Treatment

Figure 5

Figure 6. Effect of chronic ACTH treatment on adrenocortical response to ether stress and acute ACTH stimulation in young and old male rats.

Adrenocortical responses (plasma corticosterone levels in μg/100 ml) of depo-ACTH treated young and old male rats are presented as change from the comparable control response at each interval of the experiment. Responses to both ether stress and acute ACTH stimulation determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, 4 weeks and 6 weeks of depo-ACTH treatment and after 2 week recovery period from depo-ACTH treatment are presented here. Depo-ACTH treatment consisted of daily injections of ACTH (10 U/rat) in a 15% gelatin preparation. Ether stress response consisted of ether anesthetization 40 minutes, 10 minutes and just prior to orbital sinus blood sampling while acute ACTH stimulation consisted of exogenous ACTH administration (2 U/100 g body weight) 70 minutes before orbital sinus blood sampling. Young rats averaged 5 months and old rats averaged 26 months of age at the start of the experiment.

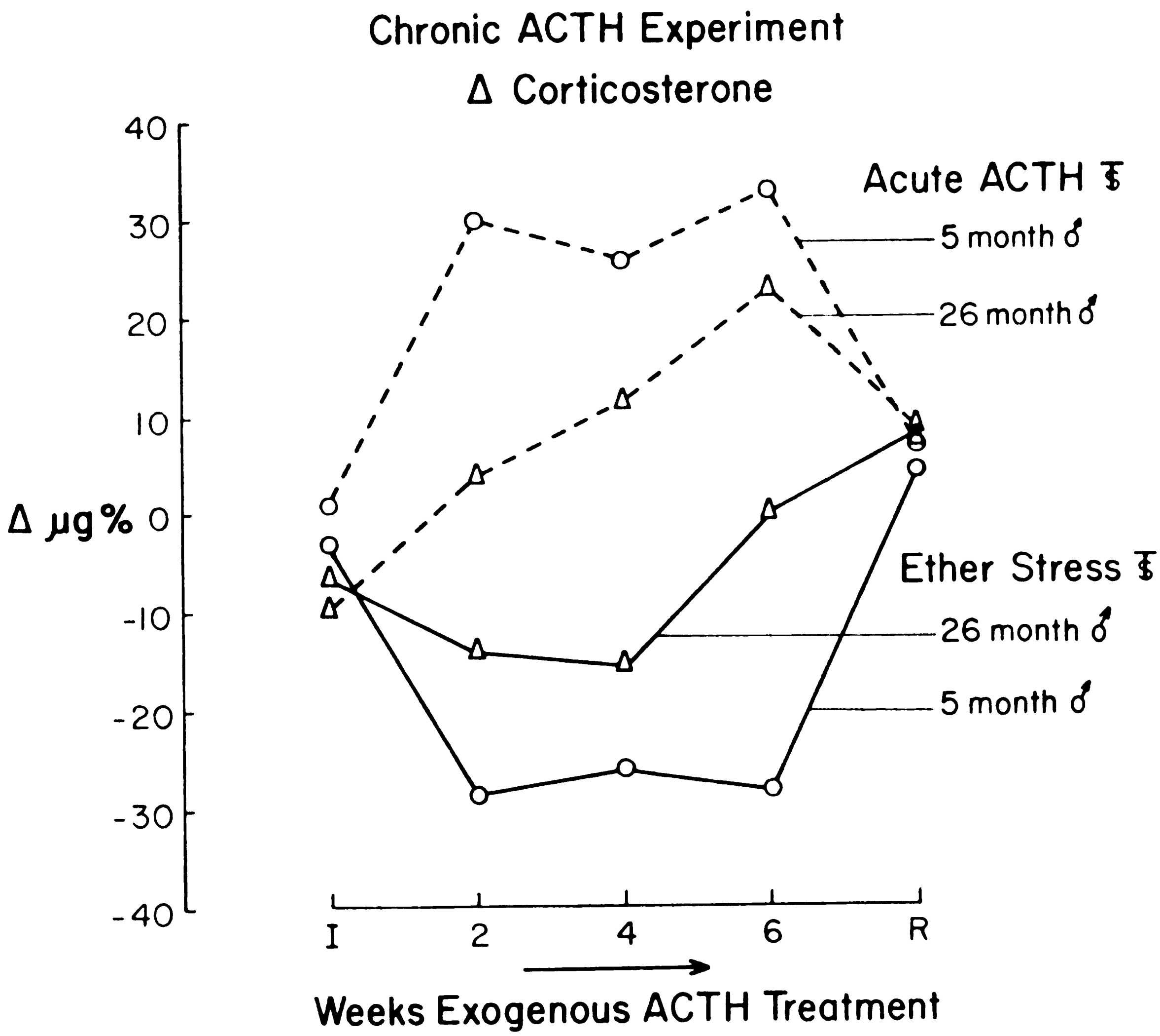

Chronic ACTH Experiment
Δ Corticosterone
40
30
20
10
Δ µg% 0
-10
-20
-30
-40
Acute ACTH
5 month ♂
26 month ♂
Ether Stress
26 month ♂
5 month ♂
I
2
4
6
R
Weeks Exogenous ACTH Treatment

Figure 6

Table 1. Adrenocortical responsiveness[1] to stress and ACTH stimulation in young and old male rats

Age	Stimulus	Treatment Group	n	Before Treatment	Weeks of ACTH Treatment[2] 2	4	6	Recovery
Young	ether[3]	depo-ACTH	6	60.4 ± 4.8	30.6 ± 3.7**	26.3 ± 5.8*	34.2 ± 5.5**	64.5 ± 8.6
		control	4	63.2 ± 4.8	59.2 ± 3.8	52.4 ± 5.0	62.1 ± 1.7	54.9 ± 2.1
	ACTH[4]	depo-ACTH	6	53.2 ± 5.3	100.0 ± 7.0*[a]	98.1 ± 4.7**[b]	91.2 ± 5.7**	62.7 ± 6.2
		control	4	54.3 ± 4.3	70.2 ± 7.0	72.1 ± 3.3	58.3 ± 3.9	55.7 ± 2.8
Old	ether[3]	depo-ACTH	6	45.1 ± 2.7	42.3 ± 6.8	42.0 ± 5.7	48.5 ± 6.7	58.3 ± 3.9
		control	4	51.7 ± 7.6	56.2 ± 6.0	57.3 ± 3.9	48.5 ± 6.5	50.3 ± 4.7
	ACTH[4]	depo-ACTH	6	44.3 ± 2.9	66.6 ± 4.5[a]	72.2 ± 6.4[b]	75.4 ± 5.7*	56.9 ± 2.6
		control	4	53.9 ± 7.6	62.7 ± 8.7	60.7 ± 6.9	53.6 ± 6.2	48.0 ± 7.7

[1] μg corticosterone/100 ml plasma presented as group mean ± S.E.M.

[2] Treated rats received 10 units ACTH subcutaneously in gelatin/day, control rats received only gelatin.

[3] Ether vapor exposure at 40 minutes before, 10 minutes before and at the time of bleeding.

[4] ACTH (2 U/100 g) in saline injected subcutaneously before blood samples.

*$p < .05$
**$p < .01$ depo-ACTH response and control response differ for same stimulus in same age group.

[a] Means having same superscript differ, $p < .01$.

[b] Means having same superscript differ, $p < .05$.

Table 2. Body weight[1] of young and old male rats at bleeding intervals of chronic ACTH experiment

Age	Treatment Group	Before Treatment	Weeks of ACTH Treatment[2] 2	4	6	Recovery
Young	depo-ACTH treated	438 ± 18	403 ± 16	397 ± 14	397 ± 12	435 ± 17
	control	395 ± 20	386 ± 16	391 ± 14	394 ± 18	410 ± 18
Old	depo-ACTH treated	483 ± 22	448 ± 23	429 ± 22	416 ± 22	420 ± 22
	control	521 ± 49	493 ± 46	484 ± 52	471 ± 57	520 ± 91

[1]Group body weight (in grams) ± S.E.M.

[2]Treated rats received 10 units ACTH in gelatin daily, control rats received only gelatin.

Experiment II: Effects of Chronic Adrenocortical Stimulation in Young and Old Female Rats

Materials and Methods

The objective and procedures followed in Experiment II were similar to those of Experiment I except that 3 month and 22 month old female rats were used as experimental subjects. Female rats received daily injections of 6 U ACTH in 15% gelatin throughout the 6 week treatment period.

Results

Adrenocortical responses of both chronic ACTH treated and control old female rats to acute ACTH stimulation at each interval of the experiment are presented in Table 3 and Figure 7. Adrenocortical response is represented as the mean plasma corticosterone level $\pm$ S.E.M. Adrenocortical response of chronic ACTH treated old rats to acute ACTH stimulation was significantly higher ($p < .05$) than that of non-treated old controls after 4 weeks and 6 weeks of chronic ACTH treatment. The adrenocortical response of chronic ACTH treated and control young female rats to acute ACTH stimulation is presented in Table 3 and Figure 8. Only after 6 weeks of chronic ACTH treatment was the adrenocortical response of chronic ACTH treated young rats to acute ACTH stimulation higher ($p < .01$) than that of non-treated young controls. Adrenocortical response of depo-ACTH treated young female rats to acute ACTH stimulation was significantly higher than that of depo-ACTH treated old female rats to acute ACTH stimulation after 6 weeks ($p < .05$) of depo-ACTH treatment.

The ether stress response of both chronic ACTH treated and control old female rats is given in Table 3 and Figure 9. After 6 weeks of treatment, the ether stress response of chronic ACTH treated old animals was significantly higher ($p < .05$) than that of old controls. By contrast, the ether stress response of chronic ACTH treated young female rats (Table 3 and Figure 10) was significantly lower ($p < .01$) than young control response after 2 weeks, 4 weeks and 6 weeks of chronic ACTH treatment. After 2 weeks recovery, treated and control responses to ether stress were not different in either young or old female rats.

Figure 11 is a composite representation of this data, in which the data is presented as change of treated response from control response at each interval of the experiment. Change is represented as μg corticosterone/100 ml plasma above or below control response.

Group body weight data (mean ± S.E.M.) for each interval of the experiment is presented in Table 4. Body weight changes of treated animals closely parallel those of non-treated controls for both age groups.

Figure 7. Adrenocortical responses of depo-ACTH treated and control old female rats to acute ACTH stimulation.

This data represents adrenococortical responses of old (22 month) female rats to acute ACTH stimulation. All rats received a saline based ACTH injection (1 U/100 g body wt) 1 hour prior to orbital sinus blood sampling. Fluorometrically determined plasma corticosterone levels (μg/100 ml) were used as an index of adrenocortical responsiveness. Depo-ACTH treated rats received subcutaneous injections (6 U daily) of ACTH in gelatin for a period of 6 weeks, during which time controls received injections of gelatin alone. Adrenocortical responses were determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, after 4 weeks and after 6 weeks of depo-ACTH treatment and after 2 weeks recovery from depo-ACTH treatment.

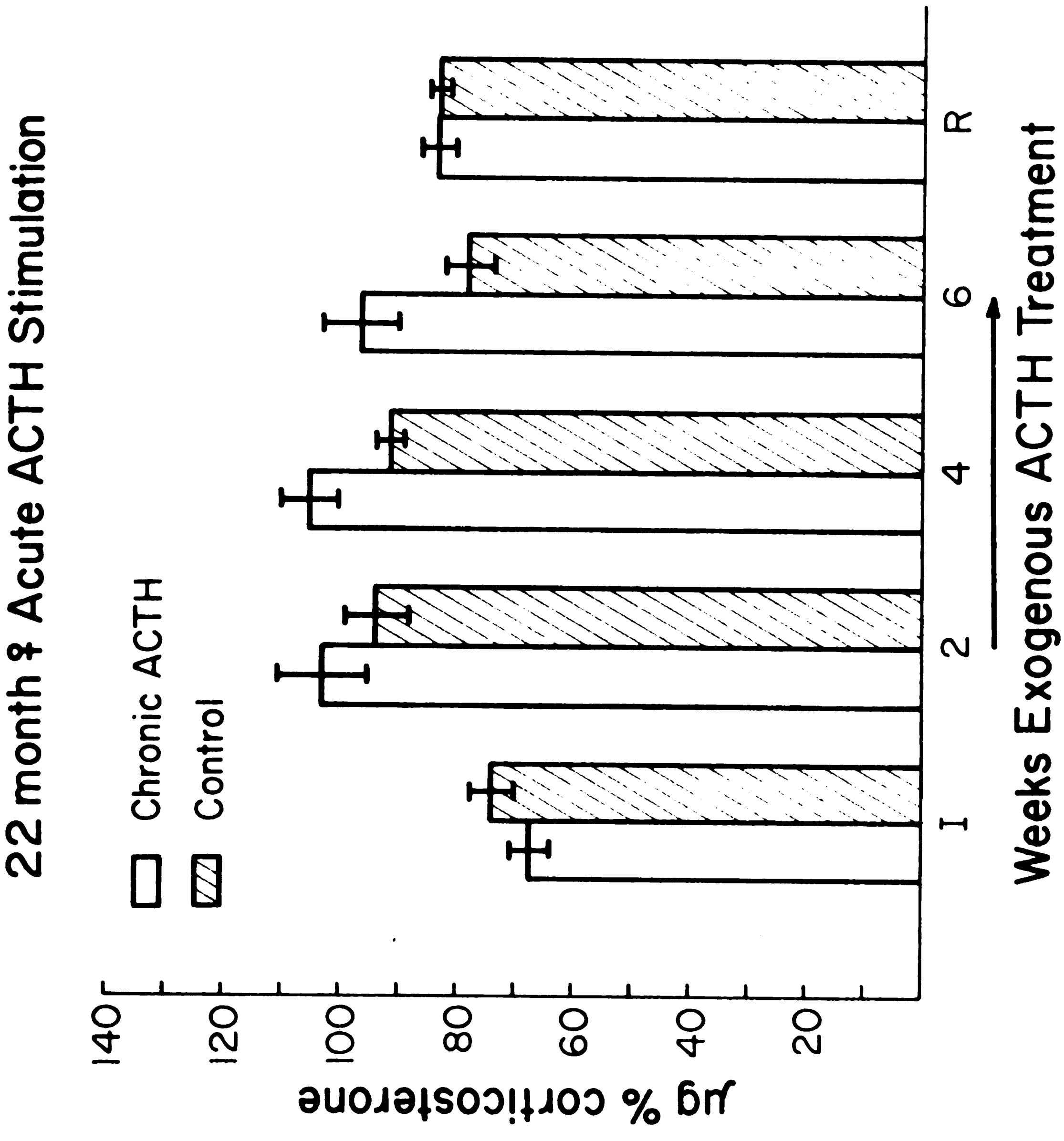

22 month ♀ Acute ACTH Stimulation
Chronic ACTH
Control
µg % corticosterone
140
120
100
80
60
40
20
I
2
4
6
R
Weeks Exogenous ACTH Treatment

Figure 7

Figure 8. Adrenocortical responses of depo-ACTH treated and control young female rats to acute ACTH stimulation.

This data represents adrenocortical responses of young (3 month) female rats to acute ACTH stimulation. All rats received a saline based ACTH injection (1 U/100 g body wt) 1 hour prior to orbital sinus blood sampling. Fluorometrically determined plasma corticosterone levels (μg/100 ml) were used as an index of adrenocortical responsiveness. Depo-ACTH treated rats received subcutaneous injections (6 U daily) of ACTH in 15% gelatin for a period of 6 weeks, during which time controls received injections of gelatin alone. Adrenocortical responses were determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, after 4 weeks and after 6 weeks of depo-ACTH treatment and after 2 weeks recovery from depo-ACTH treatment.

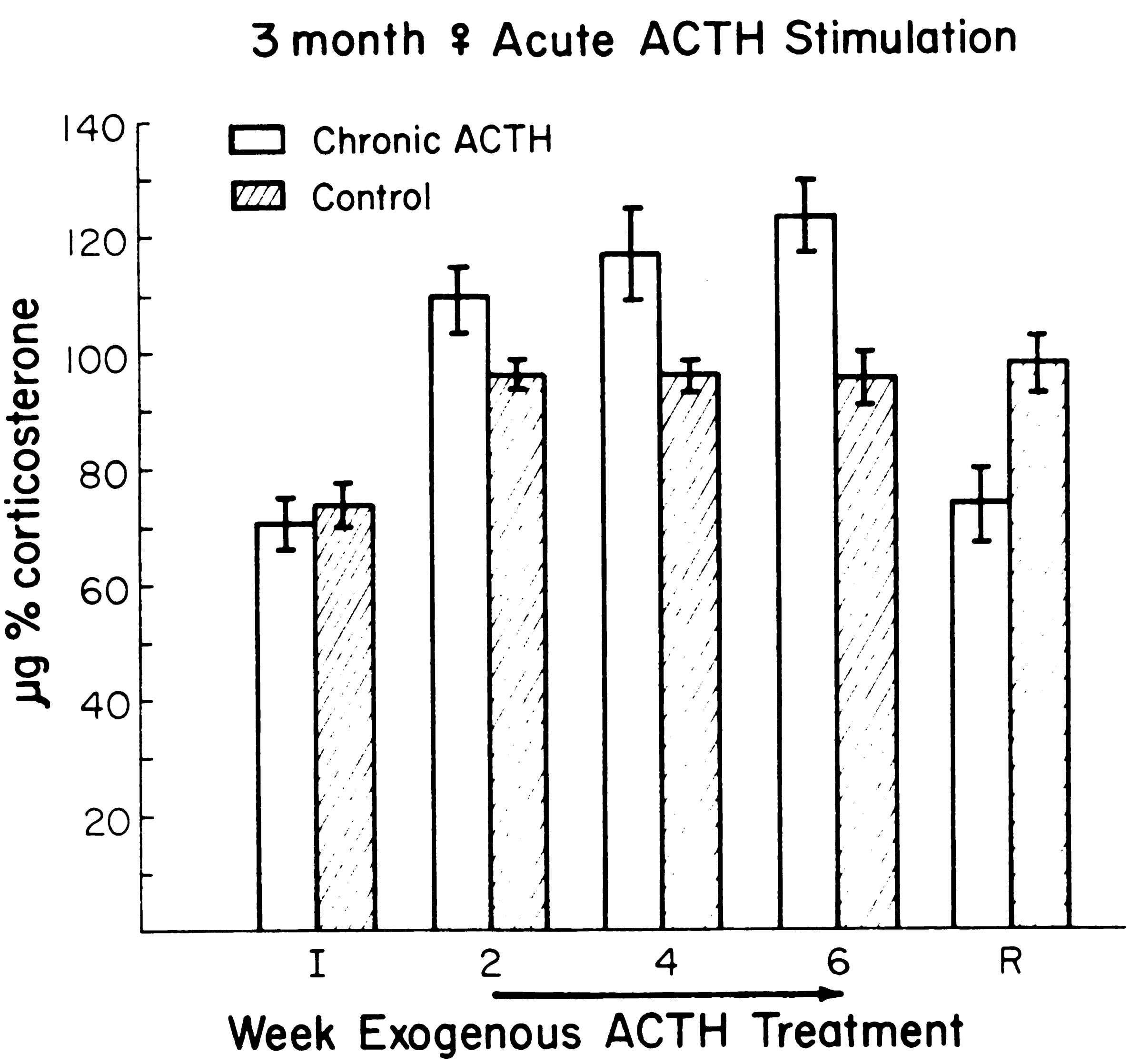

3 month ♀ Acute ACTH Stimulation
Chronic ACTH
Control
140
120
100
80
60
40
20
μg % corticosterone
I
2
4
6
R
Week Exogenous ACTH Treatment

Figure 8

Figure 9. Adrenocortical responses of depo-ACTH treated and control old female rats to ether stress.

This data represents adrenocortical responses to ether stress in old (22 month) female rats. Rats were exposed to ether anesthetization 40 minutes, 10 minutes and just prior to orbital sinus blood sampling. Fluorometrically determined plasma corticosterone levels ($\mu g/100$ ml) were used as an index of adrenocortical response. Depo-ACTH treated rats received subcutaneous injections (6 U daily) of ACTH in 15% gelatin for a period of 6 weeks, during which time controls received injections of gelatin alone. Adrenocortical responses were determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, after 4 weeks and after 6 weeks of depo-ACTH treatment and after 2 weeks recovery from depo-ACTH treatment.

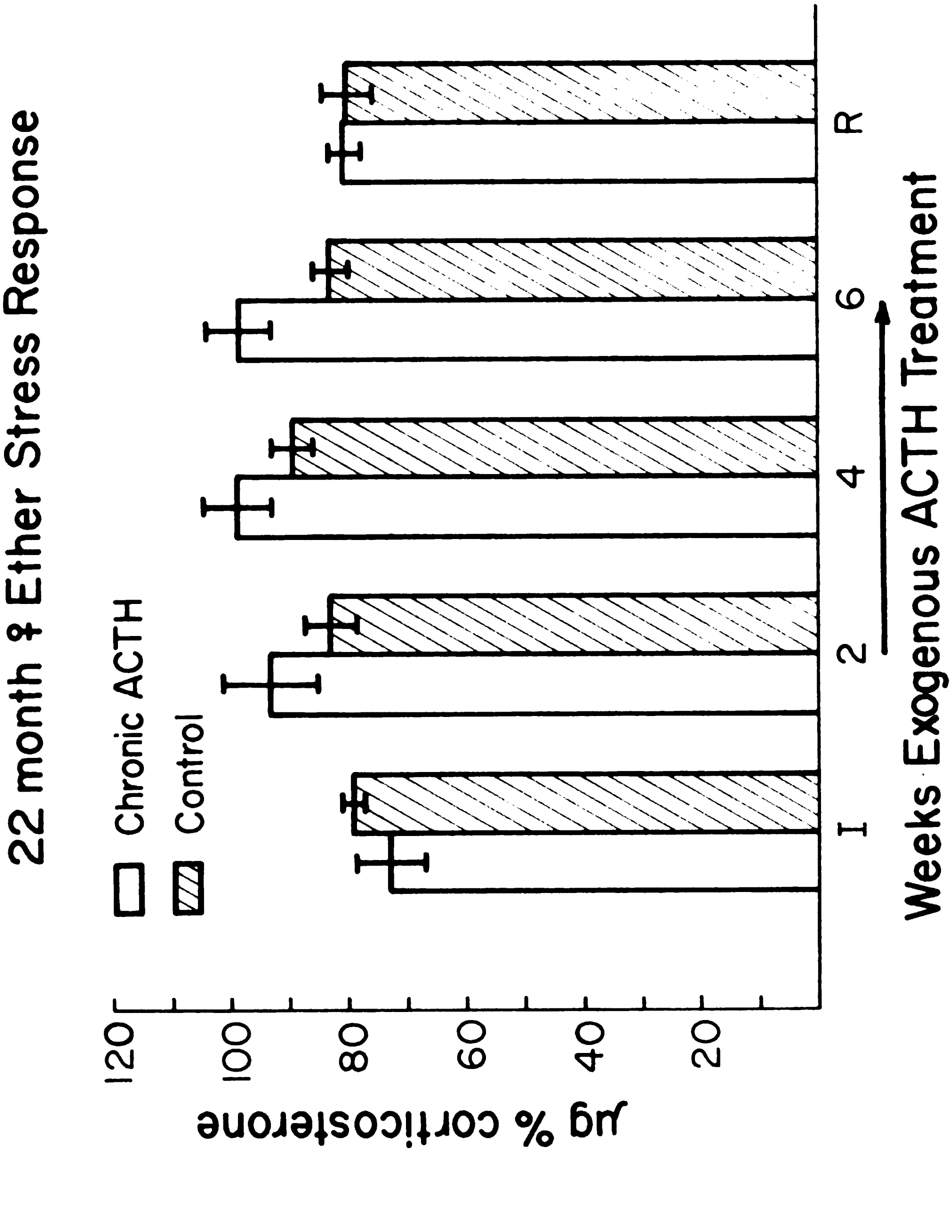

22 month ♀ Ether Stress Response
Chronic ACTH
Control
µg % corticosterone
120
100
80
60
40
20
I
2
4
6
R
Weeks Exogenous ACTH Treatment

Figure 9

Figure 10. Adrenocortical responses of depo-ACTH treated and control young female rats to ether stress.

This data represents adrenocortical responses to ether stress in young (3 month) female rats. Rats were exposed to ether anesthetization 40 minutes, 10 minutes and just prior to orbital sinus blood sampling. Fluorometrically determined plasma corticosterone levels ($\mu g/100$ ml) were used as an index of adrenocortical response. Depo-ACTH treated rats received subcutaneous injections (6 U daily) of ACTH in 15% gelatin for a period of 6 weeks, during which time controls received injections of gelatin alone. Adrenocortical responses were determined before the start of depo-ACTH treatment (designated as I on graph), after 2 weeks, after 4 weeks and after 6 weeks of depo-ACTH treatment and after 2 weeks recovery from depo-ACTH treatment.

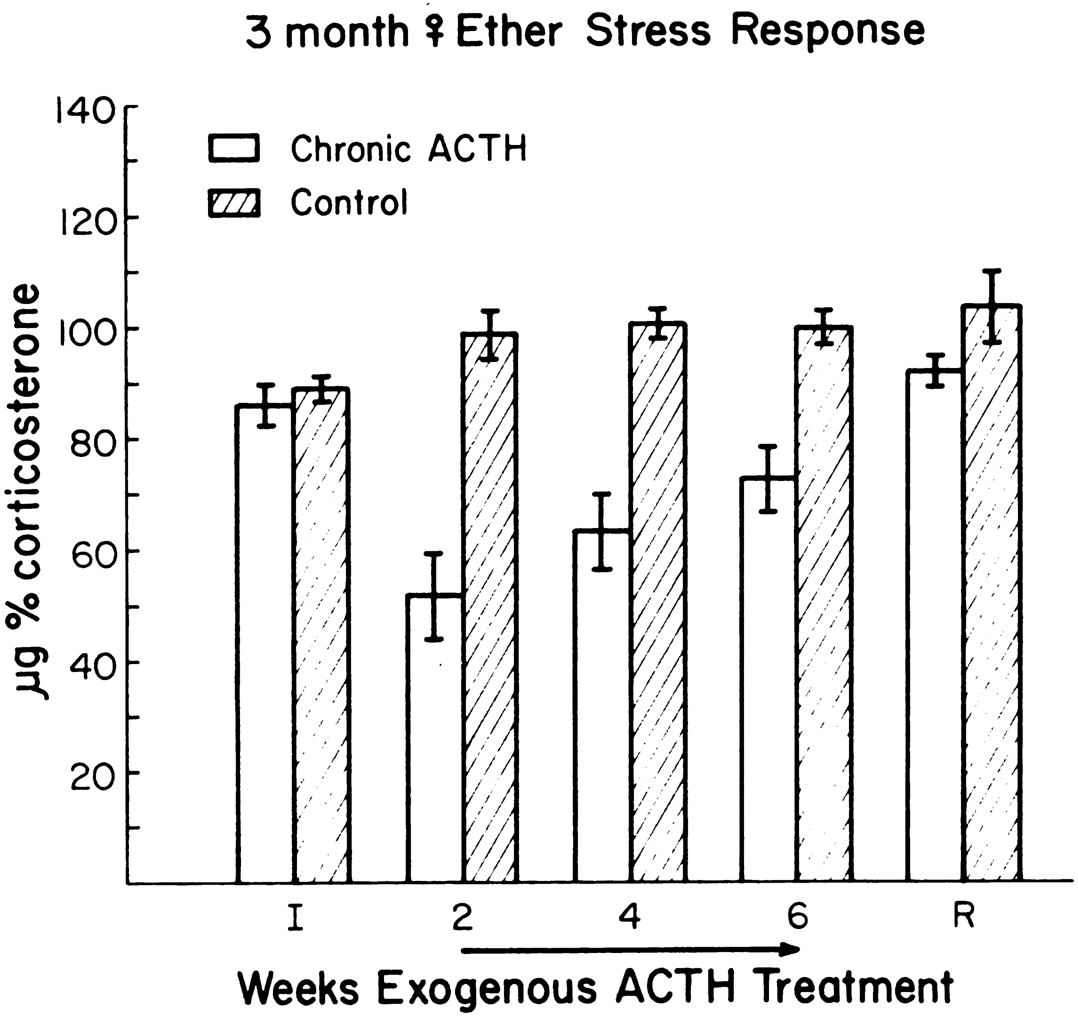

3 month ♀ Ether Stress Response
Chronic ACTH
Control
µg % corticosterone
140
120
100
80
60
40
20
I
2
4
6
R
Weeks Exogenous ACTH Treatment

Figure 10

Figure 11. Effect of chronic ACTH treatment on adrenocortical response to ether stress and acute ACTH stimulation in young and old female rats.

Adrenocortical responses (plasma corticosterone levels, μg/100 ml) of depo-ACTH treated young (3 month) and old (22 month) female rats are presented as change from the comparable control response at each interval of the experiment. Responses to both ether stress and acute ACTH stimulation determined before the start of depo-ACTH (designated as I on graph), after 2 weeks, 4 weeks and 6 weeks of depo-ACTH treatment and after a 2 week recovery period from depo-ACTH treatment are presented here. Depo-ACTH treatment consisted of daily injections of ACTH (6 U/rat) in a 15% gelatin preparation. Ether stress responses consisted of ether anesthetization 40 minutes, 10 minutes and just prior to orbital sinus blood sampling while acute ACTH stimulation consisted of exogenous ACTH administration (1 U/100 g body weight) 1 hour before orbital sinus blood sampling.

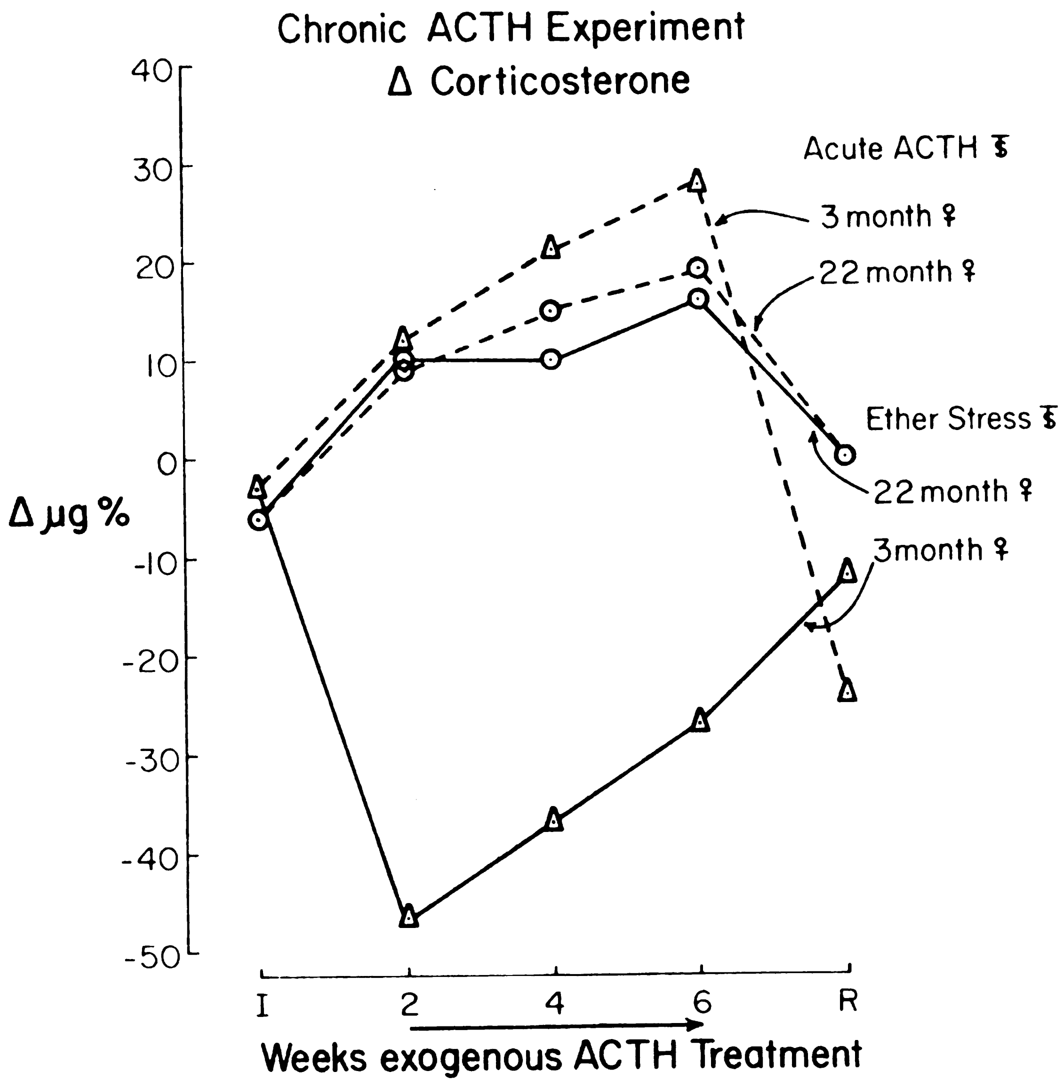

Chronic ACTH Experiment
Δ Corticosterone
40
30
20
10
0
-10
-20
-30
-40
-50
Δ μg %
Acute ACTH
3 month ♀
22 month ♀
Ether Stress
22 month ♀
3month ♀
I
2
4
6
R
Weeks exogenous ACTH Treatment

Figure 11

Table 3. Adrenocortical responsiveness[1] to stress and ACTH stimulation in young and old female rats

Age	Stimulus	Treatment Group	n	Before Treatment	Weeks of ACTH Treatment[2] 2	4	6	Recovery
Young	ether[3]	depo-ACTH	6	86.2 ± 3.7	51.8 ± 7.8**	63.5 ± 6.8**	72.7 ± 5.9**	92.3 ± 2.7
		control	6	89.2 ± 2.4	98.6 ± 4.5	100.7 ± 2.7	99.9 ± 2.9	103.6 ± 6.5
	ACTH[4]	depo-ACTH	6	70.6 ± 4.6	109.1 ± 5.6	117.3 ± 8.0	123.5 ± 6.1**[a]	73.7 ± 6.5*
		control	6	73.8 ± 3.9	96.3 ± 2.5	96.1 ± 2.9	95.5 ± 4.6	98.3 ± 5.0
Old	ether[3]	depo-ACTH	6	72.9 ± 5.8	93.1 ± 8.1	98.6 ± 5.8	98.4 ± 5.5*	80.3 ± 2.7
		control	6	79.2 ± 2.0	82.9 ± 4.4	89.3 ± 3.5	82.8 ± 3.1	79.8 ± 4.3
	ACTH[4]	depo-ACTH	6	67.4 ± 3.3	102.9 ± 7.5	105.1 ± 5.0*	96.4 ± 6.6*[a]	83.3 ± 2.8
		control	6	73.9 ± 3.7	93.7 ± 5.6	91.4 ± 2.3	77.8 ± 4.2	82.8 ± 1.7

[1] μg corticosterone/100 ml plasma, presented as group mean ± S.E.M.

[2] Treated rats received 6 units ACTH subcutaneously in gelatin/day, control rats received only gelatin.

[3] Ether vapor exposure at 40 minutes before, 10 minutes before and at the time of bleeding.

[4] ACTH (1 U/100 g) in saline injected subcutaneously 1 hour before blood sampling.

*$p < .05$, **$p < .01$ depo-ACTH response and control response differ for same stimulus in same age group.

[a] Means having same superscript differ, $p < .05$.

Table 4. Body weight[1] of young and old female rats at bleeding intervals of chronic ACTH experiment

Age	Treatment Group	Before Treatment	Weeks of ACTH Treatment[2] 2	4	6	Recovery
Young	depo-ACTH treated	233 $\pm$ 16	238 $\pm$ 18	250 $\pm$ 19	257 $\pm$ 23	270 $\pm$ 20
	control	235 $\pm$ 10	243 $\pm$ 11	253 $\pm$ 11	260 $\pm$ 11	267 $\pm$ 12
Old	depo-ACTH treated	255 $\pm$ 10	254 $\pm$ 13	254 $\pm$ 10	251 $\pm$ 11	263 $\pm$ 10
	control	272 $\pm$ 10	271 $\pm$ 11	267 $\pm$ 12	266 $\pm$ 12	264 $\pm$ 11

[1]Group body weight (in grams) $\pm$ S.E.M.

[2]Treated rats received 6 units ACTH in gelatin daily, control rats received only gelatin.

Discussion of Experiments I and II

Chronic ACTH stimulation of adrenocortical function in young and old, male and female rats.--Although the role of the adrenal cortex in the stress response of animals is not completely understood, functional adrenal cortices are essential for survival in the face of many stressful stimuli. An index commonly used to measure adrenocortical function related to stress response is the functional reserve capacity of the adrenal cortices (the capacity of the stimulated adrenal cortices to secrete glucocorticoids in excess of normal resting steady state levels). Studies in aged cattle (Riegle and Nellor, 1967) and in aged goats (Riegle et al., 1968) provided evidence that adrenocortical functional reserve capacity was lower than in young animals. Decreased adrenocortical responsiveness to exogenous ACTH stimulation has also been observed with increasing age in rats (Hess and Riegle, 1970).

These findings in cattle, goats and rats suggested that exhaustion of adrenocortical function might occur in old rats if they were exposed to prolonged periods of adrenocortical stimulation. This possibility was investigated in this study by exposing both young and old rats to chronic depo-ACTH treatment for 6 weeks. Actual experimental results did not support the hypothesis of adrenocortical exhaustion in either young or old rats after 6 weeks of chronic depo-ACTH treatment.

Adrenocortical response of chronically stimulated adrenal glands to an acute ACTH injection (sufficient ACTH to maximally stimulate the adrenal cortex) was elevated in young and old male and female rats following long-term daily injections of depo-ACTH (depo-ACTH concentrations sufficient to maintain elevated plasma corticosterone levels for at least 8 hours). Although adrenocortical responsiveness to acute ACTH stimulation increased in both young and old groups the response was significantly higher in young than in old rats of each sex.

Adrenocortical response to ether stress stimulation represents adrenocortical stimulation by endogenously released ACTH and hence reflects the influence of feedback control effects on hypothalamic-pituitary regulation of adrenocortical function. Ether stress results of the present study suggested that there was a marked age-related difference in feedback sensitivity of the adrenocortical control system. In old depo-ACTH treated male rats, plasma corticosterone levels following ether stress were not significantly different from those of controls after 2 weeks, 4 weeks and 6 weeks of depo-ACTH treatment. In young male rats, plasma corticosterone levels of depo-ACTH treated animals were significantly lower than those of controls after 2 weeks, 4 weeks and 6 weeks of treatment.

In old female rats, adrenocortical response to ether stress was higher in depo-ACTH treated than in control rats after 6 weeks of treatment although treated and control responses did not differ after 2 weeks and 4 weeks of treatment. In young female rats, ether stress response of depo-ACTH treated animals was lower than that of control rats after 2 weeks, 4 weeks and 6 weeks of treatment.

The increased responsiveness of young and old rat adrenal cortices to acute ACTH stimulation and the markedly decreased responsiveness to stress in young rats suggest that the hypothalamic-pituitary corticotropin control center (s) of the young rats are responding differently to the elevated blood corticoid levels following prolonged adrenocortical activation than those of the aged animals.

Direct observation of age-related differences in the feedback sensitivity of endocrine control systems is relatively new to experimental gerontology. Some observations of endocrine functional changes with aging support this concept. Ascheim (1965) showed that regular estrous cycles could be induced in aged constant estrous rats by injections of LH, suggesting that hypothalamic control of the pituitary failed in aging rats. Clemens et al. (1969) investigated the possibility that hypothalamic changes may account for the loss of normal reproductive function in aged rats. Ovulation was induced in aged constant estrous rats by electrical stimulation of the preoptic area as well as by treatment with epinephrine or progesterone. These findings suggested that reproductive failure in aged rats was not due to ovarian tissue senescence alone. Although observed in the gonadal rather than the adrenocortical system, the findings of Ascheim and Clemens _et al_. provide suggestive evidence for age-related changes in endocrine control systems. The present data implies that hormone sensitivity of the feedback control system is changed in aged rats. Recognizing the similarities of control system dynamics between gonadal and adrenocortical systems, it is assumed that similar altered sensitivities to gonadal steroid feedback could at least partially explain the data of Ascheim and Clemen's _et al_.

The anatomical site or sites of the age change in adrenocortical feedback sensitivity cannot be established from the data of the present study. Evidence from other investigators suggests at least two mechanisms of adrenocortical feedback control that could have been involved. One possibility is that exogenous ACTH acted at short loop feedback receptor sites to exert a feedback control effect on adrenocortical function. Hodges and Vernikos (1958, 1959), Vernikos-Danellis and Trigg (1967) and Motta _et al_. (1968) have demonstrated such a feedback effect of ACTH on adrenocortical function in rats. In these studies, plasma ACTH levels were markedly elevated by adrenalectomy or ACTH secreting pituitary tumors in order to produce the observed effects. Although injected depo-ACTH may have elevated plasma corticotropin levels enough to produce hypothalamic inhibition of the ether stress response in the present study, the slow release of ACTH from the gelatin and the short biological half-life of ACTH (Syndor and Sayers, 1953) suggest that the increase would have been of small magnitude. Although suppression of the adrenocortical stress response by ACTH acting at short loop feedback receptors remains a possibility, it seems unlikely that exogenous ACTH was directly responsible for the age-related difference in feedback sensitivity observed in the present study.

A more likely possibility is that chronically elevated plasma corticosterone levels, produced by the slowly released exogenous ACTH, were responsible for suppression of the adrenocortical stress response in young male and female rats. Halasz et al. (1967), Zimmerman and Critchlow (1969b), Smelik (1969) and others have shown that the corticosteroid sensitive feedback control of the pituitary-adrenal axis is located primarily in the hypothalamus. More specifically, Halasz et al. (1967) have shown that corticosteroid sensitive feedback control of the adrenocortical stress response is located in the median eminence area of the hypothalamus. Whether suppression of the adrenocortical stress response in young male and female rats was caused by elevated plasma corticosteroid levels or by direct action of exogenous ACTH on short loop feedback receptors, the age difference in feedback sensitivity remains apparent.

The physiological significance of the age-related difference in corticosteroid feedback sensitivity is difficult to assess. Zimmerman and Critchlow (1969a) concluded that no level of corticoid treatment will inhibit the adrenocortical stress response, provided that an adequate stress stimulus is employed. This concept was supported by Yates et al. (1969), who described the saturation characteristics of the steroid sensitive adrenocortical control mechanism. Although this relationship may hold under the conditions of their acute experiments, it is reasonable to conclude that the ether stress response can be suppressed by long-term elevation of corticosterone levels, as in the present study.

The apparent age-related change in hormone sensitivity suggests that the adrenocortical control mechanism has undergone alterations which in turn have compromised the precision of adrenocortical regulation. Such changes could represent an important link in the progressive deterioration of homeostasis with increasing age. Changes in the ether stress feedback control imply that the control system for "resting" adrenocortical function probably also becomes less sensitive to corticosteroid feedback with increasing age.

Similar conclusions concerning the corticosteroid feedback sensitivity of adrenocortical control systems have been reached in studies (Riegle, Wallace and Hess, unpublished observations) in which young and old rats were exposed to exogenous corticosteroid (Dexamethasone) treatment for 2 weeks. Apparently, feedback sensitivity to both endogenous and exogenous corticosteroids decreases with increasing age in rats.

Experiment III: Influence of Gonadal Steroids on Adrenocortical Function in the Rat

Materials and Methods

The purpose of this experiment was to test the hypothesis that age-related changes in adrenocortical function arise from changes in gonadal steroid production. Gonadal hormones (particularly the estrogens) are known to influence adrenocortical function in the rat (Kitay, 1963b) and increasing age is known to alter gonadal function in the rat (Eckstein, 1955; Krohn, 1955). Two methods were used to assess the influence of gonadal steroids on adrenocortical response to both ether stress and exogenous ACTH stimulation in young adult and aged rats. Both young adult and aged rats were gonadectomized, to determine if the loss of endogenous gonadal hormone production would alter adrenocortical responses. Subsequently, the influence of exposure to a gonadal hormone treatment regime (utilizing exogenous hormones) on adrenocortical responses was determined in young and old rats.

The first experiment employing this design was conducted in a group of young (6 month) female rats and another group of old (24 month) female rats. Adrenocortical response to both ether stress and exogenous ACTH was tested in both young and old female rats prior to ovariectomy, 4 days post-ovariectomy and 2 weeks post-ovariectomy. Adrenocortical responses of intact control young and old female rats were also determined at each of these intervals. During the third week of the experiment all animals received daily injections of progesterone (800 μg in 0.1 ml olive oil, s.c.) for a total of 7 days. Adrenocortical responses to both ether stress and exogenous ACTH were determined at the end of progesterone treatment. During the 4th week of the experiment, young and old control rats and one group each of young and old ovariectomized rats received estrogen replacement therapy for 7 days (30 μg estradiol in 0.1 ml olive oil, s.c., daily). A second group of young ovariectomized rats and a second group of old ovariectomized rats received injections of the olive oil vehicle (0.1 ml, s.c., daily) alone during the 4th week of the experiment. The treatment regimes of the 4th week were reversed during the 5th week, so that all of the experimental animals were exposed to both estradiol and oil injections during the course of the experiment. Adrenocortical responses to ether stress and exogenous ACTH were determined in all rats at the end of the 4th and the 5th week of the experiment.

A similar experiment was conducted in young adult (5 month) and aged (24 month) male rats. Adrenocortical response to exogenous ACTH stimulation was determined prior to castration, after 2 weeks of castration and after 6 weeks of castration. Adrenocortical responses of intact controls of both age groups were also determined at these three intervals throughout the experiment. During the 7th and 8th week of the experiment, all of the rats were treated with testosterone propionate (1 mg daily in olive oil, s.c.).

Adrenocortical response to exogenous ACTH was again determined at the end of the treatment period.

A third experiment was conducted to test the effect of prepubertal gonadectomy on adrenocortical function in male and female Long-Evans rats. Ovariectomy of female rats and castration of male rats was performed at 30 days of age. Sham operations were also carried out on control male and female rats at 30 days of age. Adrenocortical responses to exogenous ACTH stimulation were determined 6 weeks post-surgery. Body weights were recorded at the time of surgery and at the time of bleeding, six weeks later. Experimental results of each part of this study were analyzed by Student's t-test.

Results

Ovariectomy of young adult female rats did not affect adrenocortical response to exogenous ACTH or ether stress stimulation either 4 days or 2 weeks post-surgery (Tables 5 and 6). Similarly, no significant changes in adrenocortical response to ether stress or exogenous ACTH were observed 4 days or 2 weeks following ovariectomy of old female rats (Tables 5 and 6).

Both ovariectomized and intact rats of both age groups were exposed to gonadal hormone replacement during the 3rd, 4th and 5th week post-surgery. All rats received progesterone treatment (800 μg daily) during the 3rd week. Both young and old intact rats and a group each of young and old ovariectomized rats received estradiol treatment (30 μg/day) during the 4th week and received oil vehicle injections during the 5th week of the experiment. The treatment regime was reversed in the second group of young and old ovariectomized rats. These rats received the oil vehicle injections during the 4th week and estradiol (30 μg/day) during the 5th week of the experiment.

The only effect of progesterone treatment in young and old female rats was reduced adrenocortical responsiveness ($p < .05$) to exogenous ACTH stimulation in old intact rats compared to the corresponding pretreatment response. However, the adrenocortical response of old intact rats following progesterone treatment was not different from that of old ovariectomized rats following progesterone treatment (Table 8).

Following estradiol treatment (30 μg/day for 7 days), adrenocortical response to exogenous ACTH was significantly lower than the similar response prior to the gonadal hormone treatment regime in young intact ($p < .05$) and old intact ($p < .01$) rats (Tables 7 and 8). Estradiol treatment did not alter adrenocortical response to exogenous ACTH in either young or old ovariectomized rats (Tables 7 and 8). Adrenocortical response to ether stress following estradiol treatment was lower than the ether stress response prior to the gonadal hormone treatment regime in young intact rats ($p < .05$), but was not altered by estradiol treatment in old intact and young and old ovarectomized rats (Tables 9 and 10).

Injections of oil vehicle alone elevated the ether stress adrenocortical response ($p < .05$) of one group of young ovariectomized rats (Table 9), but had no significant effect on adrenocortical responses of any other groups.

In a related study, adrenocortical response to exogenous ACTH stimulation following castration of young and old male rats was considered (Table 11). Although adrenocortical responses of young male rats were higher ($p < .05$) than those of old male rats 6 weeks after castration, adrenocortical responses of young and old male rats 2 weeks and 6 weeks after castration were not significantly different from pre-castration responses. Testosterone replacement therapy (1 mg/day) was given during the 7th and 8th weeks of the experiment. After 2 weeks of testosterone, adrenocortical response of young male rats was lower ($p < .01$) than the 6 week response but was not significantly different from the 6 week response in old male castrate rats (Table 11).

Adrenocortical response to exogenous ACTH was also studied in prepubertally ovariectomized female rats. Plasma corticosterone levels 6 weeks after ovariectomy were significantly lower ($p < .01$) than those of sham-operated control female rats (Table 12). In male rats, adrenocortical response was not different between castrate and intact animals 6 weeks after prepubertal castration (Table 12).

Table 5. Effects of ovariectomy on adrenocortical response[1] to exogenous ACTH[2] in young and old female rats

		n	Before Ovariex	4 Days Ovariex	2 Weeks Ovariex
Young[3]	Ovariex	9	109.6 ± 3.7	111.0 ± 4.4	110.0 ± 5.7
	Control	4	99.1 ± 6.5	100.4 ± 4.8	114.4 ± 6.5
Old[3]	Ovariex	9	90.2 ± 6.9	73.7 ± 7.4	80.8 ± 6.3
	Control	4	89.9 ± 5.3	82.5 ± 2.6	87.1 ± 3.6

[1]Plasma corticosterone (μg/100 ml ± S.E.M.).

[2]1 U ACTH/100 g 1 hour before blood sampling.

[3]Ovariectomized rat response did not differ from comparable control response at any interval of the experiment.

Table 6. Effect of ovariectomy on adrenocortical response[1] to ether stress[2] in young and old female rats

		n	Before Ovariex	4 Days Ovariex	2 Weeks Ovariex
Young[3]	Ovariex	9	97.8 ± 5.7	98.9 ± 5.7	97.1 ± 5.2
	Control	4	89.0 ± 3.2	97.5 ± 5.3	95.8 ± 3.9
Old[3]	Ovariex	9	88.8 ± 4.9	74.9 ± 7.4	77.4 ± 7.7[4]
	Control	4	88.8 ± 5.6	84.4 ± 2.8	86.2 ± 8.8

[1]Plasma corticosterone (μg/100 ml ± S.E.M.).

[2]Exposed to ether anesthesia 40 minutes, 10 minutes, and 0 minutes before blood sampling.

[3]Ovariectomized rat response did not differ from comparable control response at any interval of the experiment.

[4]n = 8.

Table 7. Adrenocortical response of young female rats to ACTH[1] following gonadal hormone treatment[2]

	Ovariex[3]	Intact[3]	Ovariex[4]
n	5	4	4
Pretreatment (2nd week	105.4 ± 8.9	114.4 ± 6.5	115.8 ± 6.6
3rd week	109.8 ± 5.8	97.3 ± 3.4	123.6 ± 5.7
4th week	95.7 ± 6.9	89.8 ± 2.0*	123.5 ± 5.1
5th week	129.6 ± 8.8	108.6 ± 4.7	106.7 ± 6.4

[1]Plasma corticosterone (μg% ± S.E.M.) 1 hour after ACTH injection (1 U/100 g body weight).

[2]All rats were exposed to the following hormones for 1 week:

Progesterone (800 μg/day)
Estradiol (30 μg/day).

[3]Treatment regime as follows:

Progesterone, 3rd week
Estradiol, 4th week
Oil vehicle, 5th week.

[4]Treatment regime as follows:

Progesterone, 3rd week
Oil vehicle, 4th week
Estradiol, 5th week

*Response differs from pretreatment (2nd week) response of same group ($p < .05$).

Table 8. Adrenocortical response of old female rats to ACTH[1] following gonadal hormone treatment[2]

	Ovariex[3]	Intact[3]	Ovariex[4]
n	4	4	4
Pretreatment (2nd week)	76.4 ± 9.2	87.1 ± 3.6	85.1 ± 9.4
3rd week	65.4 ± 6.1	67.0 ± 6.3*	64.8 ± 6.3
4th week	63.7 ± 6.3	61.0 ± 3.3**	76.4 ± 6.1
5th week	74.7 ± 4.1	87.4 ± 4.7[5]	74.1 ± 12.0

[1]Plasma corticosterone (μg% ± S.E.M.) 1 hour after ACTH injections (1 U/100 g).

[2]All rats were exposed to the following hormones for 1 week:

Progesterone (800 μg/day)
Estradiol (30 μg/day)

[3]Treatment regime as follows:

Progesterone, 3rd week
Estradiol, 4th week
Oil vehicle, 5th week.

[4]Treatment regime as follows:

Progesterone, 3rd week
Oil vehicle, 4th week
Estradiol, 5th week.

[5]n = 3.

*Response differs from pretreatment (2nd week) response of same group ($p < .05$).

**Response differs from pretreatment (2nd week) response of same group ($p < .01$).

Table 9. Adrenocortical response of young female rats to ether stress[1] following gonadal hormone treatment[2]

	Ovariex[3]	Intact[3]	Ovariex[4]
n	5	4	4
Pretreatment (2nd week)	89.2 ± 6.4	95.8 ± 3.9	107.0 ± 6.1
3rd week	95.6 ± 3.3	89.2 ± 5.7	102.2 ± 3.3
4th week	86.2 ± 6.4	79.5 ± 4.1*	103.2 ± 7.4
5th week	107.9 ± 4.8*	99.9 ± 5.2	94.7 ± 5.5

[1]Plasma corticosterone (μg% ± S.E.M.) following exposure to ether anesthesia 40 minutes, 10 minutes and 0 minutes before bleeding.

[2]All rats were exposed to the following hormones for 1 week:

Progesterone (800 μg/day)
Estradiol (30 μg/day).

[3]Treatment regime as follows:

Progesterone, 3rd week
Estradiol, 4th week
Oil vehicle, 5th week.

[4]Treatment regime as follows:

Progesterone, 3rd week
Oil vehicle, 4th week
Estradiol, 5th week.

*Response differs from pretreatment (2nd week) response of same group ($p < .05$).

**Response differs from pretreatment (2nd week) response of same group ($p < .01$).

Table 10. Adrenocortical response of old female rats to ether stress[1] following gonadal hormone treatment[2]

	Ovariex[3]	Intact[3]	Ovariex[4]
n	4	4	4
Pretreatment (2nd week)	73.1 ± 13.4	86.2 ± 8.8	81.7 ± 9.3
3rd week[5]	57.1 ± 6.9	67.0 ± 6.3	56.7 ± 8.8
4th week[5]	46.6 ± 8.9	69.2 ± 3.5	74.1 ± 6.6
5th week[5]	49.1 ± 8.5	79.0 ± 7.1[6]	70.5 ± 8.8

[1]Plasma corticosterone (μg% ± S.E.M.) following exposure to ether anesthesia 40 minutes, 10 minutes and 0 minutes before bleeding.

[2]All rats were exposed to the following hormones for 1 week:

Progesterone (800 μg/day)
Estradiol (30 μg/day).

[3]Treatment regime as follows:

Progesterone, 3rd week
Estradiol, 4th week
Oil vehicle, 5th week.

[4]Treatment regime as follows:

Progesterone, 3rd week
Oil vehicle, 4th week
Estradiol, 5th week.

[5]Treatment means did not differ from 2 week (pretreatment) responses.

[6]n = 3.

Table 11. Effect of castration of adult male rats on adrenocortical response[1]

	Young[2]	n	Old[3]	n
Before castration	65.4 ± 2.9	6	55.8 ± 3.2	5
2 weeks castrate	55.0 ± 4.7	6	49.1 ± 1.9	5
6 weeks castrate	66.1 ± 2.9	6	53.3 ± 4.7	5
2 weeks testosterone	48.4 ± 5.6**	3	38.7 ± 5.7	3

[1]Adrenocortical responses reported as plasma corticosterone levels (μg% ± S.E.M.), determined 70 minutes after injection of ACTH (2 U/100 g).

[2]5 months old.

[3]24 months old.

[4]1 mg/day, testosterone propionate.

**Mean differs from 6 week castrate response, ($p < .01$).

Table 12. Effect of prepubertal gonadectomy[1] on adrenocortical response[2]

		n	µg% Corticosterone	Body Weight[3]
Female	Ovariex	12	71.0 ± 3.5[a]	225 g
	Intact	11	92.4 ± 6.0[a]	180 g
Male	Castrate	3	66.6 ± 4.9	223 g
	Intact	3	59.0 ± 5.3	270 g

[1]Gonads removed at 30 days of age.

[2]Response to exogenous ACTH 6 weeks post surgery, 1 U ACTH/100 g in female, 2 U ACTH/100 g in male, adrenocortical response reported as plasma corticosterone level (µg ± S.E.M.) 1 hour after ACTH.

[3]Average weight of group (grams).

[a]Means with the same superscript differ, ($p < .01$).

Discussion

The influence of estrogens on adrenocortical function of the female rat has been studied by several investigators including Telegdy (1964), Richard (1965/66) and D'Angelo (1968). Kitay (1961) presented evidence for an estrogenic influence in the sex difference of adrenocortical function in the rat. Kitay (1963b) also found reduced adrenocortical responsiveness to stress and exogenous ACTH stimulation in prepubertally ovariectomized rats.

The relationship of estrogen to adrenocortical function in the aged female rat was studied by subjecting young adult and aged female rats to ovariectomy. Adrenocortical responsiveness to ether stress and ACTH stimulation was not altered by either of these treatments, indicating that in the adult rat ovarian secretions exert no detectable influence on these parameters of adrenocortical steroidogenesis.

The failure to produce reduced adrenocortical responsiveness to stress following ovariectomy was in conflict with the well-known observations of Kitay (1963b) and others. In an attempt to resolve this conflict, the effect of ovariectomy at 30 days of age was studied using an experimental regime identical to the one used by Kitay (1963b). Adrenocortical responsiveness of these prepubertally ovariectomized rats was lower than that of sham-operated controls similar to the data reported by Kitay (1963b).

These results suggested that the principal influence of estrogens on adrenocortical function in the female rat occurs by the onset of puberty with little additional influence of physiological dosages of estrogens on adrenal steroidogenesis during the adult lifespan of the rat.

The second phase of this experiment was conducted to study the effects of gonadal hormone replacement therapy on adrenocortical function of both intact and overiectomized rats of both age groups. The influence of progesterone on adrenocortical function has not been extensively studied. Telegdy _et al_. (1962) reported that daily doses of 100 μg progesterone/100 g body weight for 8 days significantly increased corticosterone levels in adrenal venous blood. On the other hand, Pincus and Hirai (1964) found that although adrenal corticosterone levels were increased following 5 days of progesterone treatment (500 μg/day), progesterone treatment did not significantly affect corticosterone secretion into adrenal venous blood.

Progesterone treatment utilized in the present study (800 μg/day for 1 week) was not effective in increasing adrenocortical response. Apparently progesterone does not influence adrenocortical function of the rat, at least when stimulated plasma corticosterone levels are used as an index of adrenocortical function. The discrepancy between the findings of this study and those of previously reported studies could result from different methods of estimating adrenocortical response and the increased age of experimental subjects in this study.

Kitay (1963b) has shown that estrogen replacement therapy for 2 weeks in prepubertally ovariectomized rats will return adrenocortical response to control levels. Telegdy *et al*. (1962) reduced adrenal venous corticosterone levels of intact female rats significantly with daily doses of estrone (100 μg/100 g B.W. for 8 days). Corticosterone secretion from the adrenal vein was also decreased significantly following estradiol-17 β treatment (5 μg/day for 5 days) in studies by Pincus and Hirai (1964). D'Angelo (1968) treated female rats with either 0.1, 1, 10, or 50 μg estradiol benzoate/day for 2 weeks. Plasma corticosterone levels were significantly lower in female rats receiving 50 μg estradiol benzoate/day than in oil injected controls. Female rats receiving lower doses of estradiol benzoate showed no change in adrenocortical response. Although plasma corticosterone levels represented neither resting nor maximally stimulated adrenocortical responses, this study by D'Angelo (1968) showed that adrenocortical responses of intact rats can be depressed by high levels of estradiol benzoate. A dosage of 30 μg estradiol daily for 7 days was chosen for the present study.

Although the adrenocortical response to exogenous ACTH and stress was significantly lower in both young and old estrogen-treated intact rats, estrogen treatment did not affect adrenocortical response in either young or old ovariectomized rats. This observation suggested that the combination of injected estrogens and endogenous ovarian estrogens was adequate to suppress adrenal steroidogenic responses but that the injected estrogens in the castrate animals were not effective. The similarity of response to injected ACTH and stress induced release of ACTH in these studies indicated that the site of action of estrogen is not related to hypothalamic-pituitary corticotropin control.

The study of the effect of male gonadal function on the adrenal cortex in the rat indicated no influence of castration in young or old rats. On the other hand, testosterone propionate replacement therapy (1 mg daily for 2 weeks) showed evidence of adrenocortical suppression in young but not in old rats. This could be due to testosterone acting at steroid sensitive feedback centers of the adrenocortical control system. If this is the case, the age-related difference in feedback sensitivity to steroids is in agreement with previously reported observations in the chronic ACTH stimulation experiment.

Castration of male rats at 30 days of age had no effect on adrenocortical response to exogenous ACTH stimulation 6 weeks post-surgery. Similar findings have been reported by Kitay (1963b).

In summary, these experiments indicate that gonadal hormone deficiencies in aged rats are of limited importance with respect to the age-related changes which exist in adrenocortical function of the rat.

Experiment IV: Adrenocortical Stress Response of Male and Female Rats Between One and Six Months of Age

Materials and Methods

The purpose of this experiment was to determine the adrenocortical response characteristics of male and female Long-Evans rats between 1 and 6 months of age. Response to ether stress was determined in female rats at nine intervals ranging from 23 to 200 days of age, and in male rats at seven intervals ranging from 26 to 154 days of age. Age at the onset of puberty was also determined for female rats (day of vaginal opening). Age at puberty was also determined in a group of control rats whose adrenocortical response to ether stress was determined at 90 days of age and compared to that of the experimental group at the same age.

Adrenocortical response to exogenous ACTH was determined in both female (203 day) and male (158 day) rats to ascertain whether previous results were affected by accommodation of the rats to the ether stress procedure. Body weights were recorded at the time of each adrenocortical response determination. Statistical analysis of experimental results was based on Student's t-test.

Results

Experimental evidence from this study supports the hypothesis that adrenocortical response to stress changes during the first 6 months of age in rats. Adrenocortical responses of male rats at seven intervals between 26 and 154 days of age are shown in Table 13 and Figure 12. The peak response of adrenocortical function, which occurred at 44 days of age, was significantly higher ($p < .01$) than the response at 26 days of age. Adrenocortical response at 76 days was significantly lower ($p < .01$) than that at 44 days of age but did not change significantly between 76 and 154 days of age. Adrenocortical response of this group of rats to exogenous ACTH stimulation at 158 days of age was not significantly different from the ether stress response at 154 days of age (Table 16). Mean body weights of this group of rats rose from 54.6 g at 23 days to 373.1 g at 160 days of age (Table 13).

Adrenocortical responses of female rats to ether stress stimulation at 9 intervals between 23 and 200 days of age are shown in Table 14 and Figure 12. The peak adrenocortical response in female rats occurred at 115 days of age. The 115 day response was significantly different from the 23 day response ($p < .01$). Although adrenocortical responses were significantly lower at 160 days ($p < .05$) and at 200 days ($p < .01$) than at 115 days of age, they remained within the range of values previously reported for adult female rats (Hess, 1968). The 95 day adrenocortical response was not different from that of a group of rats whose adrenocortical response was tested for the first time at 90 days of age (Table 15). The 200 day adrenocortical response to ether stress was not significantly different from that to exogenous ACTH stimulation several days later (Table 16). The onset of puberty, defined as the day of vaginal opening, occurred at an average age of 41.8 days. Body weight rose from 59.2 g at 23 days to 295.5 g at 200 days of age (Table 14).

Figure 12. Adrenocortical responses of rats between 23 and 200 days of age to ether stress stimulation.

Data presented here represents adrenocortical response to ether stress stimulation. Plasma corticosterone levels (μg corticosteron/100 ml plasma) were used as the index of adrenocortical function. Rats were exposed to ether anesthetization 40 minutes, 10 minutes and just prior to the orbital sinus blood sampling procedure. The responses of the female rats at increasing calendar ages are connected by the solid line while responses of the male rats at increasing calendar ages are connected by the broken line.

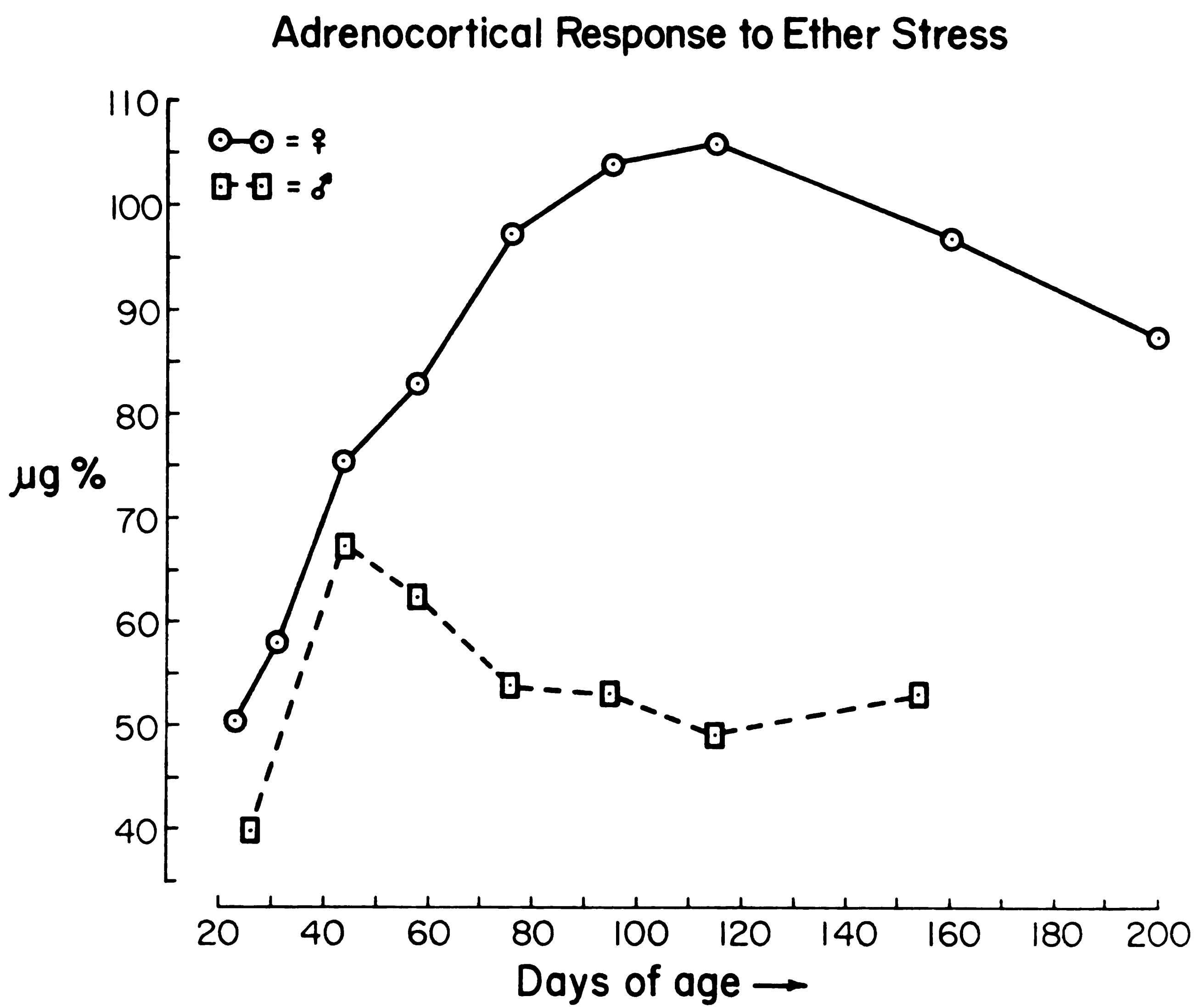

Adrenocortical Response to Ether Stress
= ♀
= ♂
µg %
110
100
90
80
70
60
50
40
20
40
60
80
100
120
140
160
180
200
Days of age →

Figure 12

Table 13. Adrenocortical response of male rats to ether stress between 26 and 154 days of age[1]

Age (days)	Plasma Corticosterone	Body Weight (g)	n
26	40.2 ± 2.2[a,b]	54.6 ± 2.2	13
44	67.4 ± 2.4[a,c]	115.4 ± 3.5	13
58	62.3 ± 2.9	161.9 ± 6.4	13
76	53.8 ± 1.6[b,c]	226.2 ± 8.9	13
95	53.2 ± 1.1	274.6 ± 9.7	13
115	49.3 ± 1.8	326.2 ± 9.8	13
154	53.2 ± 1.1	373.1 ± 10.1	13

[1]Plasma corticosterone (μg% ± S.E.M.) following ether stress.

[a,b,c]Means with same superscript are significantly different, ($p < .01$).

Table 14. Adrenocortical response of female rats to ether stress between 23 and 200 days of age[1]

Age (days)	Plasma Corticosterone	Body Weight (g)	n
23	50.4 ± 1.8^{a}	59.2 ± 1.7	12
31	57.7 ± 1.8	85.8 ± 2.2	12
44	75.3 ± 3.6	132.5 ± 3.4	12
58	82.8 ± 3.0	194.2 ± 3.4	12
76	97.4 ± 3.5	230.0 ± 3.2	11
95	104.0 ± 5.6	240.9 ± 6.7	11
115	$106.2 \pm 5.1^{a,b,c}$	262.3 ± 6.2	11
160	96.8 ± 5.9^{c}	289.9 ± 9.5	11
200	87.5 ± 3.9^{b}	295.5 ± 8.2	11

[1]Plasma corticosterone (μg% ± S.E.M.) following ether stress.

[a,b]Means with the same superscript are significantly different, ($p < .01$).

[c]Means with the same superscript are significantly different, ($p < .05$).

Table 15. Adrenocortical response of female rats to ether stress[1]

	n	Plasma Corticosterone[2]	Age at Puberty[3]
Experimental rats	11	104.0 ± 5.6	41.8
Control rats	12	103.8 ± 2.5	40.6

[1]Ether stress adrenocortical response of experimental group at 95 days of age compared to adrenocortical response of control group exposed to ether stress for first time at 90 days of age.

[2]μg/100 ml plasma ± S.E.M.

[3]Average age of onset of puberty (day of vaginal opening).

Table 16. Comparison of adrenocortical response[1] to ether stress and ACTH stimulation

	n	Ether[2] Response	Age	ACTH[3] Response	Age
Female	11	87.5 ± 3.9	200 day	81.9 ± 4.5	203 day
Male	13	53.2 ± 1.1	154 day	50.2 ± 1.6	158 day

[1]Response of same rats given as μg corticosterone/100 ml plasma ± S.E.M.

[2]Exposed to ether anesthetization 40 minutes, 10 minutes and just before bleeding.

[3]1 U ACTH/100 g body weight in female, 2 U ACTH/100 g body weight in male, 1 hour before bleeding.

Discussion

Studies of adrenocortical responsiveness to ACTH and stress in the rat have used a substantial range of animal ages. The present experiment was undertaken to determine if calendar age of the maturing rat was involved in adrenocortical responsiveness. The data presented in Figure 14 illustrates that adrenocortical response to other stress stimulation does change between 23 and 200 days of age in the rat. Adrenocortical response in male rats reached a peak at 44 days of age. Adrenocortical responses were similar in male rats between 76 and 154 days of age. To be sure that these results were not just reflecting accommodation to ether stress, adrenocortical response to exogenous ACTH was tested at 158 days of age. The close similarity of this response to the 154 day ether stress response indicated that the animals were not accommodating to ether stress.

Although it has been shown that the effect of ovarian secretion on adrenocortical function occurs by puberty in the rat, male-female difference in adrenocortical responsiveness was not different until 58 days of age. The peak response of adrenocortical function in female rats occurred at 115 days of age. The possibility that rats were accommodating to stress with repeated sampling was tested by measuring adrenocortical response of a group of female rats bled for the first time following ether stress stimulation at 90 days of age. The similarity of stress response of this group of non-accommodated rats to the experimental group and the parallel response of the experimental group to ACTH stimulated response at 203 days of age indicated that the female rats were also not accommodating to the experimental procedure in this study. Although adrenocortical responsiveness was reduced at 160 and 200 days in these female rats, the response remained within the range of response for adult female rats (Hess, 1968).

The results of this study suggested that the sex difference in adrenocortical response to ether stress in the rat is closely associated with the onset of puberty. In addition, these results emphasize the fact that adrenocortical response to ether stress does change in the maturing rat and should be considered when studies of adrenocortical function are conducted in rats with experimental animals chosen accordingly. Young mature male and female rats chosen for the studies of this thesis were in the age ranges characterized by small changes in the adrenocortical response curves of Figure 12.

Experiment V: Thyroid Hormone Influences on Adrenocortical Function in Young Adult and Aged Male Rats

Materials and Methods

This experiment was designed to test the effect of different levels of thyroid hormone (Protamone) stimulation on adrenocortical function in male rats. Quantities of Protamone equivalent to .02%, .04% and .08% of the diet were carefully mixed with the standard rat diet used in our rat colony. In the initial phase of the experiment, 24 three month old male rats were divided into four groups (six rats/group). One group was maintained on each level of Protamone and a fourth group was maintained on the normal diet for a period of 3 weeks. Rats were caged in groups of three. Feed consumption of each group was estimated daily and body weights were taken at weekly intervals throughout the experiment. Adrenocortical response (plasma corticosterone level) to a standardized ether stress was determined after three weeks of treatment.

A similar experiment was conducted in a group of male rats averaging 23 months of age. Because of anticipated variation in the adrenocortical response of old rats, response to ether stress was also determined prior to the start of Protamone treatment. The rats were divided into four treatment groups (4-6 rats/group), each of which received one of the three Protamone levels or the regular rat diet. Rats were housed two or three per cage. Daily food consumption was estimated and body weights were determined at weekly intervals. Adrenocortical response to ether stress stimulation was determined after three weeks of Protamone treatment.

Results

Ether stress adrenocortical responses of young male rats treated with different levels of Protamone are given in Table 17 and Figure 13. Plasma corticosterone levels following ether stress were compared between the four experimental groups by Duncan's Multiple Range Test subsequent to Analysis of Variance. Adrenocortical responses of rats receiving the .04% and .08% diets were significantly different from those of non-treated controls and from one another ($p < .01$).

Adrenocortical response of old male rats to ether stress was determined prior to the start of Protamone treatment as well as at the end of the 3 week treatment period. The resulting data is given in Table 18 and Figure 13. Adrenocortical responses of the four experimental groups of old male rats were not significantly different either before the start or after 3 weeks of Protamone treatment.

Plots of adrenocortical response versus log-dose Protamone for both young and old rats suggested that adrenocortical response was related to the level of Protamone treatment in young but not in old male rats (Figure 14). Average food consumption for all treatment groups of young and old male rats is presented in Table 19. Feed consumption was similar for all treatment groups of young and old male rats. Group body weight averages (± S.E.M.) of young and old male rats are given in Table 20. Body weight after Protamone treatment was not significantly different from pre-treatment body weight in any of the eight treatment groups of young and old male rats.

Figure 13. Adrenocortical responses of young and old male rats following thyroid hormone treatment.

The data presented here represents adrenocortical response of male rats to ether stress stimulation. Plasma corticosterone levels (μg/100 ml) were used as an index of adrenocortical function. Rats were exposed to ether anesthetization 40 minutes, 10 minutes and just before orbital sinus blood sampling. The left panel represents adrenocortical response of young (3 month) male rats to different levels of Protamone treatment while the right panel represents adrenocortical response of old (23 month) male rats to different levels of Protamone treatment. The cross hatched portion of the bars represents adrenocortical response prior to Protamone treatment while the open portion of the bars represents adrenocortical response after 3 weeks of Protamone treatment. The four groups in each age group represent animals which were fed the regular diet (C), a diet containing .02% Protamone (L), a diet containing .04% Protamone (M) and a diet containing .08% Protamone (H) for 3 weeks prior to determination of adrenocortical response.

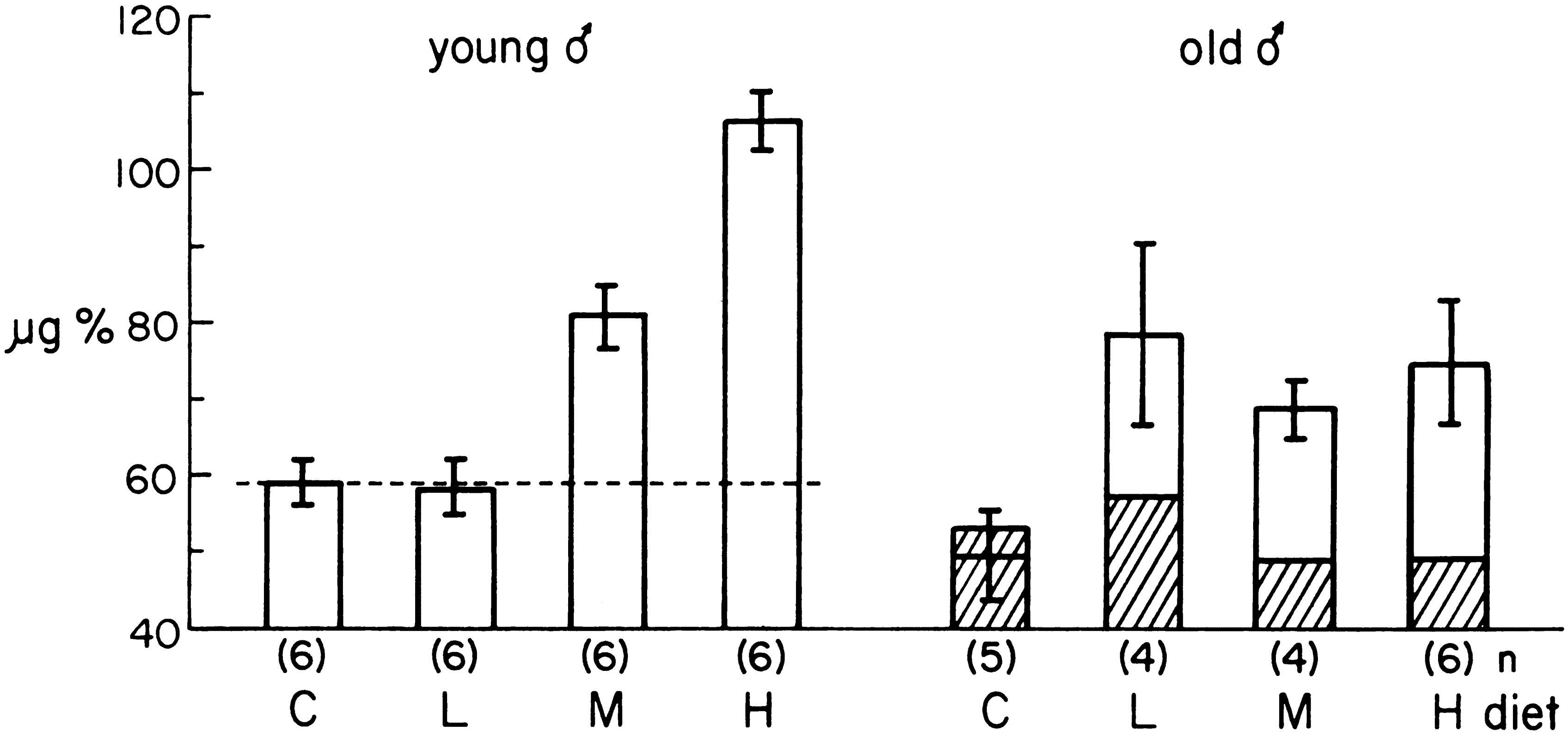


Figure 13

Figure 14. Adrenocortical response versus level of Protamone treatment in young and old male rats.

Adrenocortical response (μg% plasma corticosterone) to ether stress is plotted as a function of the log-dose of Protamone (g/100 g) contained in the regular rat diet. The least squares line fitting the three points of each age group is given.

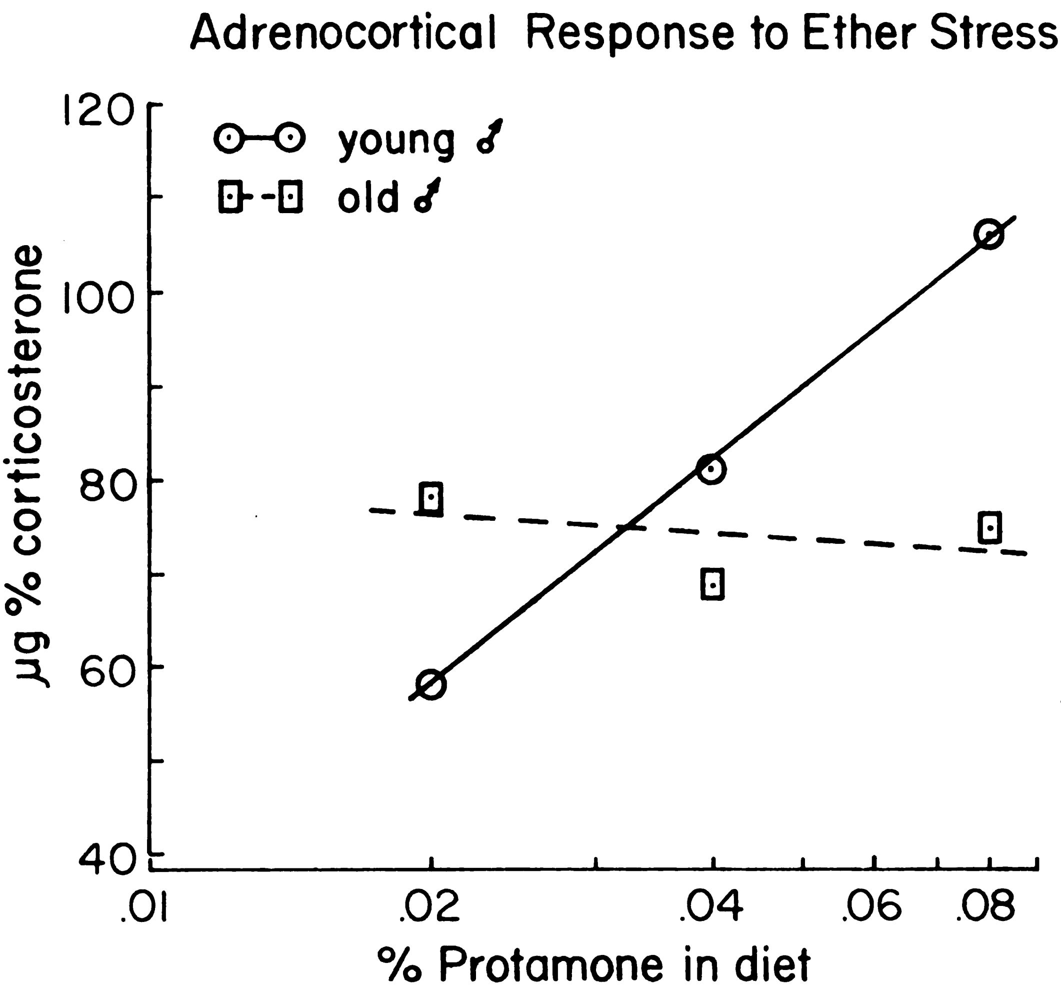

Adrenocortical Response to Ether Stress
young ♂
old ♂
120
100
80
60
40
µg % corticosterone
.01
.02
.04
.06
.08
% Protamone in diet

Figure 14

Table 17. The effect of Protamone supplement on adrenocortical function of young male rats[1]

	Level of Protamone[2]			
	0	.02%	.04%	.08%
n	6	6	6	6
Adrenocortical response[3]	$59.0 \pm 3.1^{a,b}$	$58.3 \pm 3.2^{c,d}$	$80.8 \pm 3.2^{a,c,e}$	$106.2 \pm 3.5^{b,d,e}$

[1] 3 months old.

[2] Protamone added to regular diet, dosage given as % Protamone in feed.

[3] Plasma corticosterone level (μg/100 ml ± S.E.M.) following ether stress, after 3 weeks of Protamone treatment.

[a,b,c,d,e] Means bearing the same superscript are significantly different from one another ($p < .01$).

Table 18. The effect of Protamone supplement on adrenocortical function of old male rats[1]

	Level of Protamone[2]			
	0	.02%	.04%	.08%
n	5	4	4	6
Adrenocortical[3] response before Protamone	52.8 $\pm$ 6.2	57.2 $\pm$ 7.3	48.8 $\pm$ 2.9	49.2 $\pm$ 4.4
Adrenocortical[4,5] response after Protamone	49.4 $\pm$ 6.0	78.4 $\pm$ 11.9	68.6 $\pm$ 3.7	74.6 $\pm$ 8.2

[1]23 months old.

[2]Protamone added to regular diet, dosage given as % Protamone in feed.

[3]Plasma corticosterone (μg/100 ml $\pm$ S.E.M.) following ether stress, prior to start of Protamone treatment.

[4]Plasma corticosterone (μg/100 ml $\pm$ S.E.M.) following ether stress, after 3 weeks of Protamone treatment.

[5]Treatment means after Protamone were not significantly different.

Table 19. Feed consumption of male rats on Protamone treatment[1]

	Level of Protamone[2]			
	0	.02%	.04%	.08%
Young	19.7	19.9	18.7	21.6
Old	21.1	22.6	21.7	23.7

[1]Average feed consumption (grams/rat/day), during 3 week treatment period.

[2]% Protamone contained in feed.

Table 20. Body weight[1] of young and old male rats of Protamone experiment

		Level of Protamone[2]			
		0	.02%	.04%	.08%
Young	Initial[3]	300 ±19	316 ±24	296 ±10	330 ±13
	Final[4]	348 ±21	365 ±26	328 ±14	343 ±11
Old	Initial[3]	482 ±22	530 ±36	445 ±33	541 ±16
	Final[4]	488 ±23	525 ±38	449 ±29	533 ±16

[1]Average weight of group in grams ± S.E.M.

[2]% Protamone in diet.

[3]Weight prior to start of Protamone treatment.

[4]Weight after 3 weeks of Protamone treatment.

Experiment VI: Thyroid Hormone Influences on Adrenocortical Function in Young and Aged Female Rats

Materials and Methods

This experiment was designed to test the effect of different levels of thyroid hormone (Protamone) stimulation on adrenocortical function in young adult and aged female rats. Protamone treatment levels were .02%, .05%, and .08% of the diet. A group of 23 young adult rats averaging 4 months and a group of 22 old rats averaging 25 months of age were used in this study. Each age group was divided into four treatment groups (5 or 6 rats/group), with each receiving one of the three Protamone treatment levels or the regular rat diet. Rats were housed in groups of two or three with group food consumption being estimated daily. The rats were weighed at weekly intervals throughout the course of the 3 week treatment period. Adrenocortical response to ether stress was determined prior to Protamone treatment and at the end of the 3 week treatment period.

Results

Ether stress adrenocortical responses of young female rats treated with different levels of Protamone are given in Table 21 and Figure 15. Plasma corticosterone levels between the four experimental groups after 3 weeks of Protamone treatment were compared by Duncan's Multiple Range Test subsequent to Analysis of Variance. Adrenocortical responses of rats fed the .08% Protamone diet were significantly different ($p < .01$) from those of non-treated controls. Adrenocortical responses of these four experimental groups to ether stress were not significantly different prior to the start of Protamone treatment.

Ether stress adrenocortical responses of old female rats treated with different levels of Protamone are given in Table 22 and Figure 15. Adrenocortical responses of the four experimental groups of old female rats were not significantly different either before the start or after 3 weeks of Protamone treatment.

Plots of adrenocortical response versus log-dose Protamone for both young and old female rats suggested that adrenocortical response was related to the level of treatment in young but not in old rats (Figure 16). Although the response of old rats showed a linear relationship to log-dose Protamone, adrenocortical responses to .02%, .04%, and .08% Protamone treatment were not significantly different from one another. On the other hand, adrenocortical response to .08% Protamone was significantly higher ($p < .01$) than the response to .02% Protamone in young female rats. Average feed consumption for all treatment groups of young and old female rats is given in Table 23. Feed consumption differences between young and old rats were not considered large enough to have influenced the experimental results. Average body weights ($\pm$ S.E.M.) for each treatment group of young and old female rats at the start and at the end of Protamone treatment are given in Table 24. Post-treatment body weights did not differ from pretreatment body weights for any treatment group of either young or old rats.

Figure 15. Adrenocortical responses of young and old female rats following thyroid hormone treatment.

The data presented here represents adrenocortical response of female rats to ether stress stimulation. Plasma corticosterone levels (μg/100 ml) were used as an index of adrenocortical function. Rats were exposed to ether anesthetization 40 minutes, 10 minutes and just before orbital sinus blood sampling. The left panel represents adrenocortical response of young (5 month) female rats to different levels of Protamone treatment while the right panel represents adrenocortical response of old (26 month) female rats to different levels of Protamone treatment. The cross hatched portion of the bars represents adrenocortical response prior to Protamone treatment while the open portion of the bars represents adrenocortical response after 3 weeks of Protamone treatment. The four groups in each age group represent animals which were fed the regular diet (C), a diet containing .02% Protamone (L), a diet containing .04% Protamone (M), and a diet containing .08% Protamone (H) for 3 weeks prior to determination of adrenocortical response.

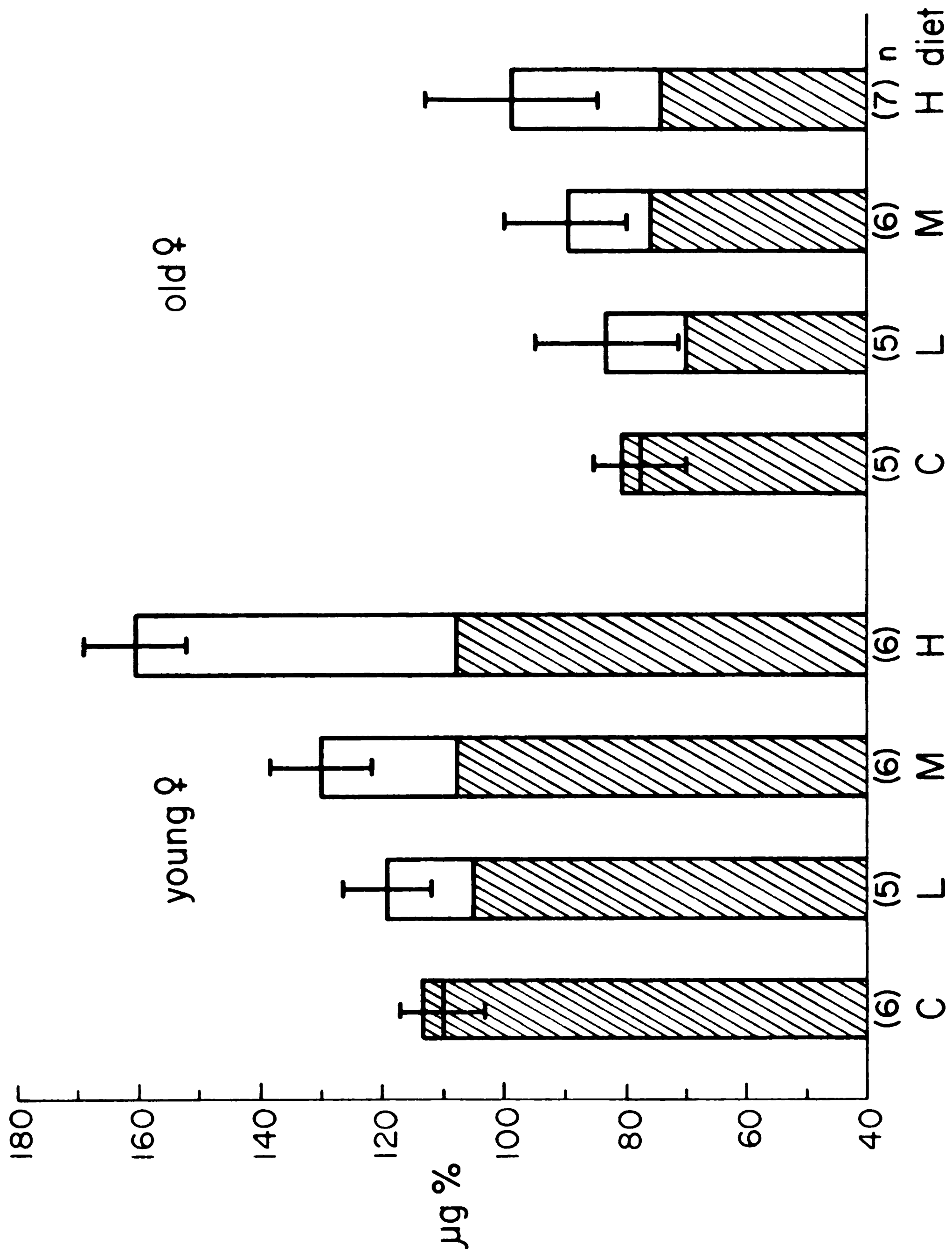
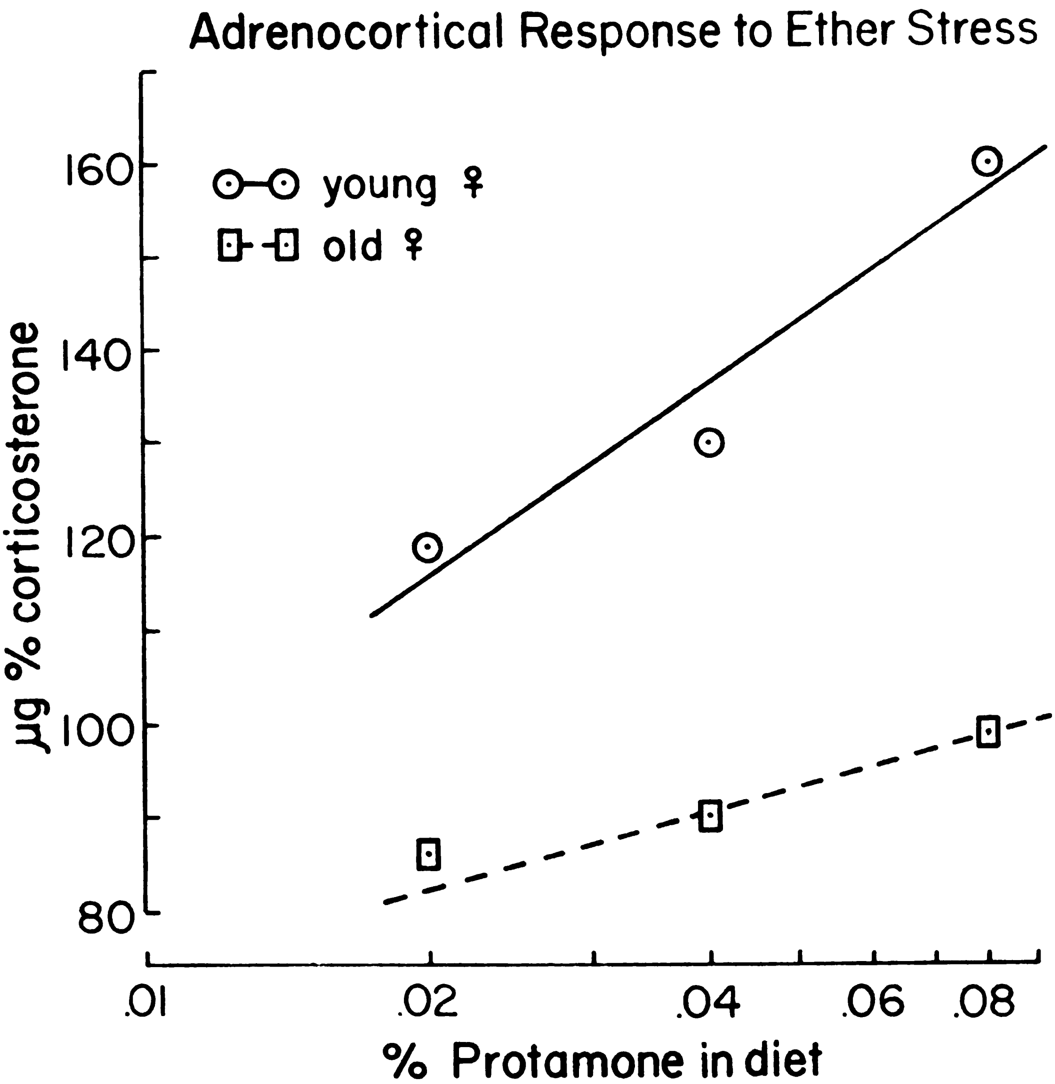

µg %
180
160
140
120
100
80
60
40
young ♀
old ♀
(6)
C
(5)
L
(6)
M
(6)
H
(5)
C
(5)
L
(6)
M
(7)
H
n
diet

Figure 15

Figure 16. Adrenocortical response versus level of Protamone treatment in young and old female rats.

Adrenocortical response (μg% plasma corticosterone) to ether stress is plotted as a function of the log-dose of Protamone (g/100 g) contained in the regular rat diet. The least squares line fitting the three points of each age group is given.



Adrenocortical Response to Ether Stress
young ♀
old ♀
μg % corticosterone
160
140
120
100
80
.01
.02
.04
.06
.08
% Protamone in diet

Figure 16

Table 21. The effect of Protamone supplement on adrenocortical function of young female rats[1]

	Level of Protamone[2]			
	0	.02%	.04%	.08%
n	6	5	6	6
Adrenocortical[3] response before Protamone	113.4 $\pm$ 2.2	105.0 $\pm$ 5.9	107.5 $\pm$ 2.2	107.3 $\pm$ 4.3
Adrenocortical[4] response after Protamone	110.0 $\pm$ 6.8[a]	119.1 $\pm$ 7.4[b]	130.0 $\pm$ 8.3	160.5 $\pm$ 8.7[a,b]

[1] 4 months old.

[2] Protamone added to regular diet, dosages given as % Protamone in feed.

[3] Plasma corticosterone (μg/100 ml $\pm$ S.E.M.) following ether stress, prior to start of Protamone treatment.

[4] Plasma corticosterone (μg/100 ml $\pm$ S.E.M.) following ether stress, after 3 weeks of Protamone treatment.

[a,b] Means bearing the same superscript are significantly different ($p < .01$).

Table 22. The effect of Protamone supplement on adrenocortical function of old female rats[1]

	Level of Protamone[2]			
	0	.02%	.04%	.08%
n	5	5	6	7
Adrenocortical[3] response before Protamone	80.6 $\pm$ 4.6	70.1 $\pm$ 7.6	76.0 $\pm$ 4.1	74.2 $\pm$ 7.8
Adrenocortical[4,5] response after Protamone	77.5 $\pm$ 7.8	82.9 $\pm$ 12.0	89.7 $\pm$ 10.2	98.8 $\pm$ 14.1

[1] 25 months old.

[2] Protamone added to regular diet, dosages given as % Protamone in feed.

[3] Plasma corticosterone (μg/100 ml $\pm$ S.E.M.) following ether stress, prior to start of Protamone treatment.

[4] Plasma corticosterone (μg/100 ml $\pm$ S.E.M.) following ether stress, after 3 weeks of Protamone treatment.

[5] Treatment means after Protamone were not significantly different.

Table 23. Feed consumption of female rats on Protamone treatment[1]

	Level of Protamone[2]			
	0	.02%	.04%	.08%
Young	15.9	15.5	17.2	16.9
Old	14.4	14.3	12.2	12.9

[1]Average feed consumption (grams/rat/day), during 3 week treatment period.

[2]% Protamone contained in feed.

Table 24. Body weight[1] of young and old female rats of Protamone experiment

		Level of Protamone[2]			
		0	.02%	.04%	.08%
Young	Initial[3]	253 ± 9	249 ± 9	261 ±10	253 ±11
	Final[4]	272 ±12	262 ± 8	277 ±13	270 ±13
Old	Initial[3]	322 ±24	319 ±24	293 ±14	305 ±15
	Final[4]	314 ±22	309 ±25	275 ±14	278 ±14

[1]Average weight of group in grams (± S.E.M.).

[2]% Protamone in diet.

[3]Weight prior to start of Protamone treatment.

[4]Weight after 3 weeks of Protamone treatment.

Discussion of Experiments V and VI

Thyroid hormone influence on adrenocortical function in young adult and aged male and female rats.--The interaction of thyroidal and adrenocortical function in the rat has been reported by a number of investigators including Wallach and Reineke (1949), Kawai (1962), Steinetz and Beach (1963) and D'Angelo and Grodin (1964). The present study was designed to consider the effect of different levels of thyroid hormone treatment on adrenocortical function in young and old rats.

Protamone, a thyro-active protein, mixed in the normal rat diet at concentrations of .02, .04 and .08% of the diet, was used to induce hyperthyrodism in the experimental subjects. Protamone contains thyroxine (1% by weight) of which approximately 33% is absorbed when administered by the oral route (E.P. Reineke, personal communication). For an average feed consumption of 5 g/100 g body weight/day, rats fed the .02% diet absorbed approximately 3 μg thyroxine/100 g body weight/day. Reineke and Singh (1955) reported a daily thyroid secretion rate of 2.21-2.56 μg L-thyroxine/100 g in adult female rats and 2.15 μg L-thyroxine/100 g in adult male rats. According to this data (Reineke and Singh) for thyroid secretion rate, the .02% diet provided a near physiological level of exogenous thyroxine on a daily basis. This level of treatment was expected to induce only a minimal level of hyperthyroidism during the 3 week treatment period. The .04% diet supplied approximately 6 μg thyroxine

100 g body weight daily and was expected to induce a moderate degree of hyperthyroidism. The .08% diet provided approximately 12 μg thyroxine/100 g body weight daily and was expected to induce a definite state of hyperthyroidism in the rat. The .08% diet represents approximately four times the normal thyroxine secretion rate in the rat. This approaches the limit for a non-toxic dose in the rat (E.P. Reineke, personal communication).

A plot of adrenocortical response versus log-dose thyroxine treatment (Figure 14) suggested that adrenocortical response was related to the level of thyroid hormone treatment in young male rats. Following 3 weeks of thyroid hormone treatment, adrenocortical response to ether stress was greater than that of non-treated controls in rats which had received the .04% and the .08% diet. Adrenocortical response of rats on the .08% diet was also significantly different from that of rats receiving the .04% diet. From these results it was concluded that thyroid hormone stimulation of the pituitary-adrenal axis occurs in the young male rat, provided that a sufficient level of hyperthyroidism was achieved.

Similar Protamone levels in old male rats did not alter the adrenocortical response to ether stress. Adrenocortical response in this study showed no relationship to the level of Protamone used (Figure 17). Differences in feed consumption (Table 16) between young and old rats were not considered to be sufficient to influence the experimental results.

A similar study was conducted to consider the effect of different levels of thyroid hormone treatment on adrenocortical function in young and old female rats. In young female rats treatment with the .08% Protamone diet for 3 weeks increased adrenocortical response to ether stress above the response of non-treated controls. Adrenocortical response to ether stress in old female rats was not altered by 3 weeks of Protamone treatment at any of the levels included in this study. Although plots of adrenocortical response versus log-dose Protamone (Figure 17) suggested that adrenocortical response to thyroid treatment was dose-related in both young and old female rats, adrenocortical responses to different levels of Protamone were significantly different only in young female rats. Feed consumption differences between young and old female rats were not considered to be sufficient to account for the age-related differences in the experimental results (Table 16).

The mechanism of thyroidal stimulation of the pituitary-adrenal axis in the young rat has not been investigated in the present study. Evidence from other investigators suggests that thyroidal stimulation exerts its effect at several sites in the pituitary-adrenal axis. Kawai (1962) reported that pituitary ACTH depletion following stress appeared greater in hyperthyroid than in control rats.

D'Angelo and Grodin (1964) concluded that adrenocortical stimulation in hyperthyroid rats is mediated primarily by way of ACTH secretion. Other changes such as increased steroidogenic capacity of the adrenal cortices (Freedland and Mrad, 1969) and reduced corticosterone distribution volume (Steinetz and Beach, 1963) have also been reported in hyperthyroid rats.

The results of this study suggested that the pituitary-adrenal axis of aged rats is less sensitive to thyroid hormone stimulation than that of young rats. This conclusion is in agreement with the previous hypothesis concerning age differences in corticoid feedback influence.

Experiment VII: The Effects of Age and Chronic ACTH Stimulation on Biological Half-Life, Distribution Volume and Production Rate of Corticosterone in Female Rats

Materials and Methods

The first part of this study was conducted to compare functional data on adrenocortical response exclusive of plasma corticosterone levels in young (5 month) and old (27 month) female rats. Biological half-life, distribution volume and production rate of corticosterone in young and old female rats were estimated by the H^3-corticosterone disappearance technique.

In the second part of this study, biological half-life, distribution volume and production rate of corticosterone were estimated in depo-ACTH treated young adult (5 month) female rats and compared to these same parameters of adrenocortical function in non-treated control rats. The H^3-corticosterone disappearance technique was used to estimate these parameters of adrenocortical function in both treated and control rats. Rats in the treated group were exposed to 3 weeks of depo-ACTH treatment (9 U ACTH daily) in 15% gelatin while control rats received the gelatin vehicle alone prior to determination of adrenocortical functional parameters. Adrenocortical responses to ether stress and acute ACTH stimulation were determined before the start and after 2 weeks of depo-ACTH treatment in both treated and control rats. Experimental results of both parts of this study were analyzed by Student's t-test.

Results

In the first part of this study, H^3-corticosterone disappearance curves were obtained for a group of young (5 month) and a group of old (27 month) female rats. Chromatographic isolation of radioactive corticosterone showed that 75% of the extractable radioactivity in the circulation 15 minutes after H^3-corticosterone injection was related to cortisterone. Disappearance curves were calculated for the disappearance of total radioactivity from the plasma. Biological half-life, distribution volume and production rate of corticosterone, based on the disappearance curve data, were compared between young and old rats. The single compartment model of steroid dynamics was used in calculating these parameters of adrenocortical function.

The estimated biological half-life of corticosterone in young rats was less than that of old rats ($p < .05$) but estimated corticosterone distribution volumes (Table 25) showed no age difference in this study. The calculated production rate of corticosterone (μg corticosterone/minute) in young rats was significantly greater ($p < .01$) than that of old rats. Plasma corticosterone levels during the period of blood sampling were significantly higher in young ($p < .05$) than in old rats.

In a subsequent study, data derived from H^3-corticosterone disappearance curves was used to calculate biological half-life, distribution volume and production rate of corticosterone in a group of female rats (5 month) exposed to 3 weeks of chronic ACTH stimulation and a comparable group of non-treated control rats (Table 26). After 2 weeks of chronic ACTH treatment, ether stress plasma corticosterone levels were significantly lower ($p < .01$) than control levels. Response to acute ACTH stimulation after 2 weeks chronic ACTH treatment was significantly higher ($p < .01$) than control response.

Biological half-life of corticosterone and estimated corticosterone distribution volume were not different between chronic ACTH treated and control animals. Calculated corticosterone production rate was significantly lower ($p < .05$) in chronic ACTH treated animals than in controls, although mean corticosterone levels during blood sampling were not significantly different between treated and control animals.

Table 25. Functional parameters of adrenocortical response in young and old female rats

	Young[1]	Old[2]
n	11	11
$t_{1/2}$[3]	9.3 ± 0.4[a]	10.6 ± 0.3[a]
Distribution volume[4]	63.0 ± 3.9	61.2 ± 3.8
Plasma corticosterone[5]	76.9 ± 2.6[b]	66.5 ± 2.5[b]
Production rate[6]	11.4 ± 0.7[c]	7.8 ± 0.4[c]

[1]5 months old.

[2]27 months old.

[3]Biological half-life of corticosterone (minutes).

[4]Estimated volume of corticosterone distribution (percent of body weight).

[5]Average plasma corticosterone level (μg%) during serial blood sampling.

[6]Corticosterone production rate (μg/minute).

[a,b]Means with same superscript differ, $p < .05$.

[c]Means with same superscript differ, $p < .01$.

(Values ± S.E.M.)

Table 26. Functional parameters of adrenocortical response in female rats: effect of chronic ACTH treatment

	Treated[1]	Control[2]
n	6	6
$t_{1/2}$[3]	7.5 ± 0.6	7.4 ± 0.3
Distribution volume[4]	49.8 ± 4.0	58.9 ± 4.3
Plasma corticosterone[5]	40.9 ± 9.9	61.6 ± 6.2
Production rate[6]	4.9 ± 1.4[a]	9.1 ± 1.2[a]
Ether stress response[7]	55.3 ± 11.0[b]	100.9 ± 1.7[b]
Acute ACTH stimulation response[8]	144.3 ± 8.2[c]	108.7 ± 4.2[c]

[1]Treated with daily injections of ACTH (9 μ/day) in 15% gelatin, for 3 weeks.

[2]Treated with 15% gelatin alone.

[3]Biological half-life of corticosterone (minutes).

[4]Estimated distribution volume of corticosterone (percent of body weight).

[5]Average plasma corticosterone level (μg%) during serial blood sampling.

[6]Corticosterone production rate (μg/minute).

[7]Adrenocortical response to ether stress after 2 weeks of depo-ACTH treatment, reported as μg corticosterone/100 ml plasma.

[8]Adrenocortical response to exogenous ACTH stimulation after 2 weeks of depo-ACTH treatment, reported as μg corticosterone/100 ml plasma.

[a]Means with same superscript differ, $p < .05$.

[b,c]Means with same superscript differ, $p < .01$.

(Values ± S.E.M.)

Discussion

This study was undertaken to provide functional adrenocortical data concerning age-related alterations not exclusively based on plasma corticosterone levels. Serial blood samples were obtained following rapid intravenous injection of H^3-corticosterone under sodium pentobarbital anesthesia. Experimental results showed that 75% of the circulating radioactivity 15 minutes after H^3-corticosterone injection was present as unmetabolized corticosterone in the present study. Glenister and Yates (1961) observed a similar percentage (at least 66%) of corticosterone related radioactivity 20 minutes after injection of H^3-corticosterone into rats. Although Saroff and Wexler (1969) based their study on the disappearance of radioactive corticosterone, they concluded that a similar interpretation of their results could have been made using the disappearance of total radioactivity from the plasma. The results of the present study were based on the disappearance of total radioactivity from the plasma. When log cpm/0.1 ml plasma was plotted against time, a predictable linear decline in radioactivity was noted. The least squares line relating the log cpm/0.1 ml plasma to time elapsed was used to calculate steroid dynamics based on the single compartment model described by Yates and Urquhart (1962).

Extrapolation of this line to time zero produced an estimate of the initial hormone concentration in the miscible pool. Comparison of this concentration to the total amount of isotope injected allowed calculation of distribution volume. Distribution volumes in this study showed no significant difference between young and old rats, suggesting that this parameter does not change with increasing age.

Biological half-life of corticosterone was shorter in young than in old rats in this study, suggesting that corticosteroid turnover time was more rapid in young than in old rats. Corticosterone production rates for old rats in the present study were also lower than those of young rats. Plasma corticosterone levels from individual rats, on which corticosterone production rate calculations were based, remained relatively constant during serial blood sampling. Plasma corticosterone levels during sampling were higher in young than in old rats although they were not maximally stimulated levels. Using techniques similar to those used in the present study, Saroff and Wexler (1969) found that corticosterone production rate was lower in breeder rats than in virgin rats. Although their breeder rats were probably younger than aged rats in this study, the results of the two studies are in agreement concerning corticosterone production rate.

Results of the present study are in agreement with those of West _et al_. (1961) who found a progressive decrease in the rate of cortisol removal from the circulation in aging men but no change in cortisol distribution volume. Samuels (1956) also found that cortisol removal rate was lower in old than in young men but an age-related decrease in cortisol distribution volume was observed in his studies. Moncloa et al. (1963) reported that daily corticosteroid excretion decreased progressively with increasing age, which also implies an increased biological half-life of corticosterone in aged subjects.

In summary, no age-related difference was observed in corticosterone distribution volume in the female rats used in this study. Biological half-life and production rate of corticosterone were altered in aged animals, suggesting that the secretory dynamics of adrenocortical function do change with increasing age in the rat.

The H^3-corticosterone disappearance technique was also used to study steroid dynamics in female rats following 2 weeks of chronic ACTH stimulation of the pituitary-adrenal axis. Two weeks of ACTH injection resulted in increased responsiveness to a maximal ACTH stimulation and decreased ether stress responsiveness similar to that observed in previous experiments.

Despite changes in adrenocortical response to both endogenous and exogenous ACTH stimulation in these chronically ACTH stimulated rats, there was no detectable effect of chronic ACTH treatment on biological half-life and distribution volume of corticosterone in this study. This suggested that "resting" adrenocortical function was not altered by chronic ACTH treatment although maximally stimulated responses were altered as the result of this treatment.

The similarity of biological half-life and distribution volume of corticosterone between these groups suggests that the animal is not compensating for the hypercorticoidism to which it is exposed.

Only minor emphasis is justified for the difference in corticosteroid production rate between groups in this study. This difference reflects plasma corticosterone levels during serial blood sampling in a stressful sampling regime. Variation of these levels between the five blood samples from each rat in the present study was sufficient to weaken the assumption of steady-state conditions, on which calculation of production rate is based.

From this study, it is concluded that chronic ACTH stimulation of the pituitary-adrenal axis does not alter biological half-life and distribution volume of corticosterone in female rats.

Experiment VIII: The Influence of Thyroid Hormone Treatment on Corticosterone Distribution Volume in the Male Rat

Materials and Methods

The purpose of this study was to investigate the possibility that alterations in corticosteroid distribution volumes might account for changes in stimulated plasma corticosterone levels following thyroid hormone treatment. Ten young adult male rats (7 month) were fed a .08% Protamone diet for 3 weeks with eight rats serving as non-treated controls. The distribution volume, biological half-life and production rate of corticosterone were estimated by procedures of the H^3-corticosterone disappearance technique. Ether stress and resting plasma corticosterone levels in both .08% Protamone treated and non-treated control rats were determined following several days of recovery from the H^3-corticosterone disappearance procedures. Statistical significance of age differences in this study was determined by Student's t-test.

Results

Corticosterone distribution volume was estimated in a group of Protamone treated male rats by extrapolation of H^3-corticosterone disappearance curves to zero time. The results were compared to those obtained in a similar manner from a comparable group of non-treated control rats.

The estimated H^3-corticosterone distribution volume in Protamone treated rats was significantly lower ($p < .01$) than that of the controls (Table 27). Subsequent determination of adrenocortical response to ether stress confirmed that adrenocortical response was higher ($p < .01$) in treated than in control animals (Table 27). Average plasma corticosterone concentration (mean of five determinations) during the course of serial blood sampling for the disappearance curve was higher ($p < .01$) in Protamone treated rats than in non-treated control animals. Despite these differences, neither biological half-life nor calculated production rate of corticosterone gave evidence of any significant treatment effect in this experiment. Resting plasma corticosterone levels were not significantly altered by Protamone treatment in this study (Table 27).

Table 27. Effects of Protamone treatment on functional parameters of adrenocortical response in male rats

	Treated[1]	Control[2]
n	10	8
$t_{½}$[3]	15.4 ± 0.8	14.5 ± 0.4
Distribution volume[4]	36.1 ± 2.2[a]	47.9 ± 2.6[a]
Plasma corticosterone[5]	70.3 ± 2.5[b]	49.1 ± 3.5[b]
Production rate[6]	4.8 ± 0.2	5.0 ± 0.6
Ether stress response[7]	79.8 ± 4.0[c]	56.4 ± 2.7[c]
Resting response[8]	5.2 ± 1.3	6.4 ± 1.8

[1]Fed diet containing .08% Protamone for 3 weeks prior to experiment.

[2]Fed regular diet.

[3]Biological half-life of corticosterone (minutes).

[4]Estimated distribution volume of corticosterone (percent of body weight).

[5]Average plasma corticosterone level (μg%) during serial blood sampling.

[6]Corticosterone production rate (μg/minute).

[7]Adrenocortical response to ether stress after 3 weeks of Protamone treatment.

[8]Resting adrenocortical response following 3 weeks of Protamone treatment.

[a,b,c]Means with the same superscript differ, $p < .01$.

(Values ± S.E.M.)

Discussion

Steinetz and Beach (1963) studied the influences of thyroid hormone on the adrenocortical function in rats. They observed significantly higher plasma corticosterone levels following ACTH injection in thyroid-treated rats than in controls but attributed this increase to a 50% reduction in the corticosterone distribution volume of thyroid-treated animals.

The present study was designed to consider the relationship between increased adrenocortical responsiveness associated with thyroid hormone treatment and corticosterone distribution volume. Distribution volumes were determined from H^3-corticosterone disappearance curves. In the present study corticosterone distribution volume was lower in thyroid-treated than in control rats, although it was reduced by 25% rather than the 50% reduction reported by Steinetz and Beach (1963). Steinetz and Beach were unable to estimate distribution volume with the precision obtained in the present study since they did not use a radioactively labelled hormone for their determination of distribution volume. Differences in animal size and methods for inducing hyperthyroidism could partially account for the lower magnitude of volume reduction in the present study. Steinetz and Beach fed 70 g rats a diet containing 0.15% USP Thyroid for 8 days while a .08% Protamone diet was fed to 350 g rats for 3 weeks in the present study.

Despite differences in experimental technique and magnitude of response, the results of the present study supported the observation by Steinetz and Beach (1963) that corticosterone distribution volume is reduced in hyperthyroid rats. Calculated corticosterone production rates were similar in thyroid-treated and control animals. This implies that adrenocortical secretion is altered less by thyroid treatment than the elevated plasma corticosterone levels suggest. Biological half life of corticosterone and resting levels of plasma corticosterone were also unaffected by thyroid treatment in this study. These observations are in agreement with those of Kawai (1962) who reported that resting plasma corticosterone levels were not altered in hyperthyroid rats. On the other hand, evidence that adrenal weights are increased (Steinetz and Beach, 1963; D'Angelo and Grodin, 1964) and that pituitary ACTH depletion following stress is increased in hyperthyroid rats (Kawai, 1962) suggests that hyperthyroidism does alter functional characteristics of the pituitary-adrenal axis even though these changes are not reflected in resting plasma corticosterone levels.

In summary, results from the present study indicated that corticosterone distribution volume was reduced following thyroid hormone treatment in the rat. In spite of this change, resting plasma corticosterone levels were not altered by thyroid hormone treatment.

GENERAL DISCUSSION

The purpose of all studies in experimental gerontology is to provide experimental evidence of changes which occur in living organisms during their adult life which might be related to the aging process. This information applied to current theories of aging, provides the basis for further investigation into the causes of aging. In theory, this process will eventually lead to an understanding of the aging process, such that its progress can be controlled or at least retarded. Recent studies in experimental gerontology have concentrated on investigation of the aging process at the molecular level. Sacher (1968) has warned against overcommitment to the molecular approach in aging studies and emphasized the importance of studies at the whole system level. Studies at the molecular level are important, in that changes observed at this level may be indicative of changes which are affecting a variety of physiological systems.

Carpenter and Loynd (1968) have attempted to integrate all of the current theories of aging into one composite theory of aging. All of the theories which they considered, e.g., cross-linkage, collagen, waste-product, mutation, etc., are related to the underlying observation that physiological control mechanisms which maintain homeostasis deteriorate with increasing age. Frolkis (1968) and Comfort (1970) also agree that aging is characterized by the loss of homeostatic capability in complex living organisms. They did not necessarily limit their concept of control systems to neural and endocrine control systems. Because of the extensive influence of the endocrines on body function, it is reasonable to believe that deterioration of the endocrine system could be involved as one of many causative factors in aging process. According to Frolkis (1968), the responsiveness of endocrine tissues to hormone decreases with increasing age. This means that a particular level of hormone acting on an endocrine tissue will produce a larger effect in a young individual than in an old one. A closely related observation by Frolkis indicated that the amount of hormone necessary to elicit a normal response from endocrine tissue is less in old than in young animals.

The well known involvement of adrenocortical response in the reaction to non-specific stress, first popularized by the General Adaptation Syndrome of Selye (1950), has raised the question of an adrenocortical role in the aging process. The age-related change in adrenocortical feedback control system characteristics observed in the chronic ACTH experiments of this study represents the type of change proposed by Frolkis (1968) to account for the age-related deterioration of homeostasis at the system level.

The age-related difference in adrenocortical sensitivity to thyroid hormone treatment and the decreased biological half-life and production rate of corticosterone observed in old female rats provide further evidence that age-related changes are occurring in the hypothalamic-pituitary adreno-cortical control system with age. If such changes occur in the adrenocortical control system, it is reasonable to expect that they might also be occurring in control systems for other endocrine glands. Altered functional characteristics of the endocrine system as a whole might be involved to some degree in the progressive deterioration of body function which characterizes aging.

Although there was evidence that endogenous estrogens are involved in establishing the female adrenocortical response pattern in maturing female rats, results obtained in this study suggest that age-related changes in gonadal hormone production have little influence on adrenocortical function in mature adult male and female rats.

The relationship of thyroid-adrenal interactions in the rat are not completely understood. Evidence obtained in the present study confirmed the finding of decreased corticosterone distribution volume in thyroid treated rats reported by Steinetz and Beach (1963). Decreased distribution volume of corticosterone may account for part of the increase in stress stimulated plasma corticosterone levels in hyperthyroid rate. Understanding of the thyroid-adrenal relationship is further complicated by the lack of a thyroid hormone treatment effect on resting plasma corticosterone levels observed in the present study. Similar findings with respect to resting plasma corticosterone levels in thyroid treated rats have been reported by Kawai (1962).

SUMMARY AND CONCLUSIONS

The investigation of adrenocortical function in the rat under the influence of chronic ACTH stimulation, gonadal hormones, thyroid hormones and age has shown the following:

1. The sensitivity of adrenocortical feedback control mechanisms (presumably to circulating corticosteroids) is markedly lower in aged rats than in young mature rats, as evidenced by suppression of the ether stress adrenocortical response in young but not old rats following chronic treatment with a long-acting ACTH preparation. This effect was observed in both male and female rats.
2. Chronic ACTH stimulation for 6 weeks did not produce exhaustion of the adrenocortical response to acute ACTH stimulation in young or old male or female rats.
3. Gonadal hormone deficiencies are of limited importance with respect to age-related changes of adrenocortical function in the rat. Castration of young adult and aged male and female rats did not alter adrenocortical responsiveness to stress or exogenous ACTH. Similarly, gonadal hormone replacement therapy with either progesterone or estradiol did not increase adrenocortical responses to stress or exogenous ACTH.

4. Adrenocortical response of male rats to ether stress changes between 23 and 160 days of age. Peak adrenocortical responses were observed at 44 days in the present study. Ether stress adrenocortical responses of female rats also changed between 23 and 200 days of age, with peak responses observed at 115 days of age. Experimental results also suggested that the sex difference in adrenocortical response to ether stress is closely associated with the onset of puberty.
5. Adrenocortical responses to ether stress following thyroid hormone (Protamone) treatment indicate that the pituitary-adrenal axis of aged rats is less sensitive to thyroid hormone stimulation than that of young rats. This conclusion was reached in both male and female rats and was in agreement with the previous hypothesis concerning age-differences in corticosteroid feedback influences.
6. Corticosterone distribution volume, biological half-life and production rate were compared between a group of young and a group of old female rats. Biological half-life was longer and production rate of corticosterone was lower in aged than in young rats, suggesting that the secretory dynamics of adrenocortical function do change with age in the rat.

7. Experimental results suggested that chronic ACTH stimulation of the pituitary-adrenal axis does not alter biological half-life and distribution volume of corticosterone in female rats.
8. Corticosterone distribution volume in a group of Protamone treated male rats was 25% lower than in a group of non-treated controls. Resting adreno-cortical responses determined in these rats showed no effect of Protamone treatment.

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APPENDICES

APPENDIX I

MSU REGULAR RAT DIET

Ingredient	Amount (lbs.)
Ground Shelled Corn	484.75
Soy Bean Oil Meal (49% protein)	175.00
Fish Meal	100.00
Alfalfa Meal (17% protein, dehydrated)	50.00
Dried Simmed Milk	100.00
Beet Sugar	50.00
Corn Oil	30.00
Trace Mineralized Salt	5.00
Dawes 4 B-Vitamin[a]	1.00
Vitamin A[b] and D Premix[c]	0.50
Dawes B-12 Vitamin Mix[a]	3.75
	1,000.00

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[a]Dawes Laboratories, Inc., Chicago, Ill.

[b]Chas. Pfizer and Co., Inc., New York, N.Y.

[c]Standard Brands Inc., Peekskill, N.Y.

APPENDIX II

CURRICULUM VITAE

NAME: Hess, Gerald Dale

DATE OF BIRTH: March 13, 1943

PLACE OF BIRTH: Lancaster, Pennsylvania, U.S.A.

SEX: Male MARITAL STATUS: Married

PRESENT ADDRESS: Endocrine Research Unit
Department of Physiology
Michigan State University
East Lansing, Michigan

EDUCATION:

Degree	Year	Institution	Major
B.A.	1965	Messiah College	Biology
M.S.	1968	Michigan State University	Physiology
Ph.D.	1970	Michigan State University	Physiology

HONORS:

(a) NIH Predoctoral Trainee, 1967-1970.
(b) Elected associate member Sigma Xi, 1968.
(c) Elected full member Sigma Xi, 1970.
(d) Participant, First Summer Course in Biology of Aging, sponsored by NIH, La Jolla, California, 1969.

RESEARCH PUBLICATIONS:

Hess, G. D., G. D. Riegle, and W. L. Frantz. Effects of chronic adrenocortical stimulation in the aging male rat. Federation Proc. 29:452, 1970 (Abstract).

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