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THE DEVELOPMENT OF THE ADRENAL AXIS IN THE NEONATAL RAT

by

Ronnie Guillet

Submitted in Partial Fulfillment

of the

Requirements for the Degree

DOCTOR OF PHILOSOPHY

Supervised by Dr. Sol M. Michaelson, D.V.M.

Department of Radiation Biology and Biophysics

The University of Rochester

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DISCLAIMER

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VITAE

The author was born [redacted]. She graduated from Bay Shore High School in Bay Shore, New York, in 1969. She received the B.S. degree in Biology from the State University of New York at Albany in 1973.

In September, 1973, she entered the University of Rochester School of Medicine and Dentistry as a graduate student in the Department of Radiation Biology and Biophysics. In November, 1975, she was accepted into the M.D.-Ph.D., Medical Scientist Training Program. She received the M.S. degree in Biophysics from the University of Rochester in 1976.

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ABSTRACT

Plasma corticosterone and ACTH concentrations were determined in neonatal rats 1, 7, 14, and 21 days old, under a variety of experimental conditions, to obtain more information on the postnatal development of the rat hypothalamo-adrenal (HHA) axis. Basal plasma corticosterone concentrations are high on day 1, very low on day 7, and are increased to adult levels by day 21. Adrenal responsiveness to stimulation follows a similar pattern with some response elicited by day 14 and an adult-like response by day 21. The absence of response on day 7 can be altered by treatment on days 1 - 6 with corticosterone or ACTH. Increased plasma corticosterone levels after ether exposure can be suppressed by treatment with dexamethasone injection three hours prior to stimulation on days 1, 14, and 21. Basal plasma ACTH is unchanged during the first three postnatal weeks. Direct stimulation of the pituitary with CRF results in a doubling of ACTH concentrations in all age rats tested. Ether exposure results in a doubling of ACTH levels in 14 and 21 day old rats, but not in 1 or 7 day old rats.

These results indicate that: 1) there is a diminution followed by an increase in responsiveness of the adrenal gland, but the pituitary response to direct hormonal stimulation is unchanged during the first three postnatal weeks; 2) continued stimulation of the adrenal by ACTH or of the central nervous system (CNS) or hypothalamus by corticosterone is necessary during early postnatal development to allow normal maturation of the HHA axis; and 3) feedback inhibition

is operative by birth, at least to a moderate degree. Taken together, the studies suggest that both the adrenal and pituitary glands are potentially functional at birth, but that the hypothalamic and CNS mediators of the stress response are not mature until at least the second or third postnatal week.

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INTRODUCTION/BACKGROUND

The development of a neonate to a mature, functional adult is a complex process. For the newborn rat, this process involves numerous, often critically timed, changes. Not only is the neonatal rat different from the adult in size (i.e. weight, length, surface-to-volume ratio), amount of hair, locomotor abilities, and the like, but the neonate is also very different from the adult physiologically. The rat is unable to regulate its body temperature until it is 2-3 weeks old (1-4). The rat is also not sexually mature until the 4th-6th (females) or 6th-8th (males) postnatal week (5,6). Plasma and pituitary hormone concentrations [T4 (7-10), corticosterone (11-16), growth hormone (17-19)] are different in different age rats, as is the ability of the animal to regulate these levels. These endocrine changes and many others are probably highly interrelated. As each individual system continues to evolve, the milieu in which the other systems exist changes. This will undoubtedly affect the subsequent evolution of each system.

The purpose of this study was to investigate the sequence of events that occurs during the maturation of the hypothalamo-hypophyseal-adrenal (HHA) axis. In order to do this, an initial series of experiments was designed to determine the pattern, as a function of age, of baseline and stimulated corticosterone concentrations in nonmanipulated neonatal rats. The stressors used included ether inhalation and hyperthermia. Thermal stress can be applied to the neonate in several

ways. In these experiments, exposure to microwaves was chosen as the primary mode of heating. Microwaves (2450 MHz, CW) have a penetration depth in tissue of the order of centimeters. As a result, they can provide a relatively quick, uniform, and reproducible means of heating the whole rat (20). Once the pattern was established, attempts were made to alter the pattern by various manipulations (corticosterone, ACTH, or Acthar ® injection) during early neonatal life. In this way, some of the controllers of the developmental evolution of the HHA axis could be defined or at least speculated upon. Another series of experiments was designed to ascertain the existence of feedback inhibition in neonatal rats. The presence or absence of negative feedback would further clarify the functional capabilities of the HHA axis and its components. Finally, basal and stimulated concentrations of plasma ACTH were investigated (with the technical assistance of Donna Gaudette, technician for Dr. John Kendall) in rats of different ages. The competence of the neonatal pituitary gland, as a function of age, was thereby examined. The results of all of these experiments were then consolidated to deduce which components of the HHA axis were functional at birth and which developed in the neonatal period; to consider possible interactions among components and between axis components and external factors that may influence normal development.

The HHA Axis

A. The mature rat:

The HHA axis is comprised of the hypothalamus, adenohypophysis (anterior pituitary), and adrenal glands (Figure 1).

Stimuli (neural signals) received by the central nervous system (CNS) may result in release of various neurotransmitters which control the part of the hypothalamus responsible for regulation of the secretion of pituitary hormones (21). In the case of the adrenal axis, corticotropin releasing factor (CRF) is released and transported from the median eminence to the pituitary via the portal circulation (22). CRF, in turn, stimulates the secretion of adrenocorticotropic hormone (ACTH) from the pituitary. The adrenal cortex synthesizes and releases corticosterone at an increased rate in response to elevated circulating ACTH levels (22). The secretion of corticosterone is determined by the rate of release of ACTH by the pituitary, under the stimulus of CRF from the hypothalamus. The rate of release of ACTH represents the balance between the excitatory influences of the CNS and the inhibitory influence of the circulating corticosterone on the pituitary or hypothalamus (23,24). A fall in plasma non-protein bound corticosterone leads to increased ACTH secretion, a rise inhibits ACTH secretion.

Plasma ACTH and corticosterone concentration (25) as well as CRF (26) show a cyclic variation over 24 hours. The light cycle is the main synchronizing factor in rat corticosterone circadian rhythm (27). The onset of the increasing phase of the rhythm occurs within the first 2 hours of the light phase for hypothalamic CRF content, between 2 and 4 hours after the beginning of the light phase for pituitary ACTH content, and between 4 and 6 hours for plasma corticosterone. Maximum CRF content is reached 2 hours before the end of the light period, maximum pituitary ACTH content and plasma corticosterone concentration at the end of the light phase (26).

The existence of feedback control of ACTH secretion by corticosteroids is well established, but the primary locus of inhibition is still under debate. Strong evidence has been provided implicating a direct action of corticosteroids on the pituitary (28-30). However, other findings suggest that the site of inhibition may be mainly at (31-33) or above (34-40) the hypothalamic level. Yates et al. (41) have shown that pituitary suppression due to dexamethasone, a potent glucocorticoid, saturates at a systemic dose of steroid between 5-10 µg/100 g. When the dose is increased, the pituitary sites become saturated and negative feedback sites in the brain for inhibition of CRF secretion are apparent. The sites in the brain that have been implicated in feedback control of the adrenocortical system include the hippocampus (34,35, 38-40), septum (36-38, 40), and amygdala (37, 39,40). These sites have been suggested on the basis of nuclear and neuronal binding studies (38-40), hormonal implants (32,34,36,37), and electrolytic lesions (35). Therefore, under different conditions the pituitary, hypothalamus, or higher brain centers, or any combination, may be involved in negative feedback control of the HHA axis. The suppressing effect of a given steroid on ACTH secretion may be due to the algebraic sum of the partial effects at each of the receptive sites (42). The intensity of the stress may be increased sufficiently, however, to overcome maximal corticosteroid-induced inhibition (32,41,43).

B. The neonate:

The primordia of the adrenal cortices in the rat first appear on the 13th fetal day. The cortical tissue differentiates into a thin subcapsular layer of small cells and a central mass of larger parenchymal cells arranged in irregular cords by the 17th day. Within a few days, the cortex becomes organized into three zones: a narrow zona glomerulosa, a broad zona fasciculata, and a broad reticularis. Between the 17th day and term, the cortical volume increases sevenfold, due to both cell multiplication and hypertrophy. At birth, the cortical volume drops abruptly, by approximately 25%, primarily due to cell atrophy (44). Growth resumes 7-9 days later, though at a slower rate. According to the hypothesis proposed by Deane (44), based on morphological considerations, the glomerulosa, both before and after birth, is an actively secreting tissue. Prenatally, the cells in the fasciculata and reticularis are also secretory. After birth, these cells shrink and the enclosed lipid droplets become enlarged. These alterations may be interpreted to mean that after birth the cells of the fasciculata secrete (presumably glucocorticoids) at a somewhat slower rate than prenatally and those of the reticularis decline still further in secretory activity (44).

Corticosterone, the primary circulating adrenocorticoid in the rat, varies in concentration with age (stage of maturation) of the animal. Malinowska et al. (15), using a competitive protein binding (CPB) assay, found that in the first half hour after birth, mean plasma levels are 12.6 $\pm$0.8 µg/dl. Thereafter, the plasma steroid concentration drops

steadily over the first 8 hours. A further drop occurs between days 2 - 3. Taylor and Howard (14), also using a CPB assay, determined plasma corticosterone in rats up to 29 days of age. Their results are comparable:

age (days)	0	2	3	5	7-8	14-16	27-29
plasma B µg/dl ± SEM	25.9±2.4	8.5±2.6	3.4±0.7	0.7±0.2	0.6±0.2	4.6±0.8	11.9±3.1

Much work has been done to determine whether the hypothalamo-hypophyseal-adrenal axis of the neonatal rat is sufficiently mature to respond to stress (45 - 51). In the 1950's and early 1960's, adrenal ascorbic acid (AAA) concentrations were used to determine if the animal responded to an imposed stress. In intact adult rats, the administration of ACTH, or an imposed stress which produced ACTH secretion from the pituitary, results in an immediate depletion of AAA. Jailer (45) found that the administration of ACTH to rats 4 - 6 days old caused a decrease in AAA. Administration of epinephrine did not result in a depletion of AAA until at least the 8th day, nor did hypothermia until the 16th day. Milković and Milković (46) reported similar results. However, even though newborn rats did not respond to a single injection of epinephrine until at least the 5th day, fetal rats did respond to stress (16). Therefore, they concluded that the nonresponsiveness of the neonate is not due to the supposed immaturity of the pituitary-adrenal system after birth.

Schapiro et al. (47) coined the term "stress-nonresponsive (SNR)" period to describe the early neonatal period. This term was later

modified to either absolute or relative SNR period, due to evidence that the AAA response matured earlier with respect to some stresses than to others (48).

Data which suggested that the neonate does respond to stress during the first few days of life were reported by many investigators beginning in the mid-1960's when more sensitive hormone assays became available (49 - 53). In the neonatal rat, there is a period of considerable pituitary-adrenal responsiveness to environmental stimulation immediately after birth, followed by a period of depressed responsiveness which develops between days 2 and 5 and lasts until some time prior to weaning (54). By three weeks of age, the ACTH and plasma corticosterone response to stress has been found to be functioning at approximately an adult level (54) (Figure 2). Stress-induced changes in hypothalamic CRF content occur on day 7, but not on day 2. Measurements at younger ages were not done (55). The period of depressed responsiveness has not been explained, nor has the locus of this apparent loss been determined.

Adult male rats show a circadian periodicity with respect to the secretory activity of the HHA axis (26). Experiments have been done to determine the age of onset of this rhythm. Allen and Kendall (11) found that the characteristic adult rat's circadian rhythm appeared on day 30 - 32, possibly as early as day 25. Ader (13) reported that the adrenocortical rhythm becomes evident somewhat earlier, in rats 21 - 25 days old. With appropriate stimulation (handling, daily shock beginning on day 1, etc.), the appearance of the rhythm can be accelerated to as early as day 16. Conversely, the circadian rhythm

may be suppressed at 30 days of age by administration of dexamethasone or hydrocortisone 2 - 4 days after birth. Similar doses of dexamethasone given 12 - 14 days after birth have no effect (56). More recently, Turner and Taylor (57) reported the existence of two distinct sensitive postnatal periods, one during the first postnatal week and the second during the third postnatal week. During the first week, some "facilitory substance involved in feedback regulation" may be in the process of differentiating and sustained high levels of plasma corticosterone may alter it. During the third week, the hippocampus (58) and suprachiasmatic nucleus (59), which have been shown to be involved in regulation of the adrenal diurnal rhythm are rapidly differentiating and maturing. Therefore, hormone administration at these times may alter subsequent development of glucocorticoid binding sites (57).

Administration of corticoids during the early neonatal period may also delay the onset and alter the temporal pattern of the pituitary-adrenal response to stress (60). Ulrich et al. (60) suggest that the adrenal capacity to maintain a sustained response to ACTH is impaired in cortisol-treated neonates, that pituitary ACTH and/or hypothalamic CRF release mechanisms are faulty, that pathways to the hypothalamus which transmit information about stress are permanently damaged by hormone treatment, or that differences exist in the ability of controls and treated rats to metabolize corticosterone. However, in a similar study, Schapiro (47) found that at 40 days of age, rats treated on day 2 with a single massive dose of cortisol had normal ACTH secretion in response to ether stress, even though body and adrenal weights were

significantly decreased.

Environmental conditions or stressful situations experienced during critical periods in development may also significantly affect the physiology and/or behavior of the adult animal (61 - 66). The responses of rats manipulated as neonates differ markedly from non-manipulated rats in both the temporal pattern and magnitude of their corticosterone response to acute noxious stimulation. Levine (63) has shown that manipulated rats make a more rapid and greater response to distinct and acute stressful conditions. However, when the stress is more chronic, the non-stimulated animal appears to exhibit the greater and more prolonged response. This was interpreted to mean that early stimulation endows the adult organism with the capacity to make responses more appropriate to the demands of the environment, including appropriate responses to stress (64, 65, 67). Levine and Mullins (67) contend that "handling, by causing variation of adrenal steroids in the infant animal, modifies the set point during a critical time in development so that it can vary in a gradual manner in the adult...In the nonhandled newborn rat there is less variation in adrenal steroid concentration during the critical period, and the set point develops fewer possible values."

Little is known about the development of negative feedback within the pituitary adrenal system in the neonate. Goldman et al. (54) suggest that although the mechanism controlling the acute activation of the pituitary-adrenal system is mature in weanlings, the negative feedback mechanism continues to increase in effectiveness between weaning and adulthood. Weanlings (25 days old) pretreated with

400 μg/100 g body weight (BW) dexamethasone phosphate showed a significant response to ether stress, although this response was suppressed by 50% compared to saline controls. In pretreated adults (65 days old), the response to ether was completely suppressed. This indicates that an immature corticoid feedback mechanism exists in weanlings (54). Goldman et al. (54) further suggest that the feedback deficit is due to a deficit in the extrahypothalamic inhibitory systems, one of three areas (anterior pituitary, hypothalamus, and extrahypothalamic CNS) that are involved in the sequence by which circulating adrenocorticoids inhibit secretion of ACTH.

In summary, newborn rats, 1 - 2 days old, have been shown to respond to stimuli with increased corticosterone concentration. Stress-responsiveness is depressed, however, during the latter part of the first week and most of the second week after birth. Stress-induced elevations in circulating corticosterone concentrations gradually approach adult magnitude during the third postnatal week. This pattern has been well established for the adrenal axis as a whole, but not for the isolated components or their interactions. In the papers that follow, experiments are described which provide additional information about the competence of individual elements of the axis and about their interrelationships, manifested as feedback inhibition, as a function of age. The effect on the developmental process of various early manipulations and its implications are also considered.

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FIGURE 1

Neuroendocrine Control of Adrenalcortical Function

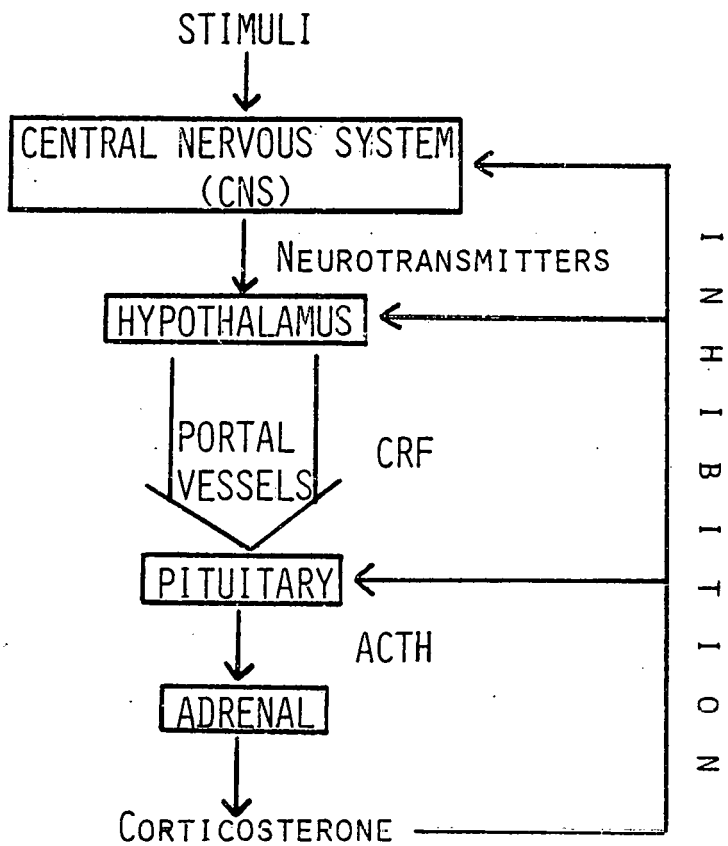
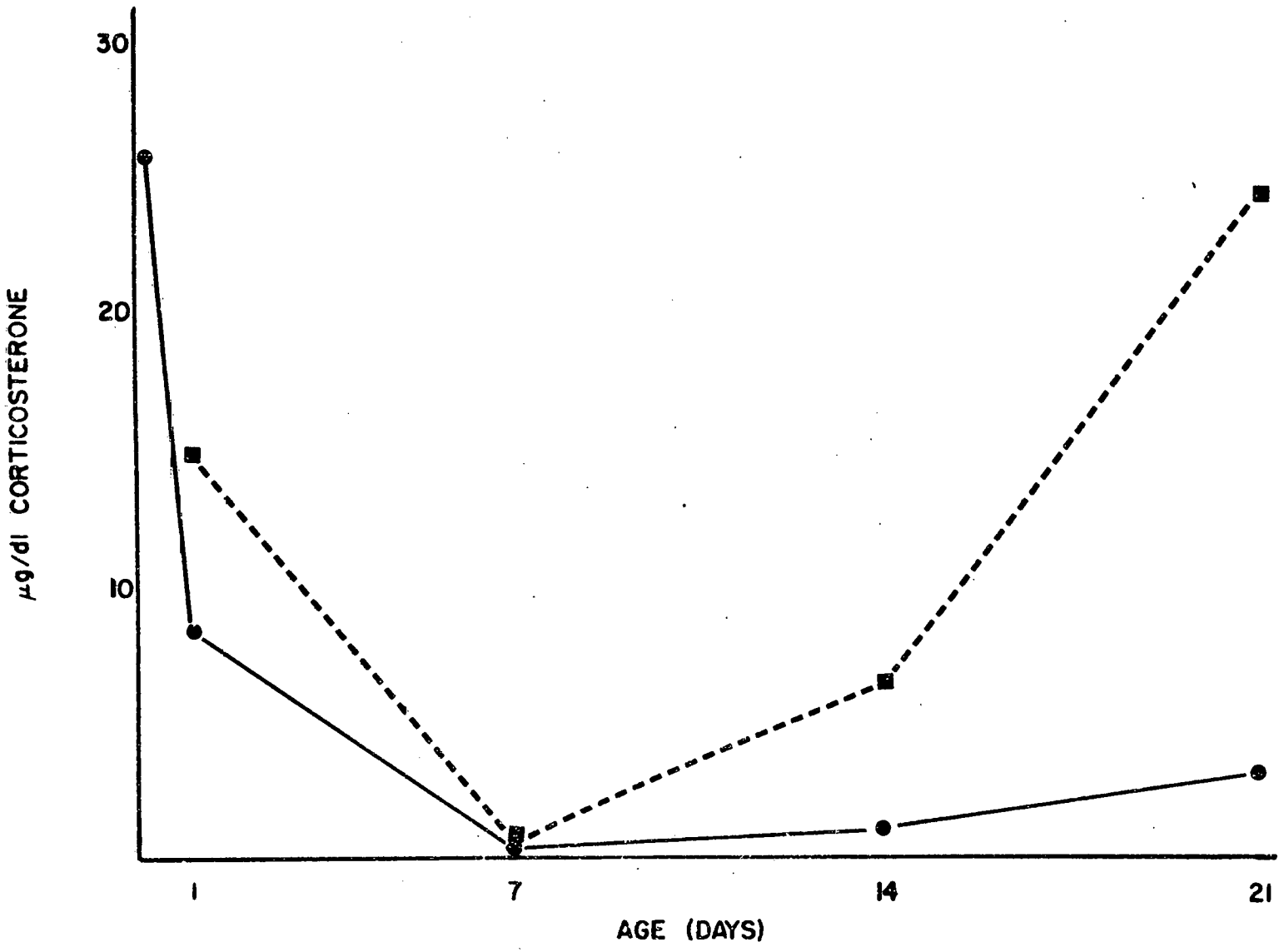

STIMULI
CENTRAL NERVOUS SYSTEM (CNS)
NEUROTRANSMITTERS
HYPOTHALAMUS
PORTAL VESSELS
CRF
PITUITARY
ACTH
ADRENAL
CORTICOSTERONE
INHIBITION

FIGURE 2

Basal (●—●) and ether-stimulated (■--■) corticosterone concentrations in rats 1, 7, 14, and 21 days old.


30
20
10
µg/dl CORTICOSTERONE
1
7
14
21
AGE (DAYS)

ABSTRACT

The Effect of Repeated Microwave Exposure in Neonatal Rats

Neonatal rats were exposed to 40 milliWatts (mW)/cm^2, 2450 MHz, CW, radiation for five minutes each day from day one to day six of life. On postnatal day seven, the rats were either sacrificed, exposed to 2450 MHz, CW, for a seventh time, or injected with ACTH. Twenty minutes after the final exposure or injection, the pups were sacrificed. Trunk blood and adrenal glands were saved. No difference was found in plasma corticosterone concentrations between the rats previously exposed to microwaves and control animals that were not exposed. Basal plasma corticosterone concentrations were less than 2 µg% in both groups. Following either microwave exposure or ACTH injection on day seven, plasma corticosterone levels remained low (< 3 µg%) in both exposed and control animals but were significantly increased in microwave exposed (2.08 µg% ± 0.85 SD) over control (0.72 ± 0.60 DS) animals. A statistically significant increase in adrenal wet weight was noted in animals exposed to microwaves on the first six postnatal days. The cause and biological significance of this enlargement are unknown. Because the first 2 - 3 weeks after birth are very critical in the development of the rat, effects of microwave exposure during this period may be readily manifested at this time or at a later stage in life.

INTRODUCTION

It is recognized that an individual may be more responsive to adverse factors during the early period in its life than at later stages. If an insult is capable of inducing subtle pathophysiologic alteration, it may be more apparent in this early period than at a later time. On the other hand, rather than leaving an imprint for later pathophysiology, an early insult may stimulate physiologic regulatory mechanisms or enhance developmental processes to compensate for the insult.

Previous studies in this laboratory (1) have provided evidence that pups from rats exposed on gestation day 16 to 40 mW/cm^2, 2450 MHz (CW) microwaves for one hour, show a higher basal corticosterone level during the first week and higher basal thyroxine level during the second and third weeks of life. It was concluded that the results of these studies suggest an uncoupling or change in set-point of hypothalmic-pituitary-adrenal or thyroid reactivity. The adrenal responsiveness of the neonate to stress was, however, not determined.

The first two weeks of life in rats were, until the mid-1960's, generally believed to constitute a relative "stress-non-responsive (SNR) period". Stress-induced increases in plasma corticosterone concentrations in neonatal rats were estimated indirectly to be minimal or entirely lacking as compared to those noted during subsequent periods of life (2,3,4,5,6). As assay techniques were refined, it became

possible to measure circulating corticosterone concentrations directly. Using a fluorometric or radiometric assay, it was found that rat pups as young as 1-2 days old could respond to a variety of stressors with increased plasma corticosterone levels (7,8,9). It is now generally acknowledged that the functional activity of the pituitary-adrenal axis is at least moderate during the first few days of life, decreases markedly and is minimal on days 4-11, then increases from day 11 to near adult levels by day 21 (10,11,12). Since functional changes in the adrenal axis are apparently occurring during the first week of life in the rat, a study was undertaken to determine if repeated acute stresses during this period would alter the response pattern. Microwave-induced hyperthermia was chosen as the stressor because exposure parameters could be well defined and the stress could be applied and removed abruptly. The resultant body temperature could be measured and the stress indicator (corticosterone) "quantified".

MATERIALS AND METHODS

Pregnant Long-Evans hooded rats (Blue Spruce Farms, Altamont, N.Y.), obtained on the 15th day of pregnancy, were housed individually in metal maternity cages. They were maintained at 24 ± 1° C, light cycle 0600 - 1800 h, and allowed food and water ad libitum. On the first day after birth (day 1), the litters were culled to 9 pups each. Thereafter, the pups were left undisturbed, except as noted below.

Beginning on day 1 until day 6, each pup was individually exposed to 2450 MHz, CW, 40 mW/cm^2 for 5 minutes each day. The pups were placed in styrofoam exposure cages (2" x 4" x 4") in the far-field of a standard gain S band horn antenna. A Raytheon, CMD-5 generator was used to produce the microwaves. The field was mapped with a Narda 8321 isotropic probe. The incident power was monitored with an H-P #430C meter. The ambient temperature within the exposure chamber was controlled at 34 ± 0.5° C (Figure 1). Rats were weighed daily preceding exposure and the rectal temperatures were taken every other day immediately following exposure. Control rats were treated similarly except that they were placed in exposure cages in an incubator (34 ± 1° C) instead of in the exposure chamber for 5 minutes each day for the first six days. The selection of 34° C was predicated by the "nest" temperature.

On day 7, to test adrenal responsiveness, one third of the pups were given ACTH (10 mU/100 g) i.p. and one third (control and experimental) exposed to 2450 MHz, CW, 40 mW/cm^2 for 5 minutes. These pups were sacrificed by decapitation 20 minutes following injection or

exposure. The remaining pups were sacrificed immediately after removal from the nest. Trunk blood was collected in tubes containing Na-EDTA. The blood was spun for 20 minutes at 2500 rpm, plasma removed and frozen at -20° C until assay for corticosterone. The plasma was assayed by the competitive protein binding assay developed by Murphy (13): the sample was extracted using ethanol; the source of CBG (corticosteroid binding globulin) was dog plasma; and dextran-coated charcoal was used to separate bound from free hormone. The adrenals were removed, weighed, and preserved in buffered formalin for future study.

RESULTS

Average body weights for all exposed (n = 18) and control (n = 18) rats are shown in Figure 2. There was no significant difference in growth rate between exposed and control rats, but the exposed rats demonstrated a slightly greater growth rate during the first 6 days of life.

Colonic temperature following microwave exposure or incubation at 34^{o} C is shown in Figure 3. Although maintained at comparable ambient temperature, the microwave exposed rats had a 1.5^{o} - 2.5^{o} C higher colonic temperature than non-exposed rats.

Plasma corticosterone concentrations in control and exposed pups that were sacrificed immediately, injected with ACTH (10 mU/100 g) or exposed to 2450 MHz, CW, 40 mW/cm^2 incident energy for 5 minutes are shown in Table I. It is apparent that in the seven-day old rat the basal corticosterone level is not altered by previous exposure to microwaves. The ratlets exposed to 40 mW/cm^2 microwaves show an adrenal responsiveness not quantitatively different from that produced by ACTH administration.

As indicated in Figure 4, adrenal wet weight and adrenal-to-body weight ratios in seven-day old rats are significantly higher in microwave exposed animals in comparison to controls.

DISCUSSION

Whether exposure to microwaves during the gestational or early neonatal period perturbs or actually modifies ontogeny of neuroendocrine responsiveness, which involves neural as well as hormonal facets and is intimately involved in conjunction with blood circulation in temperature regulation, is an important consideration that should be investigated. The effects of microwaves on the developing animal have not been adequately investigated. Such studies would be of significance in obtaining knowledge of maturational biology. It is also possible that certain times in neonatal life are "critical", and the presence or absence of specific or general stress at a given moment may result in a permanent deficit or delay in development. The importance of the central nervous system and brain development in maturation processes makes them potentially more susceptible to derangement than other factors involved in maturation. Assessment of stress responsiveness provides a tool for dissecting out the ontogeny of a particular regulation while also pinpointing perturbations which could suggest possible hazards to man.

In the present study newborn rats subjected to daily 5-minute exposures to 40 mW/cm^2, 2450 MHz, CW, microwaves at 34° C ambient temperature for six days did not result in apparent deleterious effects in spite of a 1.5° - 2.5° C body temperature rise. Growth rate was comparable for exposed and control rats maintained at similar (34° C) ambient temperature. It is presumed that if such exposure to microwaves were deleterious, weight gain, which is a very sensitive indicator of

general development, would be depressed. It should be pointed out, however, that lack of evidence of influence on growth rate during this early period, does not preclude future alteration of growth rate.

Based on water calorimetry studies in this laboratory (14), 40 mW/cm^2 incident power intensity with resultant 2 - 3° C rise in temperature is equivalent to 9 - 10 W/kg absorbed energy in these animals.

Of considerable interest is the finding that six daily exposures of newborn rats to 40 mW/cm^2 incident energy did not change basal corticosterone levels. There was no change in adrenal responsiveness to ACTH injection and the adrenal response to microwave exposure was comparable to ACTH injection. In this context, we had previously noted (15) that the hematologic response of adult dogs, exposed to 100 - 165 mW/cm^2, 2880 MHz pulsed microwaves for 1 - 3 hours, "resembles that reported to occur after slow continuous ACTH injection and may be indicative of hypothalamic or adrenal stimulation (stress effect)".

As compared with control rats, the rats exposed daily to microwaves did show a slightly greater adrenal responsiveness to ACTH or microwave-induced hyperthermia and a significantly increased ($p < .05$) adrenal wet weight. The magnitude of the increased adrenal weight is similar to that seen following injected Acthar (ACTH in depot form) 5 mU/g on days 1 - 6 (unpublished results). This measured adrenal weight may be functionally significant. It will be necessary to perform histologic examination of these glands to determine whether the larger weight is due to increased numbers of functional adrenal cortical cells or connective tissue, fat, etc. Although such increased

adrenal weight and responsiveness as a result of microwave exposure may be indicative of microwave, or thermally induced enhancement of developmental processes, additional study is indicated to establish this intriguing possibility. Increased numbers of animals, histologic examination of the adrenals and follow-up study to examine late effects of early neonatal exposure are now underway in our laboratory.

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TABLE I

Plasma Corticosterone Concentration[1] in Neonatal Rats

Treatments, Days 1-6	Treatment on Day 7		
	Control	ACTH (10 mU/100 g)	2450 MHz (40 mW/cm^2) 5 min
Incubated Pups	0.73 ± 0.45 (3)	1.63 ± 0.45 (3)	0.72 ± 0.60 (3)[a]
Radiated Pups	0.67 ± 0.24 (5)[b]	2.29 ± 0.60 (6)[c]	2.08 ± 0.85 (6)[d]
Control Pups[2]	0.58 ± 0.20 (3)[e]	1.32 ± 0.28 (3)[f]	1.82 ± 0.43 (3)[g]

[1] µg/100 ml, mean ± S.D. (n).

[2] Pups remained with dams until 7th day.

[a-d] $p < .05$; [b-c] $p < .001$; [b-d] $p < .01$; [e-f] $p < .05$; [e-g] $p < .05$.

FIGURE 1

Schematic layout of the controlled-temperature system for exposing neonatal rats to 2450 MHz microwave radiation.

CONTROLLED TEMPERATURE / MICROWAVE EXPOSURE UNIT

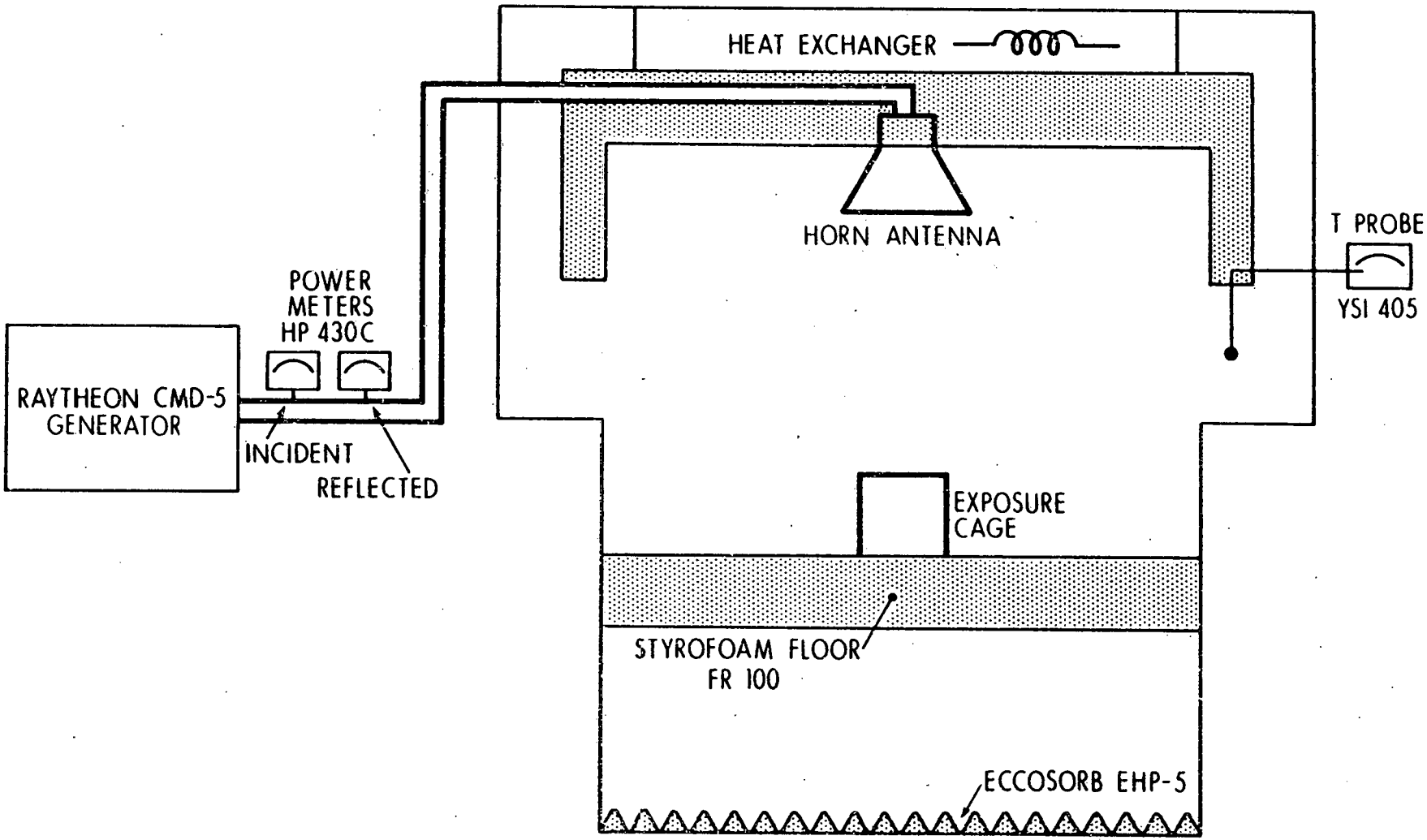


FIGURE 2

Averaged mass in grams of neonatal rats from first through seventh day of age. Eighteen pups were exposed six or seven times for five minutes to 2450 MHz CW microwave radiation at a power density of 40 mW/cm^2. Eighteen control pups were sham-radiated. All 36 pups were maintained at the "maternal temperature" of 34^{o} C during radiation or sham radiation.

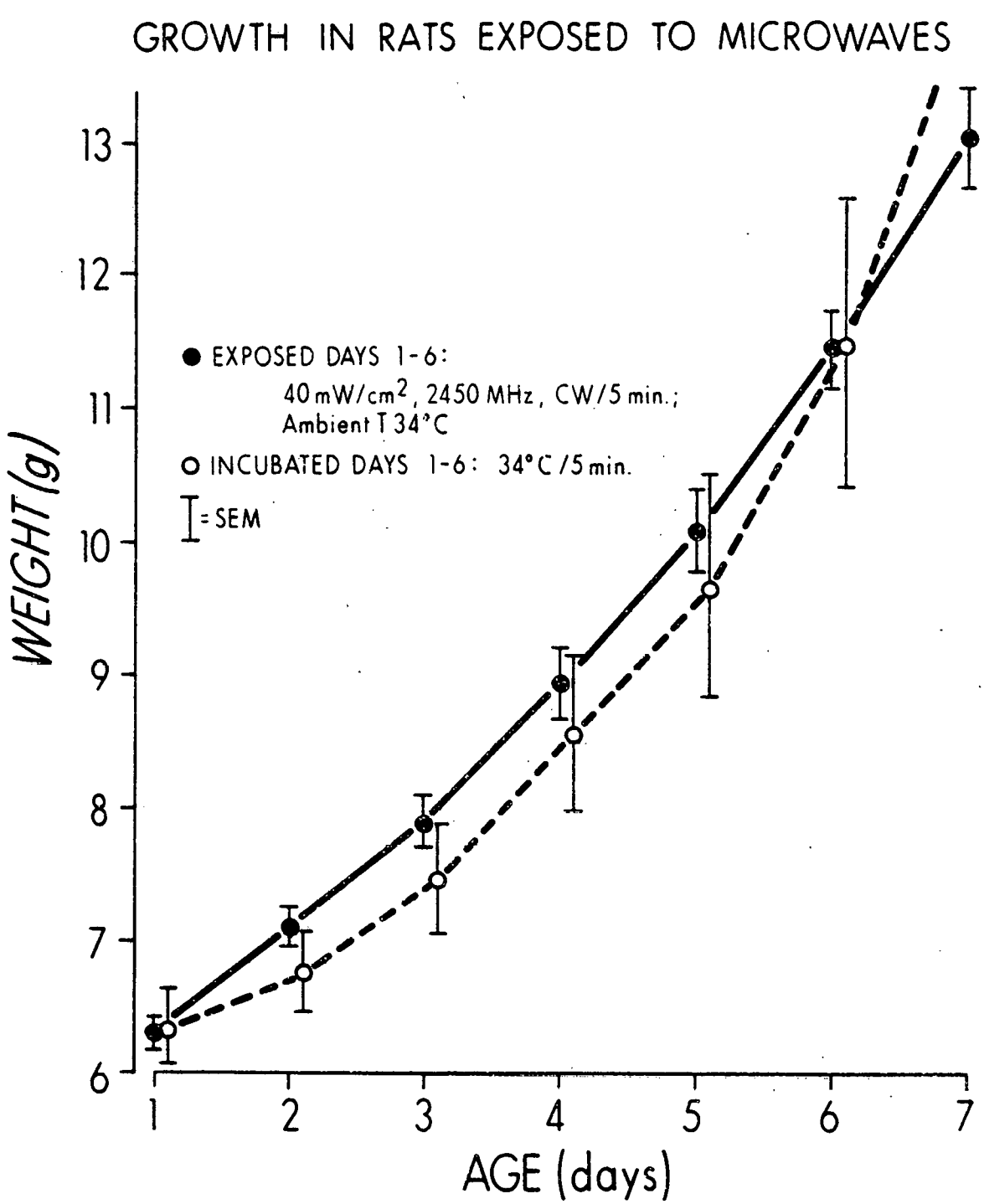

GROWTH IN RATS EXPOSED TO MICROWAVES
WEIGHT (g)
13
12
11
10
9
8
7
6
● EXPOSED DAYS 1-6:
40 mW/cm², 2450 MHz, CW/5 min.;
Ambient T 34°C
○ INCUBATED DAYS 1-6: 34°C/5 min.
I = SEM
1
2
3
4
5
6
7
AGE (days)

FIGURE 3

(Upper Portion): Averaged, post-exposure colonic temperatures of neonatal rats, 18 of which (filled circles) were radiated for five min. each day, days 1 through 6 or 7, by 2450 MHz microwaves at a power density of 40 mW/cm^2. Eighteen pups were sham-radiated (open circles) in an incubator. Environmental temperature for both conditions of exposure was 34^{o} C. (Lower Portion): Averaged increments (Δ Ts) of colonic temperature, post- vs. pre-exposure, of the 18 radiated pups.

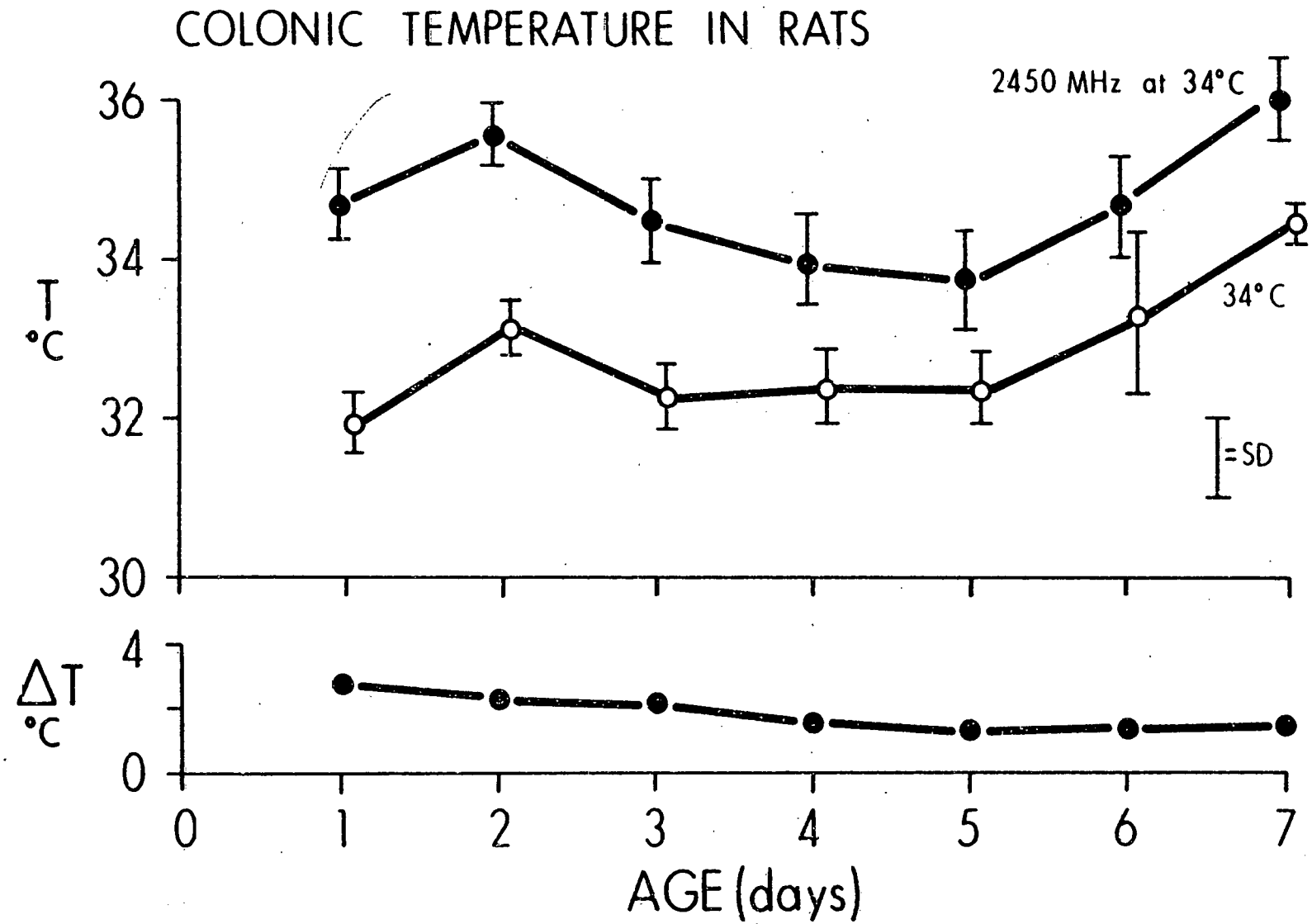
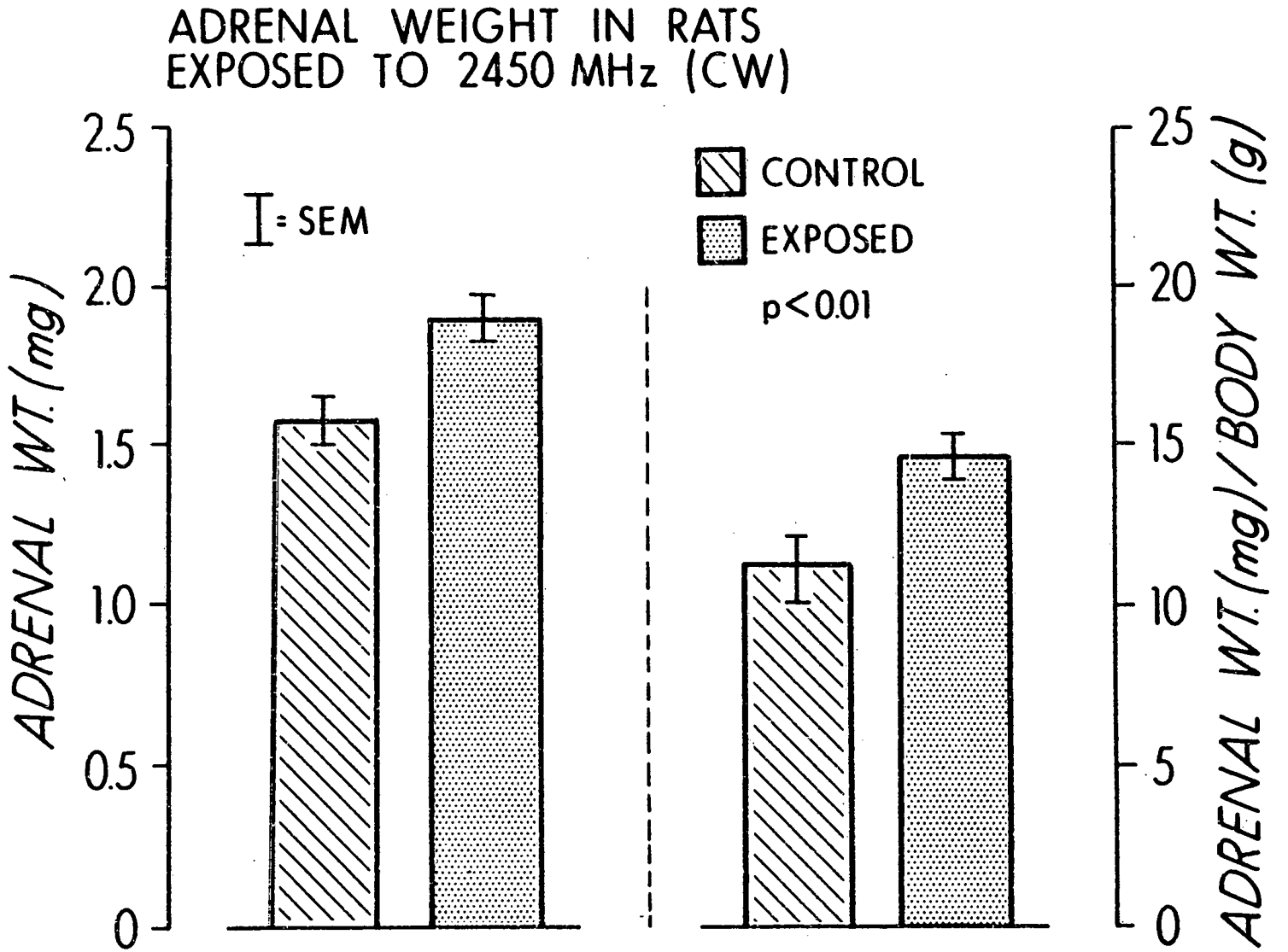

COLONIC TEMPERATURE IN RATS
2450 MHz at 34°C
34°C
= SD
T °C
ΔT °C
36
34
32
30
4
0
0
1
2
3
4
5
6
7
AGE (days)

FIGURE 4

Averaged adrenal mass (to reader's left) and averaged ratio of adrenal-to body-mass (to reader's right) of 18 sham-radiated controls and of 18 pups that had been radiated six or seven times by microwaves. The adrenal glands were harvested on post-natal day 7.


ADRENAL WEIGHT IN RATS
EXPOSED TO 2450 MHz (CW)
ADRENAL WT. (mg)
2.5
2.0
1.5
1.0
0.5
0
I = SEM
CONTROL
EXPOSED
p<0.01
25
20
15
10
5
0
ADRENAL WT. (mg) / BODY WT. (g)

ABSTRACT

Pituitary and Adrenal Responsiveness in Neonatal Rats

Neonatal rats were, until recently, believed to be relatively non-responsive to stress. With improved methods now available, rat pups as young as 1 - 2 days of age have been found to respond to stress with increased plasma corticosterone (B), are much less responsive at 4 - 11 days, and increasingly responsive thereafter, reaching adult responsiveness at about 21 days of age. We reexamined the responsiveness of neonatal rats to administration of a crude preparation of corticotropin releasing factor (CRF) and ACTH to determine the locus of the variation of the response. Plasma B increased in 1-day-old rats after ACTH or CRF. In 7-day-olds, both stimuli caused negligible increases in plasma B. Some response after these stimuli was elicited in 14-day-olds, and the responses of 21-day-olds approached those in adults. Pretreatment for the first 6 postnatal days with ACTH or coticosterone resulted in a moderate increase in plasma B after CRF or ACTH on day 7. The postnatal fall in sensitivity is partially explained by a decreased adrenal sensitivity to ACTH, perhaps due to an immature central nervous component and/or a lack of continued in utero stimulation.

INTRODUCTION

The first two weeks of life in rats were, until the mid-1960's, generally believed to constitute a relative "stress-non-responsive" (SNR) period. Stress-induced increases in plasma corticosterone concentrations in neonatal rats were estimated indirectly to be minimal or entirely lacking as compared to those noted during subsequent periods of life (1). As measurement techniques were refined, circulating hormone concentrations could be measured directly. Using a fluorometric or radiometric assay, rat pups as young as 1 - 2 days old were found to respond to a variety of stresses with increased plasma corticosterone levels (3 - 7).

The functional activity of the pituitary-adrenal axis is now generally acknowledged to be at least moderate during the first few days of life, decrease markedly and be minimal on days 4 - 11, then increase from day 11 to near adult levels by day 21 (8 - 11). Since functional changes in the adrenal axis are apparently occurring during the first week of life in the rat, a study was undertaken to determine if the rapid loss of responsiveness during this time could be prevented. Components of the axis were thus manipulated during the first week and the corticosterone response to ACTH or median eminence extract on day 7 was measured.

MATERIALS AND METHODS

Pregnant Long-Evans hooded rats (Blue Spruce Farms, Altamont, N.Y.), obtained on the 15th day of gestation, were housed individually in wire mesh maternity cages (42 x 36 x 24 cm) containing wood shavings. They were maintained at 24 ± 1 C, 40 - 60% humidity, light cycle 0600 - 1800 h and allowed food and water ad libitum. On the first day after birth (day 1), the litters were culled to 9 pups each by choosing the heaviest, healthiest appearing animals. Thereafter, the pups were left undisturbed, except as noted below.

A. Basal, ACTH- and CRF-stimulated corticosterone concentrations in rats 1 - 21 days old:

Control rats 1, 7, 14, and 21 days old were rapidly decapitated within 5 minutes of removal from their home cages. Trunk blood was collected in tubes containing Na-EDTA and the plasma was separated and frozen at -20 C until time of assay. Two additional groups of rats of similar age were injected i.p. with either 10 mU ACTH/100 g body weight or an extract with CRF activity equivalent to that in one adult rat median eminence (kindly supplied by Dr. Murray Saffran, Toledo, Ohio), and sacrificed twenty minutes later. Plasma was saved as above.

B. Basal, ACTH- and CRF-stimulated corticosterone concentrations in rats 7 days old, treated on days 1 - 6 or day 4 only:

After being culled on day 1, litters were randomly assigned to one of the following treatment regimens: a) non-manipulated:

pups in cage tray, without dam, removed to room temperature for one hour, days 1 - 6; b) handled: pups picked up daily, days 1 - 6; c) saline: pups injected with 5 µl/g, i.p. daily, days 1 - 6; d) ACTH: pups injected daily with 5 µl/g (0.1 mU/g), i.p. days 1 - 6; e) corticosterone: pups injected daily with 5 µl/g (10 µg/g), i.p. days 1 - 6; f) diluent: pups injected daily with vehicle used in g), 5 µl/g, subcutaneously in the back of the neck, days 1 - 6; g) Acthar (ACTH in depot form, Parke Davis): pups injected daily with 5 µl/g (50 mU/g), subcutaneously in the back of the neck, days 1 - 6 or day 4 only. On day 7, each litter was divided into groups of 3 - 5 pups, and subjected to one of the following: 1) sacrificed immediately upon removal from cage; 2) saline injection - 5 µl/g; 3) ACTH injection - 5 µl/g (10 mU/100 g); 4) median eminence (ME) extract injection - 1 ME/pup (in 5 µl/g). Twenty minutes later the pups were sacrificed by decapitation; trunk blood was saved. Plasma was stored at -20 C until assay.

C. Assay for plasma corticosterone:

Plasma corticosterone concentrations were measured by the competitive protein binding method of Murphy (12, 13). A minimum of 20 µl plasma was used for each determination. Where possible, 50 µl plasma were used for better sensitivity. Tritiated corticosterone (1, 2 ^{3}H-B) was obtained from New England Nuclear Co. (Boston, Massachusetts).

D. Statistical methods:

Two sample Student's t-tests were done, comparing basal or stimulated corticosterone concentrations as a function of age in non-manipulated rats. The variance within both the control and treatment groups was analyzed. These groups were then pooled and a two sample Student's t-test done to compare control and pretreated groups.

RESULTS

Basal and ACTH- or CRF-stimulated corticosterone concentrations in rats 1 - 21 days old (Figure 1):

Corticosterone levels were elevated at birth and decreased rapidly during the first 24 postnatal hours. Corticosterone levels in untreated rats remained low until at least day 14 (1.08 ± 0.46, n = 6)*, after which they reached adult levels by day 21 (3.19 ± 2.07, n = 8). Following injection of ACTH or ME, corticosterone concentrations increased ($p < .005$) in 1 day old rats (ACTH: 24.26 ± 4.69, n = 10; ME: 22.98 ± 5.69, n = 14), but not in 7 day old rats (ACTH: 1.90 ± 1.16, n = 10; ME: 1.71 ± 0.94, n = 16). Increased corticosterone ($p < .005$) in response to ACTH (3.90 ± 1.69, n = 11) or ME (4.36 ± 0.82, n = 12) reappeared by day 14 and approached adult range by day 21 (ACTH: 20.71 ± 4.68, n = 10; ME: 19.88 ± 7.34, n = 12). The older rats probably responded to the stimulus of handling or injection because response to saline injection was comparable to responses to ACTH, ME, or ether.

Basal and ACTH- or CRF-stimulated corticosterone concentrations in rats 7 days old, pre-treated on days 1 - 6 with corticosterone, ACTH, or Acthar or day 4 only with Acthar (Figure 2, Table 1):

The four control groups (a - d) were analyzed and combined when no differences using a one-way analysis of variance were found among them. Basal corticosterone levels in both the control and treated

* $\bar{x}$ ± SD, n

groups (e, f, g) were low (0.76 ± 0.58, n = 14) and there was no significant difference among them ($p > .05$). ACTH- or ME-stimulated corticosterone levels remained low in control animals (1.97 ± 0.89, n = 49) but were significantly ($p < .001$) increased following ACTH injection in animals that received pretreatment on days 1 - 6 with corticosterone (3.25 ± 1.47, n = 22), ACTH (3.26 ± 1.85, n = 25), Acthar (5.53 ± 5.03, n = 12), or Acthar on day 4 only (4.40 ± 0.94, n = 5). Corticosterone levels were also significantly ($p < .001$) increased following ME injection in animals pretreated with Acthar on day 4 only (4.73 ± 2.29, n = 20).

DISCUSSION

The adrenal axis is a complex system of cerebral, hypothalamic, pituitary, and adrenal components. The competence and coupling of these separate parts is essential for optimal functioning of the axis. The system as a whole is not active if any one of the subunits does not synthesize and secrete its appropriate hormone, if the secreted hormone does not reach the target tissue in sufficient concentrations, or if the target tissue does not respond to adequate amounts of the hormone.

In the rat, brain development continues for weeks after birth; more primitive, vital parts of the brain (e.g., brain stem) are functional at birth, while "higher centers" (e.g., cerebral cortex) are not "adult-like" until at least 4 - 6 weeks after birth (16). The rat adrenal axis is not completely competent by birth. Only when all parts of the axis have attained maturity and are functionally connected is a coordinated (stress) response possible. The time course of development of different levels of functioning as determined by intervention and measurement of hormones within the hierarchy of the adrenal axis provides a clue to the developmental sequence of the components of the axis and their interconnections. This information can then be applied to other related systems in order to learn more about development in general.

There is a striking change in both basal and stimulated plasma corticosterone concentrations during the first three postnatal weeks of life. Basal hormone levels are high ($\sim$ 20 µg/dl) at birth but

rapidly decline during the first 24 hours of life (8 - 11). The initially elevated concentration may be due to an increase in hormone secretion in the newborn rat in response to the stress of birth, maternal corticosterone that has crossed the placenta prior to birth, or a yet-undefined developmental process. By themselves, the first two possibilities cannot explain the changes that occur. Stress effects of birth, if they exist, minimally affect basal corticosterone levels. Plasma corticosterone concentrations in rat fetuses 20-1/2 and 21-1/2 days of gestation are higher than those in newborn rats (4, 6, 16). Corticosterone levels begin to decline before birth at a rate comparable to that seen during the first day postpartum. In addition, during the last two days of gestation, a gradient between maternal and fetal corticosterone concentrations exists, with higher levels in fetal blood than in maternal blood (6, 7, 16). This implies either a concentrating mechanism in the fetus or fetal production of hormone. Experiments in which pregnant rats, adrenalectomized on day 9 of gestation, as well as their fetuses showed normal basal hormone levels, support the concept of fetal production (16). Further evidence of the new-born rat's ability to synthesize and release corticosterone is the rise in hormone levels 20 minutes after injection of ACTH or ME. Both the adrenal and pituitary glands in one-day-old rats are therefore capable of responding to appropriate stimulation.

According to our studies, by seven days of age, basal plasma corticosterone concentrations are $\leq$ 1 µg/dl, which is consistently lower than those in 1, 14, and 21 day old rats. Injection of ACTH or

ME results in little rise in plasma corticosterone after 20 minutes. In contrast to those of one-day-old rats, the adrenal and pituitary glands of 7-day-old rats do not respond appreciably to appropriate stimulation. It is unlikely that all of the corticosterone synthetic and secretory machinary is lost during the first week of life and is reestablished. Rather, the gland's gradual loss of sensitivity to stimulation is perhaps due to a lack of continued trophic input. Prenatally, maternal factors may have provided necessary stimulation; an immature adrenal axis in the newborn may be unable to do so.

The second series of experiments attempted to test this hypothesis. Daily stimulation of the rat's adrenal gland with ACTH for the first six postnatal days increased the responsiveness of the adrenal on day 7. Plasma corticosterone concentrations following ACTH injection on day 7 in these pretreated animals were 4 - 5 μg/dl. While these levels are not as high as those seen in 1 or 21 day old rats, they represent a significant ($p < .001$) elevation in hormone levels. Keeping the pup's adrenals "primed" allowed a greater magnitude of response to stimulation on day 7. Curiously, this effect could also be produced using corticosterone as the pretreatment on days 1 - 6. Pups injected with corticosterone daily for the first six days postpartum had an increased plasma corticosterone concentration (3.79 ± 1.46) 20 minutes after ACTH injection.

The exogenous corticosterone probably does not act directly on the adrenal gland as was postulated in the case of ACTH pretreatment. Corticosterone, acting at the pituitary or hypothalamus in a negative feedback fashion, would be expected to decrease rather than increase

responsiveness to stimulation. Instead, corticosterone may be necessary at the level of the cerebral cortex, hypothalamus, or pituitary during critical periods for development of control and sensitivity of the adrenal axis. Brain corticosterone concentrations vary during the first three weeks of life in the mouse (17), increase beginning on day 13, peak at days 18 - 20, then decline to the 13 day level by day 30. Exogenous corticosterone during the first six postnatal days may produce changes in brain corticosterone that mimic the developmental changes that normally occur a week later. These changes, either natural or induced, may be responsible and necessary for integration of adrenal axis function. Measurement of brain corticosterone concentrations in pretreated animals is necessary to test this hypothesis.

In summary, in reexamining the neonatal rat responsiveness to stress (ether), ACTH injection, and ME injection, we have found a changing sensitivity of the adrenal gland to stimulation during the first three postnatal weeks. This change may be due to the progressive maturation of the components of the adrenal axis. Both the pituitary and adrenal glands apparently are potentially functional by the time of birth. Lack of continued tonic stimulation, due perhaps to an immature central nervous system, may result in a progressive loss of responsiveness. Either experimentally using ACTH or, oddly, corticosterone during the first week of life, or naturally during the second and third weeks, this process can be reversed and responsiveness maintained or restored.

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TABLE 1

PROBABILITY (p) VALUES FOR THE STUDIES ILLUSTRATED IN FIGURE 2

(handling + diluent + non-manipulated + saline), sacrificed vs. (corticosterone + ACTH + Acthar + Acthar day 4), sacrificed	$p < .10$
(handling + diluent + non-manipulated + saline), ACTH day 7 vs. (corticosterone + ACTH + Acthar + Acthar day 4), ACTH day 7	$p < .005$
(handling + diluent + non-manipulated + saline), sacrificed vs. (handling + diluent + non-manipulated + saline), ACTH day 7	$p < .005$
(corticosterone + ACTH + Acthar + Acthar day 4), sacrificed vs. (corticosterone + ACTH + Acthar + Acthar day 4), ACTH day 7	$p < .005$

FIGURE 1

Basal and ACTH- or CRF-stimulated plasma corticosterone concentrations in rats 1, 7, 14, and 21 days old. Shown are the mean + SD. The numbers of animals for each determination are indicated above the appropriate bar.

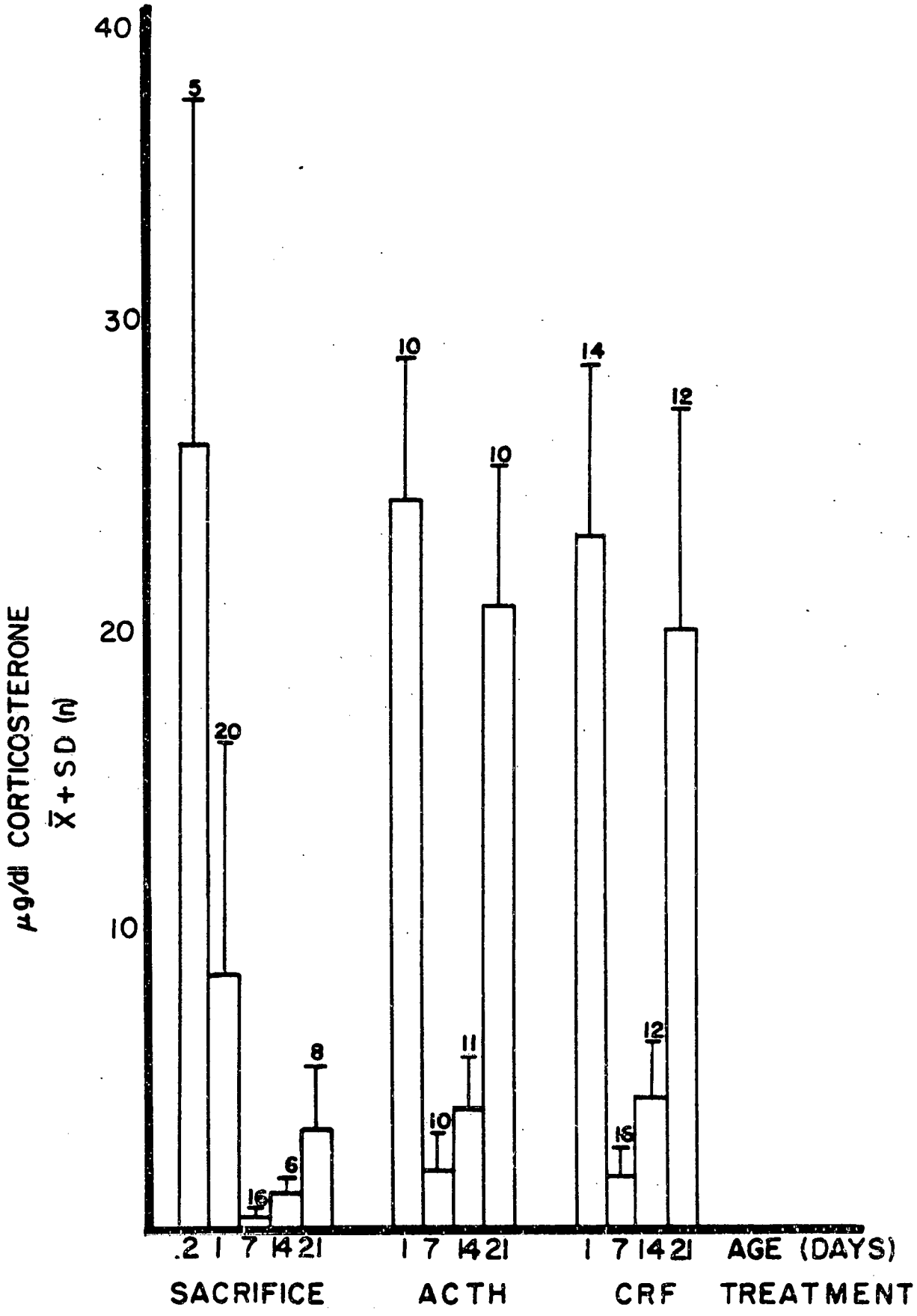
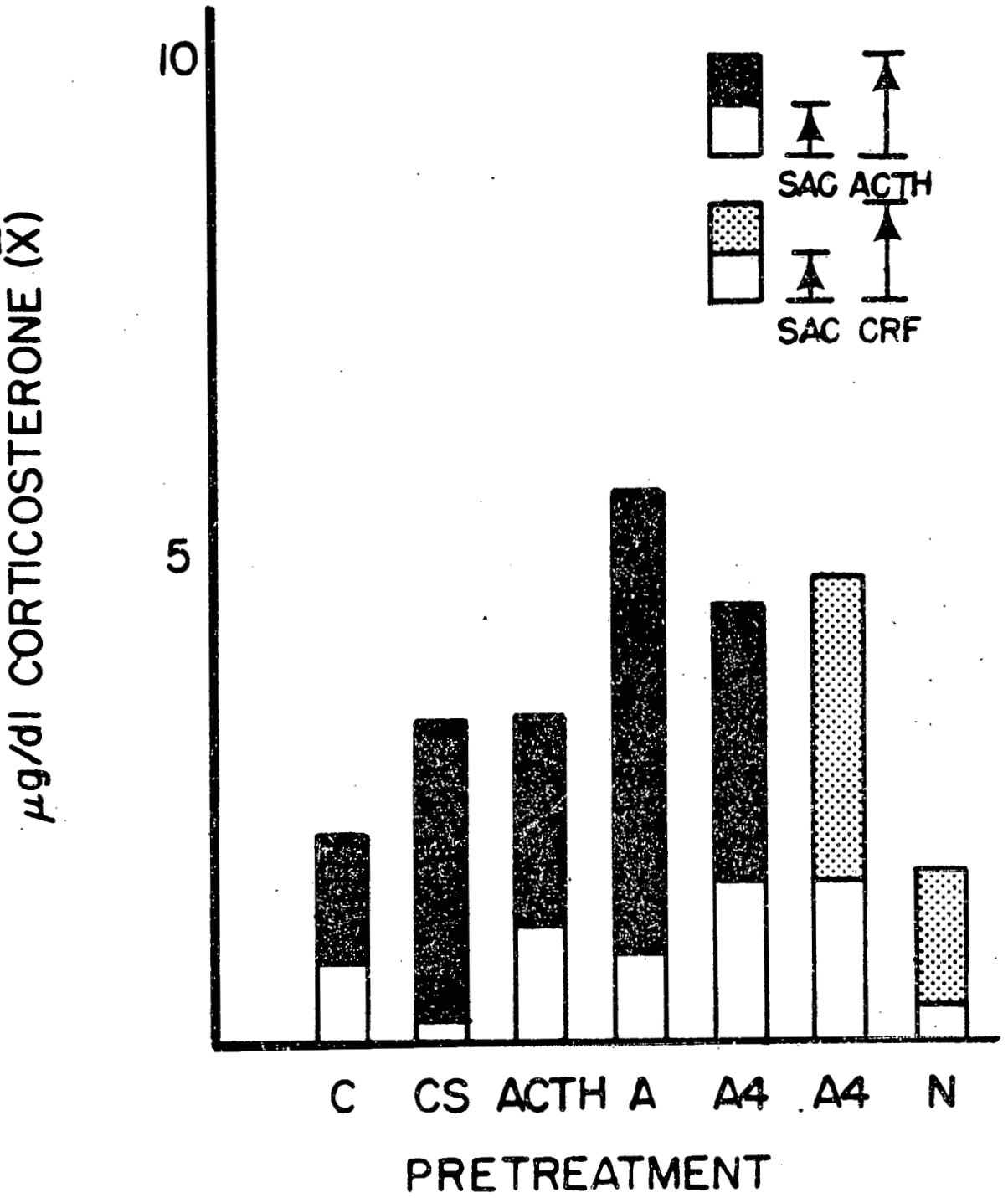

40
30
20
10
μg/dl CORTICOSTERONE
X̄ + SD (n)
5
20
16
6
8
10
10
11
14
16
12
12
.2 1 7 14 21
1 7 14 21
1 7 14 21
AGE (DAYS)
SACRIFICE
ACTH
CRF
TREATMENT

FIGURE 2

Basal and ACTH- or CRF- stimulated plasma corticosterone concentrations in 7-day-old rats, pretreated on days 1 - 6, or day 4 only, as follows: C - pooled controls (see text for details); CS - corticosterone days 1 - 6, 10 µg/g; ACTH - ACTH days 1 - 6, 10 mU/100 g; A - Acthar days 1 - 6, 50 mU/g; A4 - Acthar day 4 only, 50 mU/g; N - nonmanipulated days 1 - 6.


10
5
μg/dl CORTICOSTERONE (X̄)
SAC ACTH
SAC CRF
C
CS
ACTH
A
A4
A4
N
PRETREATMENT

ABSTRACT

Development of Feedback Inhibition in the Adrenal Axis of the Neonatal Rat

Neonatal rats were, until recently, believed to be relatively non-responsive to stress. With improved methods now available, rat pups as young as 1 - 2 days of age were found to respond to stress with increased plasma corticosterone concentrations, were much less responsive at 4 - 11 days, and increasingly responsive thereafter, reaching adult responsiveness at about 21 days of age. We reexamined the responsiveness of neonatal rats to ether stress and tested suppressibility of the response by prior dexamethasone administration. Plasma corticosterone was significantly ($p < .005$) lower in pups (1, 14, and 21 days old) injected with dexamethasone three hours before exposure to ether. Seven-day-old pups did not have a significant increase in corticosterone levels after ether exposure, so suppressibility could not be tested. Suppression of plasma corticosterone by dexamethasone demonstrates the presence of feedback control of adrenal corticosteroid secretion in rats as young as one day old.

INTRODUCTION

The negative feedback influence of corticosteroids on ACTH secretion is well established (1). Low doses of corticosteroids can inhibit ACTH secretion in the absence of stress; however, sufficiently strong, acute stressors can override this inhibitory effect (2). The primary site of feedback control is believed to be the adenohypophysis, with some evidence of hypothalamic participation (1).

The existence of feedback inhibition in the neonatal rat has been considered but has not been tested. Inhibitory effects of high circulating levels of corticosterone immediately after birth have been invoked to explain the reduction in adrenal size and weight during the first few postnatal days (3) and relative "stress-nonresponsiveness" during the same period (4). In this series of experiments, the presence of "negative feedback" was examined in rats 1 - 21 days old by measuring plasma corticosterone concentrations in rats exposed to ether, with and without prior treatment with dexamethasone.

METHODS

Pregnant Long Evans hooded rats (Blue Spruce Farms, Altamont, N.Y.), obtained on the 15th day of gestation, were housed individually in wire mesh maternity cages (42 x 36 x 24 cm) containing wood shavings. They were maintained at 24 ± 1 C, 40 - 60% humidity, light cycle 0600 - 1800 h, and allowed food and water ad libitum. On the first day after birth (day 1), the litters were culled to 9 - 10 pups each by choosing the heaviest, most healthy-appearing animals. Thereafter, the pups were left undisturbed, except as noted below.

At 1, 7, 14, and 21 days of age, animals were assigned randomly to one of four groups: 1) untreated, sacrificed; 2) ether exposure (45 - 60 sec) followed by sacrifice 3 hours later; 3) dexamethasone, i.p. (25 mg/100 g BW), followed by sacrifice 3 hours later; 4) dexamethasone, i.p. (25 mg/100 g BW), ether exposure (45 - 60 sec) 3 hours later, followed by sacrifice 20 minutes later. All dexamethasone injections were done at 0830; ether exposures at 1130; sacrifices at 1100 - 1200. Animals were rapidly sacrificed by decapitation and trunk blood saved in tubes containing Na-EDTA. The blood was centrifuged and plasma frozen at -20 C until assayed.

Plasma corticosterone concentrations were measured by competitive protein binding (5, 6). The lower limit of reliability of the assay is 2 µg/dl using 20 µl serum for each determination. Where possible, 50 µl of serum were used to lower this limit to approximately 0.8 µg/dl.

Two sample t-tests were done comparing the various groups at given ages.

RESULTS

Results are shown in Figure 1. Corticosterone concentrations are significantly ($p < .005$) elevated in pups exposed to ether as compared to pups sacrificed immediately on days 1, 14, and 21, but not on day 7. Dexamethasone pretreatment results in decreased basal corticosterone levels in 1 day old pups ($p < .01$) and 21 day old pups ($p < .005$), while they are slightly elevated ($p < .025$) in 7 day old pups and nonsignificantly elevated in 14 day old pups. Ether has no effect on corticosterone levels in dexamethasone treated pups, except on day 21 ($p < .025$). Pups (1, 14, 21 days old) pretreated with dexamethasone then exposed to ether have significantly ($p < .005$) lower corticosterone concentrations than those exposed to ether without pretreatment; however, 21 day old pups fall into two distinct groups based on their response to anesthesia*: A: 0.30 ± 0.14, n = 6; B: 15.00 ± 2.61, n = 5. The absence of an increased corticosterone concentration in response to ether on day 7 precludes analysis of the effect of dexamethasone pretreatment on these animals.

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* $\bar{x}$ ± SD, n

DISCUSSION

These results demonstrate the existence of feedback inhibition in the adrenal axis of rats as young as one day old. Dexamethasone injected intraperitoneally three hours prior to ether exposure suppresses the expected rise in plasma corticosterone in pups 1, 14, and 21 days old. The existence of negative feedback in 7 day old rats could not be tested because no increase in corticosterone levels could be elicited following stress.

The site of feedback inhibition has been suggested to lie in the hypothalamus (1) or pituitary (1). Other investigators have shown that both loci may be involved, depending upon the type, duration, and intensity of stimulation (7). Yates et al. (7), suggested that the pituitary response to dexamethasone with respect to ACTH suppression saturates at a systemic dose of 5 - 10 µg/100 g steroid. When the dose is increased and pituitary receptors are saturated, further inhibition depends on negative feedback sites in the brain for suppression of endogenous corticotropin releasing factor (CRF) release. Furthermore, the intensity of the stress stimulus may be increased sufficiently to overcome maximal corticosteroid induced inhibition (7).

The functional capability of the neonatal rat adranal axis has been shown to be limited in comparison with that of the adult rat. Until recently, the rat was assumed to be stress-nonresponsive until 2 - 3 weeks of age. However, with more sensitive hormone assays, rats as young as 1 - 2 days were shown to respond to certain stressors

with increased plasma corticosterone (8).

This responsiveness seems to decrease during days 4 - 11 and reappear during the third week of life. The locus of this diminution in activity may lie in any one of the components of the adrenal axis: the adrenal gland, the pituitary gland, the hypothalamus, some part of the cerebral cortex, or any of the interconnections (i.e., fiber tracts, hormones, neurotransmitters). We have previously demonstrated (9) that the adrenal gland is functional by the time of birth. The present experiments provide evidence that the negative feedback components of the axis (the pituitary and/or hypothalamus) are also functional at that time.

We have shown that, when injected during the first week with ACTH or corticosterone, seven day old rats have increased ($p < .005$) plasma corticosterone, following stimulation by ACTH or CRF. Daily ACTH or corticosterone injection was able to maintain a degree of activity, however daily ether exposure (unpublished results), heating or handling (9, 10) was not. The adrenal and pituitary glands, once able to synthesize and secrete hormones upon appropriate stimulation, must therefore cease to do so because of impaired sensitivity to physiological levels of CRF, ACTH or moderate stimulation, or a combination of the two.

By twenty-one days of age, pups have basal corticosterone concentrations approaching those measured in adult rats, although if unmanipulated they do not yet show a circadian variation in those levels (11). The magnitude of response to ether exposure and its suppressibility by prior dexamethasone treatment in 21 day old pups

also resembles that which is seen in mature rats. Similarly, it is possible to overcome maximal inhibition by corticosteroid administration. The dose of dexamethasone used in these experiments is one that was shown to produce maximal inhibition in adult rats (2). This dose, in mature rats, is sufficient to suppress corticosterone response to 1 minute ether, handling, etc., but not 3 minute ether stress (2). Twenty-one day old rats were exposed to ether for approximately one minute following prior dexamethasone treatment (see Methods). Some rats recovered from the anesthesia quickly, others required longer periods of time or even resuscitation. The rats in the former group (A) tended to have suppressed corticosterone levels 20 minutes after ether; in the latter group (B), corticosterone concentrations were depressed, but were significantly ($p < .005$) higher than in the former group.

The adrenal axis is a complex system composed of cerebral, hypothalamic, pituitary, and adrenal components. The competence and interconnection of these separate parts is essential for optimal functioning of the axis. The system as a whole is not active if any one of the subunits does not synthesize and secrete its hormone upon appropriate stimulation or if the secreted hormone does not reach the target tissue in sufficient concentrations. In the rat, the various parts of the adrenal axis develop at different rates and at different times, both pre- and postnatally. Growth of neurons and their processes continue for 1 - 10 days after birth (12). It is only by 6 - 12 weeks that fiber tracts are fully myelinated and the majority of the interconnections are functional. Only when all parts of the

axis have attained maturity and are functionally connected is a coordinated response possible.

The time-course of development of different levels of functioning as determined by intervention and measurement of hormones within the hierarchy of the adrenal axis may provide a clue to the developmental sequence of the components of the axis and the interconnections within it. Previous studies (9) have demonstrated that the adrenal and pituitary glands are potentially functional by the time of birth. Evidence is presented here that the components of the negative feedback system are intact and function by day 1. This suggests that the pituitary and possibly parts of the hypothalamus are mature by birth. Therefore, any functional immaturity in the neonatal adrenal axis should reside within the hypothalamus or cerebral cortex. Development must proceed to include the neurotransmitters and releasing factor stimulation, synthesis, and secretion before full activity is possible. This general scheme may also be applied to other endocrine systems in order to learn more about development in general.

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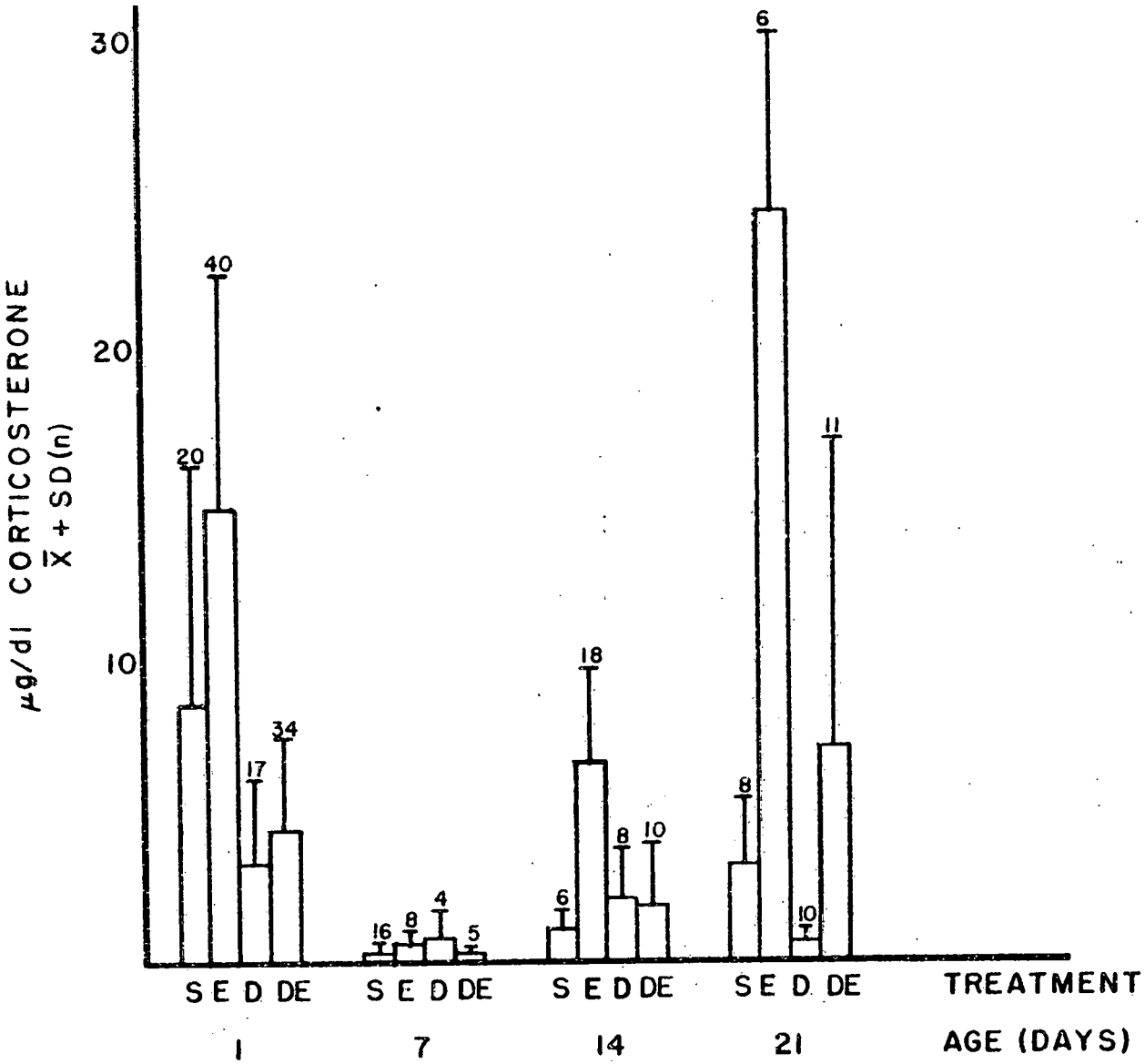
FIGURE 1

Plasma corticosterone concentrations in rats 1, 7, 14, and 21 days old sacrificed immediately (S); dexamethasone injected (D); ether exposed (E); or dexamethasone injected and ether exposed (DE). For details, see text.


μg/dl CORTICOSTERONE
X̄ + SD(n)
30
20
10
20
40
17
34
16
8
4
5
6
18
8
10
8
6
10
11
S E D DE
S E D DE
S E D DE
S E D DE
TREATMENT
1
7
14
21
AGE (DAYS)

ABSTRACT

Pituitary Responsiveness in the Neonatal Rat

Plasma ACTH concentrations were determined in neonatal rats 1, 7, 14, and 21 days old, subjected to treatment with either corticotropin releasing factor (CRF) (the equivalent of the CRF activity in one adult median eminence/rat) or to ether exposure (45 - 60 sec). CRF injection elevated plasma ACTH levels on all days tested. Ether exposure elevated ACTH levels on days 14 and 21, but not on days 1 or 7. These results indicate: 1) that there is no diminution of responsiveness of the pituitary to direct stimulation as a function of age, and 2) that the mediators of the stress response above the level of the pituitary are not functionally mature until approximately day 14. Correlations with previous studies (11) also indicate that there is a delay in the return of adrenal responsiveness until some time after these mediators attain maturity.

INTRODUCTION

In the neonatal rat, there is a period immediately after birth of considerable pituitary-adrenal responsiveness to environmental stimulation, followed by a period of depressed responsiveness which develops between days 2 and 5 and lasts until some time prior to weaning (1). By three weeks of age, the plasma corticosterone response to stress has been found to be functioning at approximately an adult level (1). This pattern has been well established for the adrenal axis as a whole, but not for the isolated components or their interactions. The period of diminished responsiveness has not been explained, nor has the locus of this apparent loss been determined.

In the mature rat, stimuli received by the central nervous system (CNS) result in the release of various neurotransmitters which cause the release of corticotropin releasing factor (CRF) from the hypothalamus (2). CRF, transported from the median eminence to the pituitary via the portal circulation (3) in turn stimulates the secretion of adrenocorticotropic hormone (ACTH). The adrenal cortex synthesizes and releases corticosterone at an increased rate in response to elevated circulating ACTH levels (3). In the neonate, this complex sequence is not fully developed; imposed stimuli do not, at all ages, result in increased circulating corticosterone concentrations.

In this study, we have used plasma ACTH as the endpoint to test the pituitary's competence as a function of age. In addition, the cause of diminished responsiveness during the first and second postnatal weeks was investigated.

MATERIALS AND METHODS

Pregnant Long-Evans hooded rats (Blue Spruce Farms, Altamont, N.Y.), obtained on the 13-15th day of gestation, were housed individually in wire mesh maternity cages (42 x 36 x 24 cm) containing nesting material. They were maintained at 25 ± 1 C, 40 - 60% humidity, light cycle 0600 - 1800 h. They were allowed food and water ad libitum. Litters were culled to the 9 - 10 largest pups on day 1 (the day after birth) then left undisturbed, except for cage cleaning, until the day of the experiment.

Litters were randomly assigned to one of the following groups: a) control (sacrificed within 1 - 3 minutes of removal from the cage); b) CRF injection (adult rat median eminence (ME) extract, generously supplied by Murray Saffran, approximately 1 ME/50 µl/pup); c) ether exposure (45 - 60 sec); at 1, 7, 14, or 21 days of age. Pups injected with CRF or exposed to ether were rapidly sacrificed by decapitation 2-1/2 - 5 minutes after treatment. Trunk blood was stored at -20 C until assay. It was necessary to pool blood samples in the youngest three age groups to obtain sufficient plasma to assay: 6 - 9 pups at 1 day old, 4 - 6 pups at 7 days old, and 2 - 3 pups at 14 days old.

Frozen plasma samples were shipped packed in dry ice and kindly assayed for ACTH in the laboratory of John Kendall (4).

Two sample t-tests were done comparing ACTH concentrations as a function of age or treatment. An analysis of variance among all four age groups as a function of treatment was also done.

RESULTS

Basal (control) ACTH concentrations were not statistically different in rats 1, 7, 14, and 21 days old (Figure 1). Stimulation of the pituitary with corticotropin releasing factor (CRF) (Figure 2) induces the same magnitude of ACTH secretion independent of age. ACTH increases from 98.6 ± 10.6 (SEM) to 210.3 ± 12.4 pg/ml within 5 minutes after CRF injection in rats 1, 7, 14, and 21 days old. There does not appear to be any diminution of responsiveness of the pituitary to direct stimulation as a function of age.

Indirect stimulation of pituitary secretion by an imposed stress (ether exposure), via CNS neurotransmitters and hypothalamic CRF, results in increased plasma ACTH concentrations in 14 and 21 day old rats ($p < .025$, $p < .005$, respectively), but not in 1 or 7 day old rats (Figure 3). CRF- and ether-stimulated increases in 14 and 21 day old rats were of the same magnitude. A summary of these results is presented in Figure 4 and Table 1.

DISCUSSION

The results of our investigation clearly demonstrate that the functional potential of the neonatal pituitary gland is virtually unchanged during the first three weeks of life. There does not appear to be any diminution of responsiveness of the pituitary to direct stimulation as a function of age, however, indirect stimulation of pituitary secretion by ether exposure results in increased plasma ACTH concentrations in rats 14 and 21 days old, but not in rats 1 and 7 days old. Since no elevation in plasma ACTH is apparent in 1 or 7 day old rats, in which the pituitary has the potential for response, CRF must not be reaching the pituitary gland following ether exposure.

This may be due to any of three factors: 1) inability of endogenous CRF to reach the pituitary, 2) absence of CRF production by the hypothalamus due to a deficiency in the synthetic process, or 3) lack of appropriate stimulation of the hypothalamus by neurotransmitters. Dupouy (5), however, has reported evidence of CRF activity in hypothalamic extracts from 20-day-old fetuses. It is unlikely that this CRF is simply unable to reach the pituitary because physiological levels of exogenous CRF reach and stimulate the pituitary. There is evidence (6, 7) that excitatory and inhibitory controls in the central nervous system do not develop simultaneously and that much of this development occurs postnatally.

The development of regulatory mechanisms for the control of CRF or ACTH may be similar to those postulated for the control of growth hormone (GH) secretion (8). The fetal pituitary has the capacity to

synthesize and secrete GH, which may be autonomous or regulated by hypothalamic growth hormone releasing factor (GRF). With further maturation of the median eminence and pituitary portal system, relatively unrestrained tonic release of GRF leads to intense stimulation of GH secretion. Then, neuroinhibitory influences become operative, possibly partly related to the maturation of the hypothalamic neuronal network and coincident with the increased development of neurophysiologic function. Finally, further maturation of regulatory mechanisms occurs and both inhibitory and excitatory effects are functional. Similarly, hypothalamic mechanisms for prolactin secretion mature postnatally (6, 8, 9).

There is a marked contrast between the continued responsiveness of the pituitary upon direct stimulation with CRF and the diminution in responsiveness of the adrenal upon ACTH stimulation during the first and second postnatal weeks (Figures 2, 5). There is evidence that the depressed adrenal responsiveness between days 4 and 12 may reside at the adrenal level (10). Studies have shown that the adrenal response to ACTH closely parallels that to histamine (10) and ether (11) stress, suggesting that adrenal competence is the limiting factor. Butte et al. (12) proposed that the high plasma corticosterone levels just prior to and during the first 2 days after birth exert an inhibitory influence on the hypothalamus or pituitary. This, in turn, may result in the gradually decreasing response of the adrenal cortex. Cote and Yasumura (10) feel that before birth, a maternal influence maintains the fetal adrenal cortex and that after birth, the adrenal cortex undergoes a functional involution involving diminished steroidogenic capacity. By

day 9, they postulate, the neonatal rat's hypothalamic-pituitary axis has matured sufficiently to maintain its own adrenal gland.

In this study, we have provided evidence that the decreased responsiveness with respect to corticosterone secretion following stress is not due to pituitary immaturity. The level of tonic ACTH secretion does not vary with age, nor does the level of ACTH secretion following direct stimulation with CRF. The depressed responsiveness may reside, at least partially, within the adrenal cortex. However, CNS and hypothalamic factors are probably also involved. It is not until day 14 that the pituitary response to ether stress is manifest, a process that would require maturation of the neurotransmitter and releasing factor apparatus.

Although the pituitary stress response appears "mature" by day 14, adrenal responsiveness lags behind. Despite similar plasma ACTH concentrations 5 minutes after ether exposure in 14 and 21 day old rats, the plasma corticosterone concentrations are strikingly different. The steroidogenic capacity of the adrenal cortex is apparently diminished and requires time and, possibly, continued stimulation to become active again.

The responsiveness of the adrenal cortex on day 1 remains puzzling. The ability to respond may be maintained, as Cote and Yasumura (10) suggest, by maternal influences. However, our data raise the question of what the stimulus for the response is in the one day old rat. Following ether exposure, plasma corticosterone concentration rises despite the fact that no increase in plasma ACTH can be measured. The one day old pituitary, though it has the capability to synthesize and

secrete ACTH, does not appear to do so after ether stimulation. A similar discrepancy has been found in the relationship between cAMP rise after histamine stress as opposed to ACTH stimulation (10). Despite increased corticosterone levels on day 2, the injection of histamine does not result in a significant rise in adrenal cAMP until day 12, whereas ACTH injection results in significant elevations of both cAMP and corticosterone on day 2. Therefore, stress responsiveness on days 1 and 2 may be due to a more primitive mechanism which is superseded by the developing adult mechanism by days 12 - 14.

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TABLE 1

PROBABILITY (p) VALUES FOR THE STUDIES ILLUSTRATED IN FIGURES 1-4

	Ages (days)		
Treatment	1 vs. 7	7 vs. 14	14 vs. 21
sacrifice	NS	.1 > p > .05	< .025
CRF	NS	.1 > p > .05	NS
ether	NS	< .005	NS

	Day 1	Day 7	Day 14	Day 21
sacrifice vs. ether	NS	NS	< .025	< .005
sacrifice vs. CRF	< .01	< .01	< .005	< .005
ether vs. CRF	< .005	< .005	NS	NS

FIGURE 1

Basal plasma ACTH concentrations in rats 1, 7, 14, and 21 days old. Shown are the mean ± SEM. The numbers of animals for each determination are indicated above the appropriate bar.

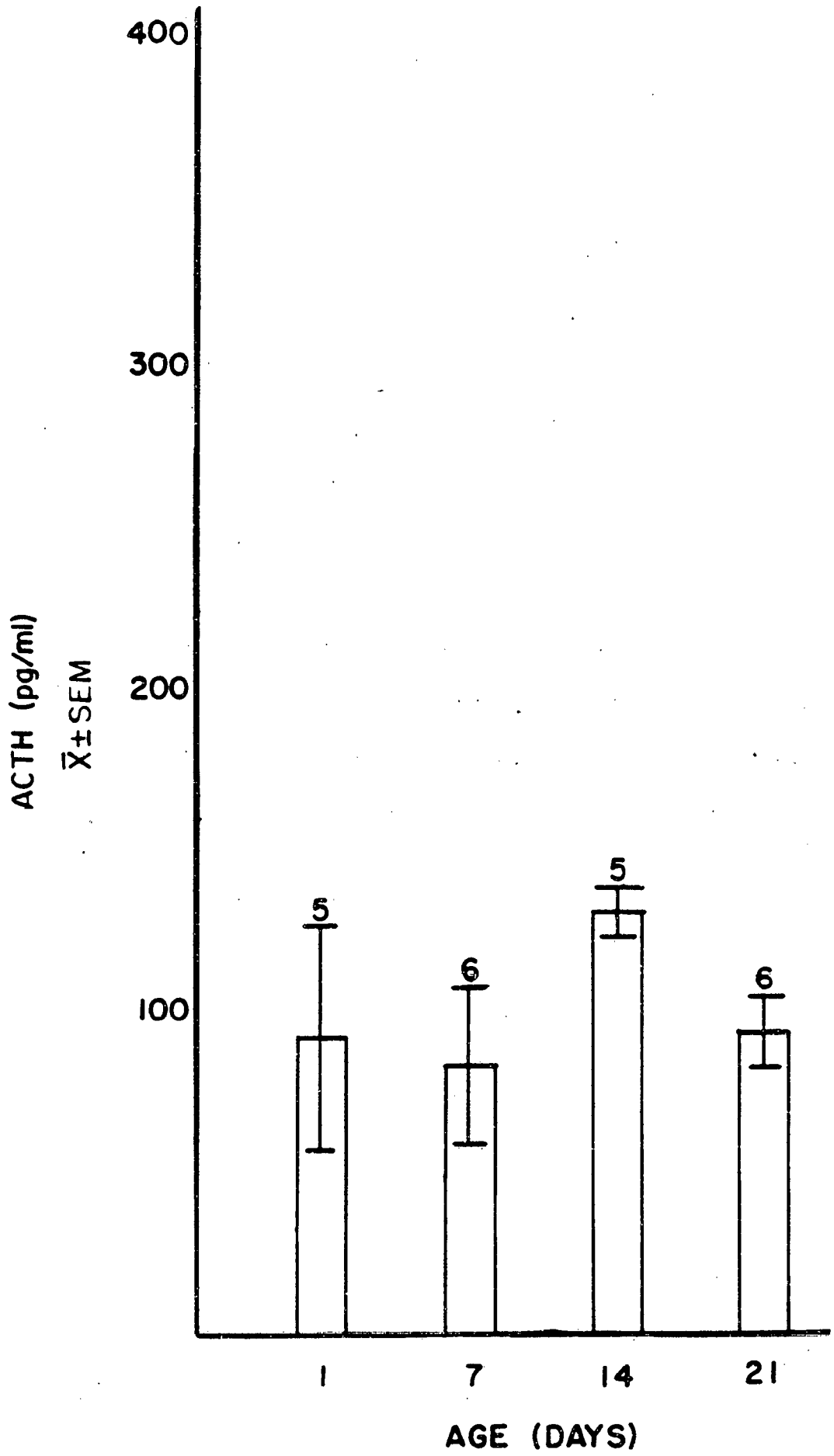

400
300
200
100
ACTH (pg/ml)
X̄±SEM
5
6
5
6
1
7
14
21
AGE (DAYS)

FIGURE 2

CRF-stimulated plasma ACTH concentrations in rats 1, 7, 14, and 21 days old. Shown are the mean ± SEM. The numbers of animals for each determination are indicated above the appropriate bar.

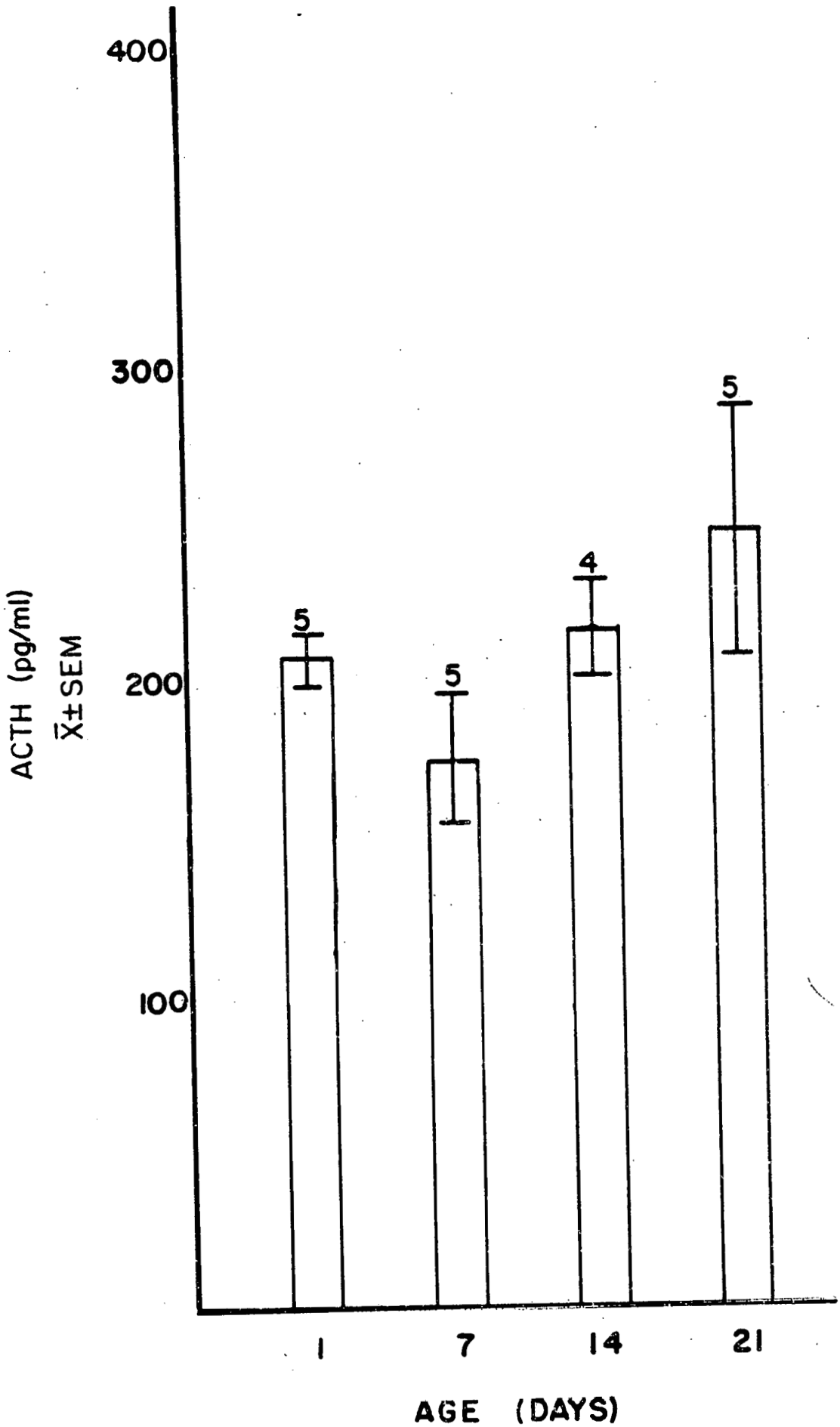

400
300
200
100
ACTH (pg/ml)
X̄ ± SEM
5
5
4
5
1
7
14
21
AGE (DAYS)

FIGURE 3

Ether-stimulated plasma ACTH concentrations in rats 1, 7, 14, and 21 days old. Shown are the mean ± SEM. The numbers of animals for each determination are indicated above the appropriate bar.

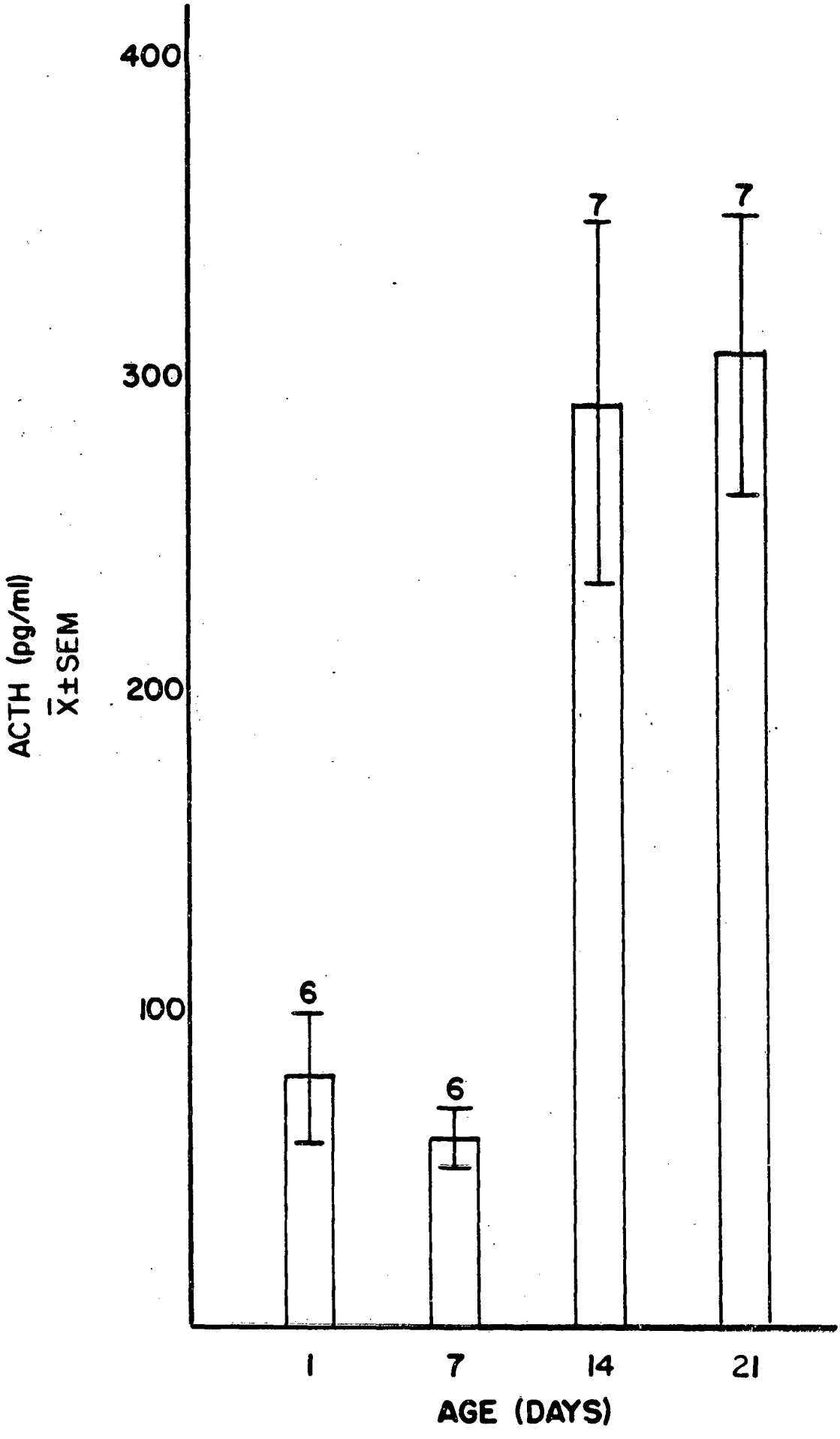

400
300
200
100
ACTH (pg/ml)
X̄±SEM
6
6
7
7
1
7
14
21
AGE (DAYS)

FIGURE 4

Basal (●—●), CRF- (■--■) and ether- (◆◆◆) stimulated plasma ACTH concentrations in rats 1, 7, 14, and 21 days old. Shown are the mean ± SEM.

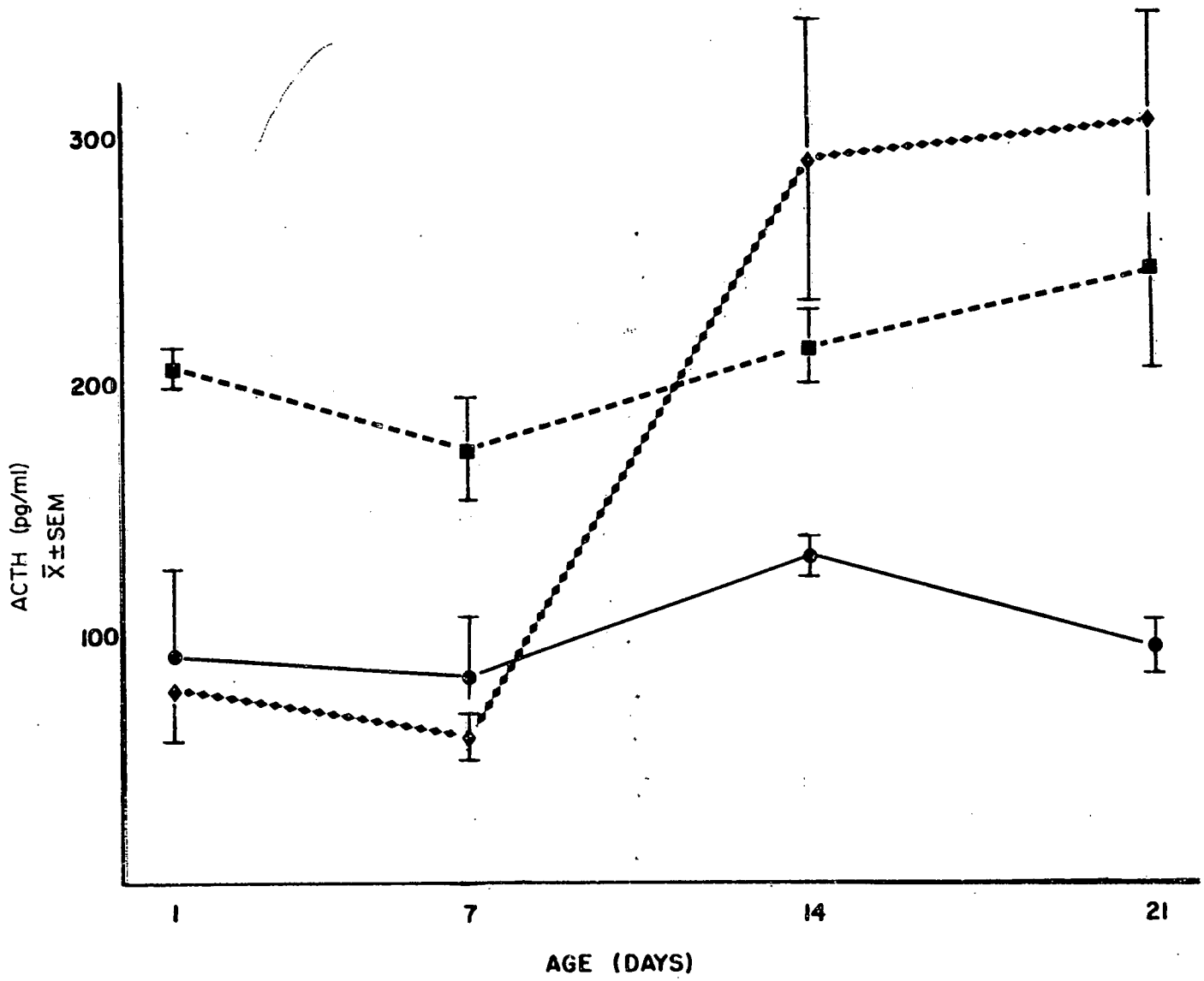
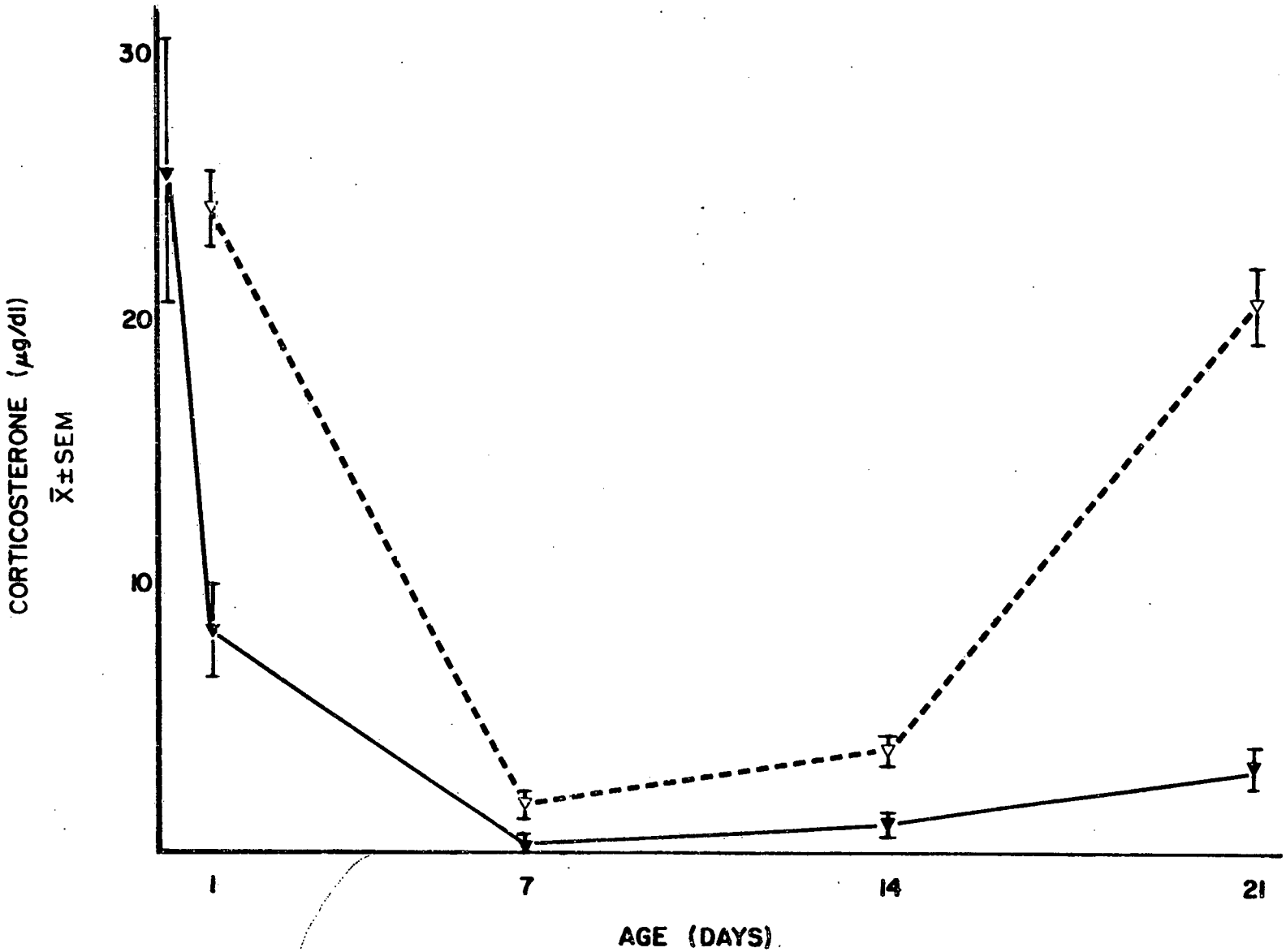

300
200
100
ACTH (pg/ml)
X̄ ± SEM
1
7
14
21
AGE (DAYS)

FIGURE 5

Basal (▼—▼) and ACTH- (▽--▽) stimulated plasma corticosterone concentrations in rats 1, 7, 14, and 21 days old. Shown are the mean ± SEM.


30
20
10
CORTICOSTERONE (µg/dl)
X̄±SEM
1
7
14
21
AGE (DAYS)

SUMMARY

The overall experimental design for this thesis is based on the hierarchical organization of the hypothalamo-hypophyseal-adrenal axis (Figure 1).

An attempt was made to dissect the adrenal axis and examine individual components and then add increasing orders of complexity. The limiting step, at a given stage of development, could thereby be deduced.

The following experiments were done (refer to Figure 1):

[1] basal corticosterone, to determine the tonic level of adrenal secretion

[2] ACTH-stimulated corticosterone, to determine adrenal responsiveness to direct hormonal stimulation

[3] CRF-stimulated corticosterone, to determine pituitary-mediated adrenal responsiveness following direct hormonal stimulation of the pituitary

[4] stress-induced corticosterone, to determine adrenal responsiveness to environmental stimulation

[5] basal ACTH, to determine the tonic level of pituitary secretion

[6] CRF-induced ACTH, to determine pituitary responsiveness to direct hormonal stimulation

[7] stress-induced ACTH, to determine pituitary responsiveness to environmental stimulation

[8] corticosterone, to determine the effectiveness of dexamethasone as a feedback inhibitor

[9] corticosterone, to determine the effect on the HHA axis of postnatal corticosterone or ACTH administration.

During the first few hours after birth, corticosterone concentrations [1] are high. Plasma corticosterone immediately begins to decrease rapidly and is very low on day 7. Thereafter, basal corticosterone gradually increases by day 14 and further by day 21 (day 1 >> day 7 < day 14 < day 21). In contrast, the tonic secretion of ACTH [5] by the pituitary is unchanged during the first three postnatal weeks (day 1 = day 7 = day 14 = day 21).

There is a postnatal fall in sensitivity of the adrenals between days 1 and 7, independent of the stimulus [2], [3], [4]. Responsiveness reappears by day 14 and reaches that of the adult by day 21 (day 1 >> day 7 < day 14 << day 21). Direct stimulation of the pituitary [6] results in a doubling of ACTH secretion in all ages of rats tested (day 1 = day 7 = day 14 = day 21). Ether stimulation of the pituitary [7] results in no increase in ACTH secretion in 1- or 7-day-old rats and a doubling of ACTH secretion in 14- and 21-day-old rats (day 1 = day 7 << day 14 = day 21).

Feedback inhibition [8] is demonstrable in rats 1, 14, and 21 days old. Seven-day-old rats could not be tested because of the absence of response to both direct and indirect stimulation.

The responsiveness of the adrenal gland in 7-day-old rats can be increased by prior treatment on days 1 - 6 with either corticosterone

or ACTH [9]. Daily treatment results in elevation of plasma corticosterone following ACTH or CRF administration on day 7 to levels seen in 14-day-old rats following similar stimulation.

These experiments demonstrate that the rat adrenal gland is functional by the time of birth but, probably due to insufficient continued stimulation, becomes refractory by the end of the first week. This may be due to a loss of sensitivity of the adrenal to ACTH. Direct stimulation with CRF resulting in increased ACTH secretion demonstrates that the pituitary is potentially responsive to stress by birth. However, because no elevation in plasma ACTH occurs following environmental stimulation until day 14, mediators of the stress response above the level of the pituitary must not be functional until the second postnatal week.

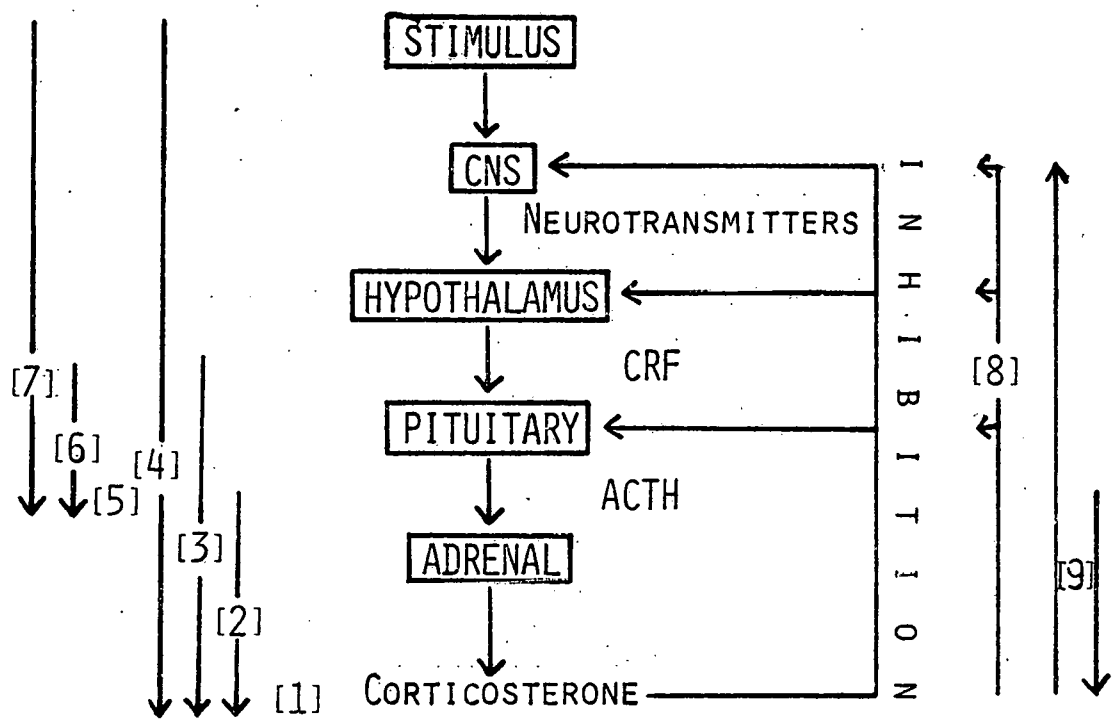
Feedback inhibition involves pituitary, hypothalamus, and CNS sites in the adult rat. At least one of these tissues must be functional by day 1 to account for dexamethasone suppression of the stress response at that age. However, they may not be fully mature even by day 21, because dexamethasone inhibition can be overridden more easily than in adult rats.

With repeated direct adrenal stimulation during the first week, a degree of responsiveness can be maintained by day 7. This is presumably due to a direct action of ACTH on adrenal steroidogenic capacity. The fact that responsiveness can also be maintained with prior corticosterone treatment suggests that corticosterone may be required at the level of the hypothalamus or CNS to allow normal maturation of the HHA axis.

In summary, evidence has been presented, indicating that both the adrenal and pituitary glands are potentially functional at birth. The central nervous system and/or hypothalamic mediators of the stress response, however, are not mature until at least the second or third postnatal week.

FIGURE 1

Experimental design for the study of neuroendocrine control of adrenocortical function in the neonatal rat.


STIMULUS
CNS
NEUROTRANSMITTERS
HYPOTHALAMUS
CRF
PITUITARY
ACTH
ADRENAL
CORTICOSTERONE
INHIBITION
[1]
[2]
[3]
[4]
[5]
[6]
[7]
[8]
[9]

APPENDIX

TABLE 1

CORTICOSTERONE LEVELS (µg/dl) IN NEONATAL RATS
1 DAY OLD

	TREATMENT					
Sacrifice	Saline	ACTH	CRF	Ether	Dexa-methasone	Dex + Ether
8.9	11.7	21.0	16.5	15.1 12.1	4.6	9.3
3.6	11.5	23.5	23.8	17.3 7.0	7.2	10.9
4.9	12.0	32.0	25.8	20.6 8.9	3.7	9.5
14.9	17.0	21.5	25.7	16.5 13.8	8.3	12.3
31.0	16.7	21.8	26.2	1.9 15.3	9.6	10.3
17.8	13.3	23.6	8.9	5.2 13.3	1.0	0.7
14.3	16.3	31.0	29.3	3.7 31.5	0.8	1.6
13.5	16.3	26.1	19.6	7.2 12.5	1.0	1.6
10.0	16.1	16.4	17.8	2.6 22.7	0.7	1.5
2.0	16.4	25.7	25.8	7.6 8.4	2.0	0
1.7	13.5		23.3	6.5 17.2	2.4	0.4
2.5			30.8	7.9 17.4	2.0	3.2
1.3			22.7	13.3 18.3	3.0	2.9
2.0			25.5	10.6 21.5	2.6	3.0
1.9				22.8 13.7	2.0	2.3
2.8				13.9 12.0	2.3	3.2
2.8				17.0 23.7	1.4	3.8
16.5				11.6 27.5		3.5
7.4				33.3 14.1		4.3
8.0				18.6 26.7		3.7
						3.8
						2.8
						4.1
						4.3
						3.0
						7.0
						5.3
						5.0
						4.3
						3.6
						4.6
						3.7
						2.5
						3.7
$\bar{x}$ 8.39	14.62	24.26	22.98	14.77	3.21	4.30
SD 7.69	2.22	4.69	5.69	7.53	2.70	2.98
SEM 1.72	.67	1.48	1.52	1.19	0.65	0.51
(n) 20	11	10	14	40	17	34

TABLE 2

CORTICOSTERONE LEVELS (µg/dl) IN NEONATAL RATS

7 DAYS OLD

	TREATMENT					
Sacrifice	Saline	ACTH	CRF	Ether	Dexa-methasone	Dex + Ether
0.4	1.0	0.1	1.4	0.2	0.2	0.4
0.4	1.3	0	0.4	0.3	0.8	0.4
0.2	0	3.4	1.3	0.8	2.0	0.4
0.2	0	3.4	1.3	0.4	0.2	0.2
0.4	0	2.3	4.5	0.1		0.1
0.4	1.7	2.2	1.3	0.1		
0.5	1.6	2.1	2.4	1.0		
0.4	1.1	2.3	1.6	1.5		
0.4	2.1	1.8	2.6			
0.5	1.0	1.4	1.7			
0	0.2		0.7			
0			2.4			
0			1.4			
0.4			1.1			
0.7			1.6			
0.7			1.6			
$\bar{x}$ 0.35	0.91	1.90	1.71	0.55	0.80	0.30
SD 0.22	0.76	1.16	0.94	0.50	0.85	0.14
SEM 0.05	0.23	0.37	0.24	0.18	0.43	0.06
(n) 16	11	10	16	8	4	5

TABLE 3

CORTICOSTERONE LEVELS (μg/dl) IN NEONATAL RATS

7 DAYS OLD

	TREATMENT					
Pre-treatment	Sacrifice	Saline	ACTH	CRF	Ether	μ Wave Exposure
Handling	1.3	0	0.1			
	0.8	0	0			
	0.8	0.8	3.4			
	0.2	5.0	2.3			
	0.02	0.8	2.2			
	0.2	1.2	2.1			
	(8.8)*	0.7	1.8			
			2.5			
			1.6			
			2.7			
			1.1			
			2.3			
			1.3			
			2.3			
			1.7			
			3.5			
			2.9			
			2.9			
			3.1			
			2.5			
			3.1			
Saline	0.5		2.4			
	0.8		1.8			
	1.0		2.0			
Diluent	0.8	0	1.7			
	2.4	0	1.6			
		1.8	1.9			
		2.4	3.7			
		2.3	1.5			
		2.9	1.7			
Incubate	1.2		1.8			0.8
	0.7		1.5			0.1
	0.3		2.2			1.3
			1.3			
Exposure	0.6		2.4			2.1
	0.5		1.9			1.8
	0.6		1.9			1.3
	0.6		3.5			2.1
	1.1		2.1			3.7
			2.1			1.7

* ( ) outliers

TABLE 3 (continued)

Pre-treatment	Sacrifice	Saline	ACTH	CRF	Ether	μ Wave Exposure
Ether	0.2		1.7		1.0	
	0.1		2.8		1.5	
	0.8		2.7		1.0	
	0.7		2.6		1.0	
	0.5		2.9		1.0	
			2.3		1.7	
			2.0		1.0	
			1.4		1.6	
			1.9		1.4	
			1.9		0.7	
			2.2			
ACTH	1.1	1.4	5.1		1.5	1.6
	5.0	5.7	3.8		1.5	2.0
	0.6	1.9	2.5		1.1	2.2
	0.6	1.9	3.0		2.7	2.0
	0.6	2.6	1.0		1.7	1.9
	(8.0)	4.3	3.8		2.3	1.4
	1.2	1.8	4.4		3.1	
	0.1	2.2	10.5		3.1	
	0.4	2.9	2.8		1.3	
			4.0		2.6	
			4.2			
			4.0			
			2.4			
			2.0			
			2.8			
			2.2			
			2.9			
			2.4			
			3.3			
			3.3			
			4.1			
			2.6			
			2.2			
			1.7			
			0.5			
Acthar 1-6	0.4	0	13.0			0.5
	(6.8)	0	5.2			0.8
	0	0.4	1.3			0.7
	0	0.8	4.9			0.4
	0.2	2.0	7.0			1.1
	4.4	0.1	4.3			0.8
	0.5	0	0.1			3.3
	0	0.3	13.3			0
	1.7		13.1			0
	0.8		1.7			0
	0.4		2.0			0
			0.4			0.3

TABLE 3 (continued)

Pre-treatment	Sacrifice	Saline	ACTH	CRF	Ether	μ Wave Exposure
						5.9 3.4 2.0 8.2 4.4 4.0
Acthar 4	0 3.4 (7.5) 4.9 0.6 0.5 0.9 1.2	2.3 2.0 1.1 1.5 2.3 1.3 1.7 1.7 1.3 2.5	5.1 3.7 3.1 5.1 5.0	5.7 2.9 7.2 7.9 9.2 4.8 5.3 6.0 4.6 6.5 4.5 5.8 4.5 5.3 7.4 6.3 3.7 3.6 2.2 3.2	2.6 2.1 2.8 1.9 3.3 2.3 2.5 2.2	
Cortico-sterone	0 1.3 0.1 0.02 0 0.1 0 0 0 0 0.1 0.8	0.2 2.8 0.3 3.3 0.9 1.3 0.4 0.4 0.4 0.8	4.4 1.8 2.1 1.9 2.9 2.3 1.4 1.6 3.2 3.7 3.0 6.0 5.3 2.7 5.1 2.8 4.0 3.4 2.5 2.3 2.3 6.8		2.9 2.8 1.3 1.7 2.2 0.8 1.1 1.6 0.9 1.4 1.3	

TABLE 4

CORTICOSTERONE LEVELS (µg/dl) IN NEONATAL RATS

14 DAYS OLD

TREATMENT

Sacrifice	Saline	ACTH	CRF	Ether	Dexa-methasone	Dex + Ether
1.2	5.2	3.1	3.4	4.4	0.1	0
1.5	5.3	3.0	4.8	2.4	0	0
1.7	6.2	3.8	3.2	4.4	0.1	0
0.5	4.6	2.6	5.5	2.1	3.1	0
0.6	2.7	4.8	4.2	1.8	3.0	0
1.0	4.0	4.4	5.5	1.6	3.4	4.6
		1.3	4.0	9.0	3.6	2.7
		4.6	4.2	8.6	2.9	2.9
		3.7	4.4	6.6		4.5
		3.6	4.7	8.0		3.3
		8.0	5.2	7.4		
			3.2	7.8		
				8.4		
				7.7		
				11.7		
				7.8		
				7.9		
				9.3		
$\bar{x}$ 1.08	4.67	3.90	4.36	6.49	2.03	1.80
SD 0.48	1.21	1.69	0.82	2.97	1.64	1.99
SEM 0.20	0.50	0.51	0.24	0.70	0.58	0.63
(n) 6	6	11	12	18	8	10

TABLE 5

CORTICOSTERONE LEVELS (µg/dl) IN NEONATAL RATS

21 DAYS OLD

	TREATMENT						
Sacrifice	Saline	ACTH	CRF	Ether	Dexa-methasone	Dex + Ether
1.5	18.9	25.0	19.6	20.7	0.3	0.3
0.6	17.1	24.2	17.3	19.4	0.1	0.5
2.1	16.8	22.2	9.9	20.4	0.5	0.3
7.0	24.7	14.8	15.5	22.7	0.5	0.2
4.5	18.9	16.6	13.5	33.6	0.8	0.1
3.7	18.2	17.8	33.2		1.0	0.4
4.2	30.0	15.1	9.7		1.0	15.5
1.9	19.3	28.4	19.5		1.0	13.7
	29.3	24.0	23.1		1.1	11.3
	29.7	19.0	21.9		1.7	18.1
	31.6		25.4			16.4
			29.9			
$\bar{x}$ 3.19	23.14	20.71	19.88	24.28	0.70	6.98
SD 2.07	5.95	4.68	7.34	5.66	0.34	7.85
SEM 0.73	1.79	1.48	2.12	2.31	0.11	2.37
(n) 8	11	10	12	6	10	11

TABLE 6

ACTH LEVELS (pg/ml) IN NEONATAL RATS

AGE	CONTROL	CRF	ETHER
1	50	200	175
	60	220	80
	40	210	40
	75	180	60
	230	225	80
			30
	91 ± 79 (SD)	207 ± 18 (SD)	78 ± 52 (SD)
	35 (SEM)	8 (SEM)	21 (SEM)
7	40	240	50
	20	200	100
	30	160	55
	150	130	45
	140	140	45
	115		60
	83 ± 59 (SD)	174 ± 46 (SD)	59 ± 21 (SD)
	24 (SEM)	21 (SEM)	9 (SEM)
14	115	220	295
	150	250	195
	150	180	220
	110	210	575
	130		400
			150
			200
	131 ± 19 (SD)	215 ± 29 (SD)	291 ± 150 (SD)
	8 (SEM)	15 (SEM)	57 (SEM)
21	90	200	200
	115	400	480
	105	200	430
	85	200	315
	45	230	270
	125		150
			300
	94 ± 28 (SD)	246 ± 87 (SD)	306 ± 117 (SD)
	11 (SEM)	39 (SEM)	44 (SEM)