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QUANTITATIVE ASPECTS OF CORTISOL FEEDBACK ON THE SECRETION OF ACTH IN DOGS

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A thesis submitted to the School of Graduate Studies of the University of Ottawa in partial fulfillment of the requirements for the degree of Master of Science in the Department of Physiology

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ABSTRACT

Seven male mongrel dogs of 8.3 - 13.1 kg. were bilaterally adrenalectomized and maintained for one week on replacement therapy, discontinued early enough to eliminate feedback effects of glucocorticoids by experiment time. Each dog, under light Nembutal anesthesia, received two stepwise primed constant infusions of cortisol (i.v.), each lasting 90 minutes. 23 venous blood samples were withdrawn for determination of plasma ACTH concentrations by adrenal cell suspension bioassay and 25 samples for plasma cortisol determination, at various times before, during and after the cortisol infusions. ACTH secretion rates were calculated using previously determined ACTH clearance and distribution volume characteristics. As a mean of 6 animals, the cortisol concentration plateaux, which averaged 4.59 ± 0.57 and 6.49 ± 0.92 µg/100 ml., caused suppression of ACTH secretion (expressed as percentage of pre-period secretion rates) from 0% before cortisol to 65.05% suppression during plateau # 1 and to 95.3% suppression during plateau # 2. 70 minutes after the cessation of cortisol infusion the plasma cortisol had fallen to 1.95 ± 0.43 µg/100 ml. with an associated 69.6% suppression of ACTH secretion rate. The suppression, associated with the two infusion periods and the delay associated with the recovery in the post-infusion period, suggests a delayed feedback of the order of 1 hour. The infused cortisol resulted in low physiological plasma levels of cortisol which had the following characteristics in these adrenalectomized dogs: metabolic clearance rates of 12.8 and 17.4 ml/kg. min associated, respectively, with mean plasma cortisol concentrations of 4.27 and 6.23 µg/100 ml. (MCR increasing with increasing cortisol concentration) and an apparent distribution volume of about 40% V (distribution volume not correlated with plasma

cortisol levels). Also, results on an as yet incomplete series of stress (histamine, anoxia) experiments showing suppression of stress induced ACTH secretion by low physiological plasma concentrations of cortisol, are included in an appendix.

INTRODUCTION

CHAPTER I

1) HISTORICAL DEVELOPMENT OF ADRENAL GLAND RESEARCH

The adrenal gland and its mechanism of informed control has had quite a long history of research development. What follows is a brief historical account of research done on this gland, its secretions and its control.

The adrenals, as anatomical entities, were first described by Eustachius (1520-1574) as early as 1563. Later, in 1849, Thomas Addison described a syndrome resulting from destruction of the adrenal glands and which was characterized by languor, debility, remarkable feebleness of heart action, irritability of the stomach and a peculiar change in the color of the skin (Addison 1855). It was later demonstrated that bilateral adrenalectomy in experimental animals resulted in death, although control animals which had been sham-operated survived (Brown-Sequard . 1856). The implication that the adrenal was a life-support organ began to take on more validity. Little progress was made in this field of research until the work of Oliver and Schafer (1894). They reported that adrenal extracts could be characterized as having "an extraordinary effect upon the tone of the heart and arteries, transcending that of any known drug". Also, adrenal insufficiency was shown to include marked weakness of the heart beat and low blood pressure. It was from findings like these that investigators began to indicate the vital function of the adrenals as sustaining cardiovascular activity (tonus). This, then lead to a "tonus theory" for adrenal function which placed the onus of importance on the adrenal

medullary secretions.

This theory, as time passed, proved to be quite untenable. Evidence began to accumulate that it was the adrenal cortex and not the medulla which was life-sustaining (Biedl, 1922). Biedl's work on the skate, in which medullary and cortical homologues are anatomically separate, showed that death resulted only when the cortical bodies were destroyed. Destruction of the medullary homologue did not have this effect. Similar results were obtained in mammals (Wheeler and Vincent 1917).

Research for many years revolved around treatment and cure of patients with adrenal insufficiency. Adrenal extracts of varying potencies were tested and used during the latter part of the 19th century and beginning of the 20th. Osler, for example, in the late 19th century, described a method for preparing an adrenal extract, in which he used fresh hog adrenals, kept cold, and which were subjected to extraction by glycerin. This glycerin extraction method was shown later to be a very effective extractant of corticosteroids.

It was these corticosteroids which were proving to have the ability to prolong the lives of adrenalectomized dogs, cats and later, in restoring patients in addisonian crisis. The treatment of adrenal insufficiency had shifted to these adrenal cortical extracts from epinephrine treatments, which for years had been used and which had only marginal effectiveness.

The adrenal cortex was now regarded as having sodium-retaining and carbohydrate regulating ability. The controversy, which occurred in the early 1930's, as to which of these features of the adrenal cortex was the more important as concerned life support was later concluded to be the

salt-retaining ability. From that point, it was only a matter of time until one of these cortical extracts was isolated and synthesized. This corticoid, deoxycorticosteroid (Doc), proved its ability in salt-retaining potency as it improved the life expectancy and means of therapy of addisonian patients (Steiger & Reichstein, 1937). However, this therapy did nothing for abnormality in carbohydrate metabolism. This situation was improved later, when a corticoid, which had some salt-retaining ability and some carbohydrate metabolic effect was made available. Unfortunately, this corticoid, corticosterone, did not have enough of these two features as was needed daily. But it did set the stage for the identification and synthesis of cortisone and hydrocortisone which are potent glucocorticoids. The break through in a potent mineralocorticoid came in 1952, when electrocortin (aldosterone) was isolated (Simpson et al 1952). For a more detailed historical development of the adrenal gland refer to some of the following references (Thorn, 1973; Bard, 1956; Hamblen, 1949; Soffer et al 1961).

This leads to the current view of the adrenal cortex, which secretes generally, two classes of steroids, mineralo and glucocorticoids, as well as some androgens and estrogens, (Tepperman, 1969). However, nothing has been mentioned about any control mechanisms.

2) HISTORICAL DEVELOPMENT OF ADRENAL-HYPOTHALAMUS-PITUITARY INTERRELATIONSHIPS.

This comprises a brief outline of the work on pituitary and hypothalamic relationships with the adrenal cortex.

The pituitary has been regarded as a distinct anatomical structure as early as the time of Galen (131-201 A.D.). Sometime later Vesalius gave

it the name "pituitaria", which lead to the long-held misinformation of its function of secreting a pharyngeal lubricant (pituita, mucus). This secretion was supposed to pass through the interstices of the cribiform plate of the ethmoid bone into the nasal cavity. This idea was disproved by Conrad Schneider in 1660. For some time thereafter this gland was regarded as a vestigial organ (Bard, 1956).

In 1912, it was first recognized that the pituitary exerted an influence on the adrenal cortical structure and function (Ascoli and Legnani 1912). They noticed that the inner zones of the adrenal cortex atrophied following hypophysectomy in dogs. This work was confirmed by Smith (1930). It was found by Mackey and Mackey (1926) that the removal of one adrenal was followed by a compensatory hypertrophy of the remaining gland. This was shown not to occur if the animal was hypophysectomized.

Until fairly recently the pituitary gland has been thought of as somewhat of an hormonal master gland, (Shumacker and Firor, 1934; Swann 1940). It was long felt that there was some neural control over this gland which made it able to respond to various stimuli. The hypothesis was that any endocrine feedback systems for any endocrine organ maintained and controlled by pituitary hormones had its hormone sensor located in the hypophysis (Russel et al 1969; Fleischer and Rawls 1970). It was felt, then, that the pituitary interacted with target gland secretions, was generally self-regulating and was influenced by the C.N.S. only on certain occasions.

It is now known that this is not the case. For one thing, there is an absence of innervation to the anterior lobe of the pituitary. Nonetheless, the pituitary gland is under nearly continuous control by the

central nervous system via the hypothalamus. This control is neurohumoral in nature and involves a group of releasing and inhibiting polypeptide hormones. These factors are stored mainly in the median eminence and infundibular stem and are transported to the adenohypophysis via specialized portal vessels. There are two groups of these portal vessels. The long portal vessels originate from the median eminence and pass down the pituitary stalk to end in the sinusoids of the anterior lobe. The short vessels originate in the neural lobe of the pituitary and pass to the anterior lobe via the intermediate lobe (Daniel, 1966). Since there is no actual blood supply to the anterior lobe, all of the blood which does pass through it, has previously passed through either the median eminence, infundibular stem or infundibular process, all of which are sources of neurosecretory products. There seems to be some anatomical specificity since individual portal vessels appear to receive blood from rather restricted regions of the median eminence and perfuse relatively restricted areas in the adenohypophysis (Adams et al 1964). This implies that releasing factors released into particular portal vessels might act on particular pituitary cells perfused by these vessels.

The earliest evidence for this hypothalamic control of the adenohypophysis involved lesion and electrical stimulation studies. Bailey and Brenner (1921) showed atrophy of dog testis following hypothalamic lesion. If lesions were restricted to the basal hypothalamus of rats (Smith 1927), atrophy of various target glands and disruption of estrus cycles were observed. Further, if lesions were restricted to the median eminence of the female guinea pig, gonadal function was found to be disrupted and if the

optic chiasma was lesioned constant vaginal estrus was induced (Dey 1943). Electrical stimulation studies such as those showing ACTH release when areas of the anterior hypothalamus and median eminence were stimulated (Goldfien and Ganong 1962), also demonstrated the role of the hypothalamus in controlling pituitary function. There was no knowledge of the means of this control until Hinsey and Markee (1933) suggested that the anterior pituitary might be controlled humorally via neurohypophysial hormones. This argument was strengthened with the discovery of the hypophysial portal venous system (Popa and Fielding 1930).

Later, injections of crude hypothalamic extracts were found to stimulate secretion of pituitary hormones. With the advent of means of evaluating pituitary secretory functioning (bioassays, immunoassays etc.), more foundation was added to the argument of a hypothalamic system controlling pituitary function.

This led to the present view of this control system as one in which the CNS controls pituitary secretions via hypothalamic releasing and inhibiting factors (Yates et al 1971a), which are themselves modulated by the secretions of the target organs (McCann et al 1974 ; Ganong et al 1974).

3) THE CURRENT VIEW OF THE BRAIN-PITUITARY-ADRENAL AXIS

The current view of this axis involves basically the release and synthesis of ACTH in response to a hypothalamic releasing factor (CRF) and the release and synthesis of glucocorticoids in response to this released ACTH. The free (unbound) portion of this glucocorticoid then feeds back negatively, primarily on the hypothalamus to inhibit CRF and less dramatically on the pituitary gland to inhibit ACTH release directly (Yates et al

1971a).

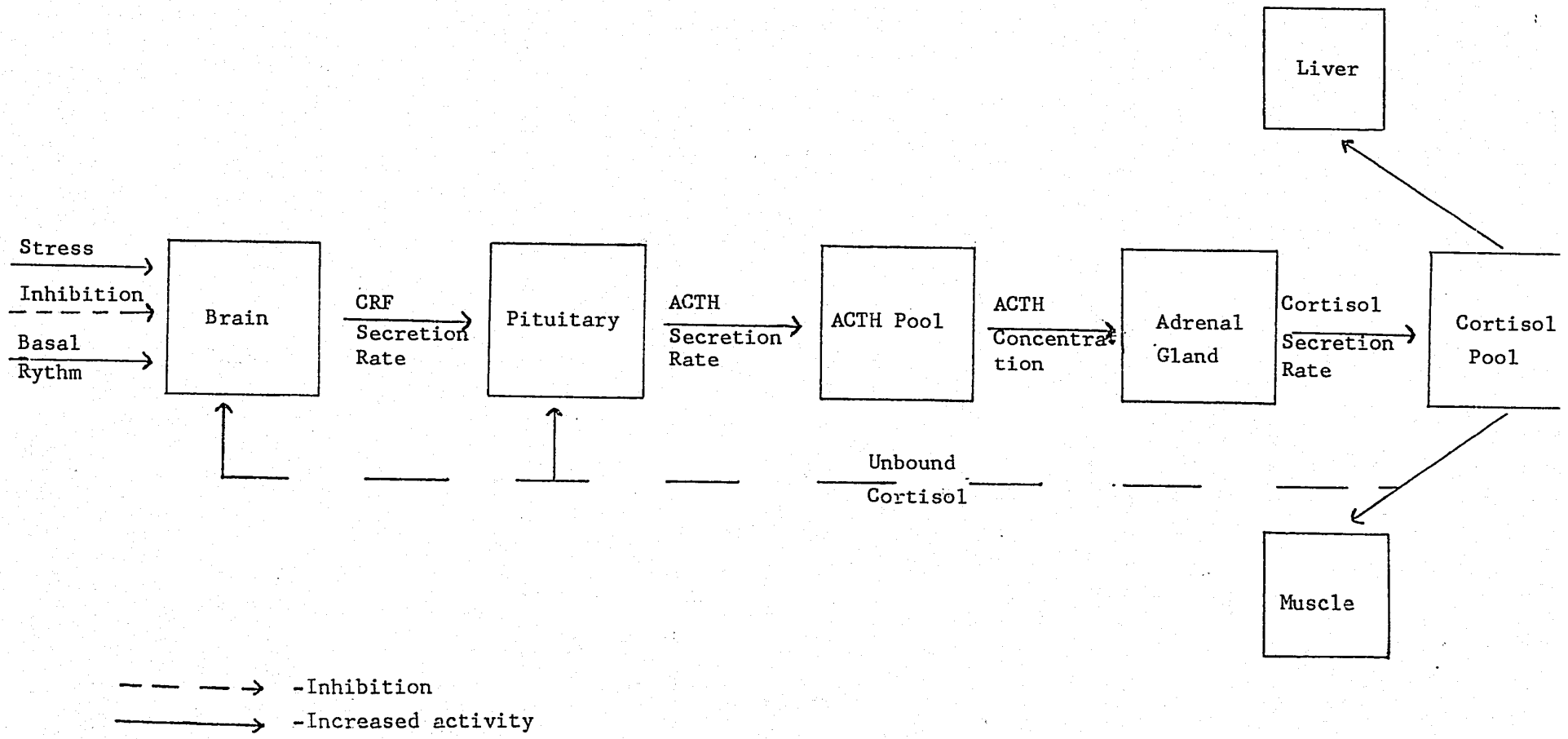
If the whole system is arbitrarily divided into subunits as Yates did (Yates and Brennan 1969), (see Figure I), then some of the existing inter-relationships can better be seen. One such subunit, the brain, generates a basal (circadian or non-stress component) secretion rate of CRF with a period of about 24 hours. This basal component seems to involve neural pathways originating from anterior parts of the brain which end in the medial basal hypothalamus (MBH), (Halasz et al 1967; Palka et al 1969; Dunn and Critchlow 1973). Although the origin of the rhythm is the brain, features such as light, temperature, wake-sleep periods etc., can alter it. Also, the rythm is still present in blind subjects or those who remain awake for several days (Migion et al 1956).

Another component, of this brain subunit, is that of stress. These stresses seem to be superimposed on the basal rythm component at various times. Here, there seems to be reason to believe that these stress components, primarily, come by different neural pathways than the rythm component. The stress component, then, seems to involve posterior brain pathways impinging on the MBH (Slusher and Hyde 1961). There is also some evidence of neural inhibition on the MBH (Taylor and Branch 1971; Slusher and Hyde 1961).

The cumulative effect of these two stimulatory and one inhibitory inputs on the MBH results in a certain secretion of CRF into the hypothalmo-hypophyseal portal system. The converging signals on the MBH are summated spatially and temporally. An amount of the small CRF polypeptide (perhaps 9-13 a.a.) reaches the adenohypophysis where it causes the release of a related amount of ACTH and more of its synthesis.

Figure 1

Hypothalamic-Pituitary-Adrenal Axis (as proposed by Yates (1969))



The ACTH released into the general circulation, then, undergoes distribution, some inactivation (metabolism) and perhaps slight binding to plasma proteins. The free portion of the ACTH is detected by the two adrenal glands, which adjust their glucocorticoid secretion rates (and rates of synthesis). The released glucocorticoids (cortisol or corticosterone) on entering the systemic circulation becomes bound to transcortin and some albumin (Gala and Westphal 1966; Acs and Stark 1973), with a certain unbound fraction (some being metabolized) in plasma generating a feedback signal inhibiting pituitary release of ACTH directly (Russell et al 1969; Gonalez-Lucue et al 1970; Kraicer and Milligan 1970; Pollock and Labella 1966; Sayers 1950). The quantitatively more important inhibition of glucocorticoids is of CRF release at the brain (Lemaire et al 1974) and specifically at the level of the MBH (Yates et al 1971a).

There seem to be two models postulating how ACTH causes secretion and synthesis of steroids from the adrenal cortices. And, since both of these theories adequately model the adrenal part of the axis, both will be briefly mentioned here.

One theory (see Figure 2) postulates that ACTH has a specific receptor in the adrenal cortical cell. The activated ACTH-receptor complex then activates adenyl cyclase which in turn causes the formation of cAMP from ATP. cAMP then activates a specific phosphorylase. The result of this process is increased generation of TPNH. The adrenal cell has a very active direct oxidative pathway (pentose shunt) for glucose. ACTH seems to increase the transport of glucose into adrenal cells as well as its utilization in the pentose shunt to produce reducing equivalents of

Figure 2

Increased Steroidogenesis By ACTH Through Increased Production of TPNH

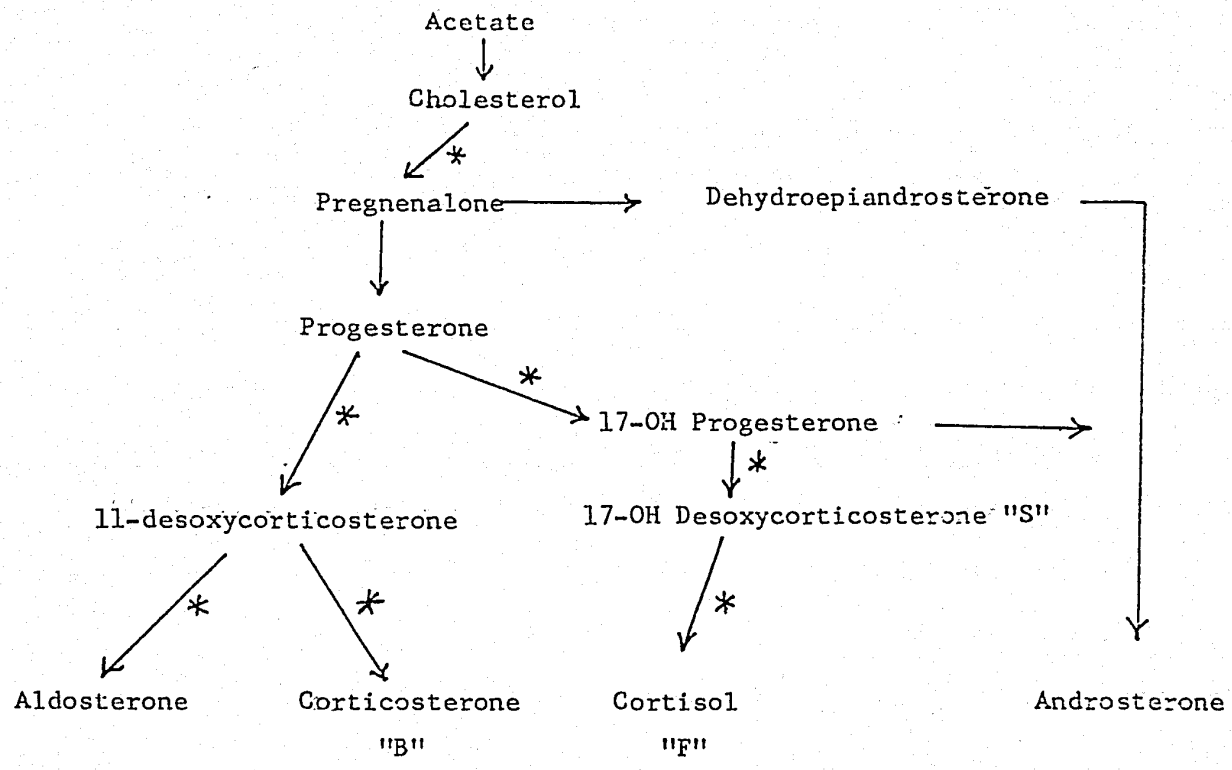
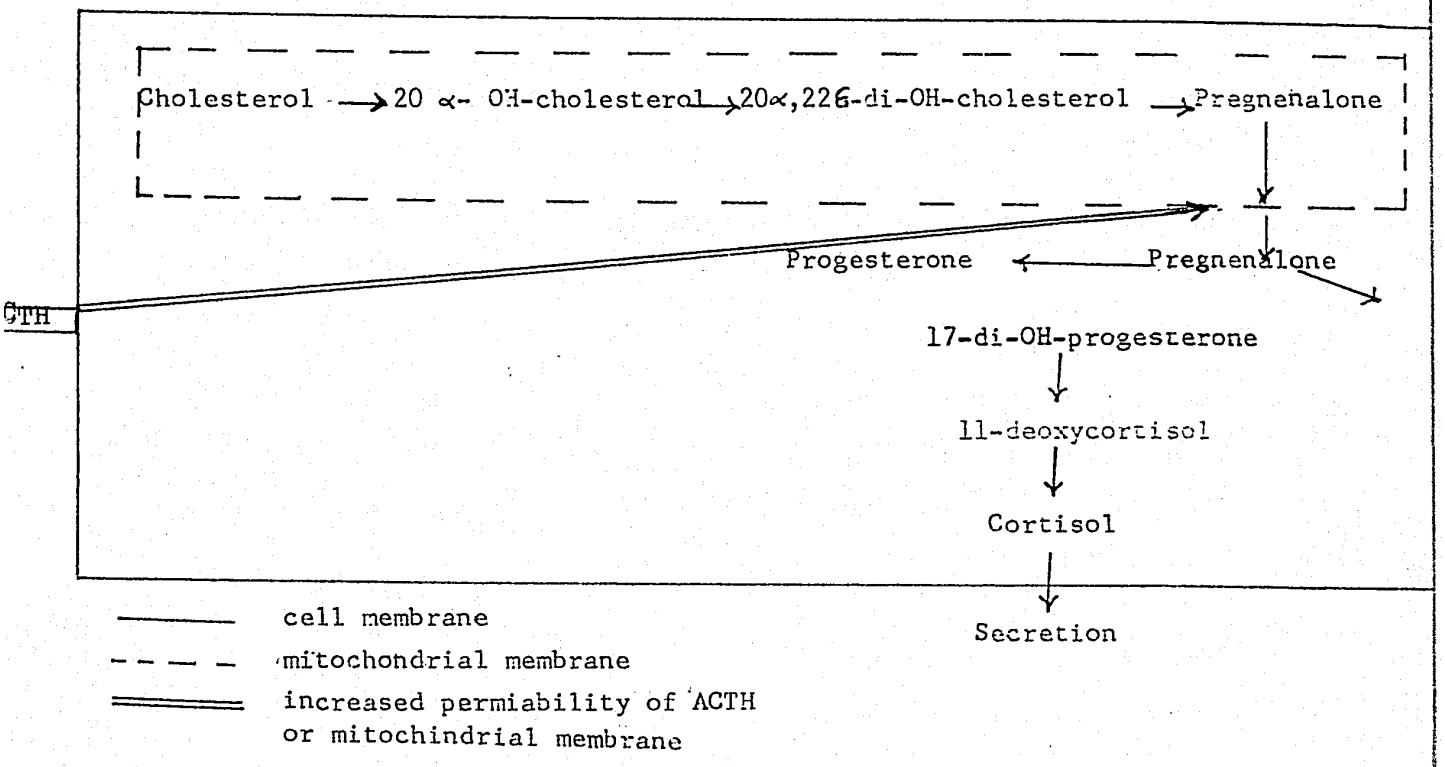


Figure 3

Increased Steroidogenesis By ACTH Through Increased Mitochondrial Membrane Permiability



TPNH. The increased amounts of TPNH, produced in this way, are believed to increase the synthesis and release of these steroids (Tepperman 1969; Haynes and Berthet 1957; Jenkins 1968).

The second theory is that of Koritz and Hall (1964) (see Figure 3). They showed that pregnenalone could inhibit cholesterol hydroxylation. Pregnenalone is formed from cholesterol in the mitochondria and pregnenalone must leave the mitochondria before any further reaction can take place. This theory, then suggests that ACTH, or some mediator, might act to increase the permeability of the mitochondrial membrane to pregnenalone. This would remove the inhibition on cholesterol hydroxylation and allow the whole pathway to proceed toward its final products (Koritz and Hall 1964: Urquhart et al 1968).

The glucocorticoids so produced undergo distribution, binding to transcortin and albumin etc. and a certain unbound fraction (Kawai and Yates 1966) feeding back to inhibit the pituitary release of ACTH and the hypothalamic release of CRF.

CRF stimulates the synthesis of ACTH (Dhariwal et al 1969) from its precursors as well as the release of ACTH into the circulation (see Figure 4). The inhibition by glucocorticoids on the pituitary (Russell et al 1969; Penn and Knight 1972), although not as important as that in the hypothalamus, probably operates to reduce secretion as well as to reduce synthesis of ACTH (whether by direct inhibition of production of ACTH from its precursors or indirectly by inhibiting the facilitory effect of CRF).

Figure 4

EFFECT OF GLUCOCORTICOID AND CRF ON ACTH SYNTHESIS AND RELEASE IN THE PITUITARY GLAND.

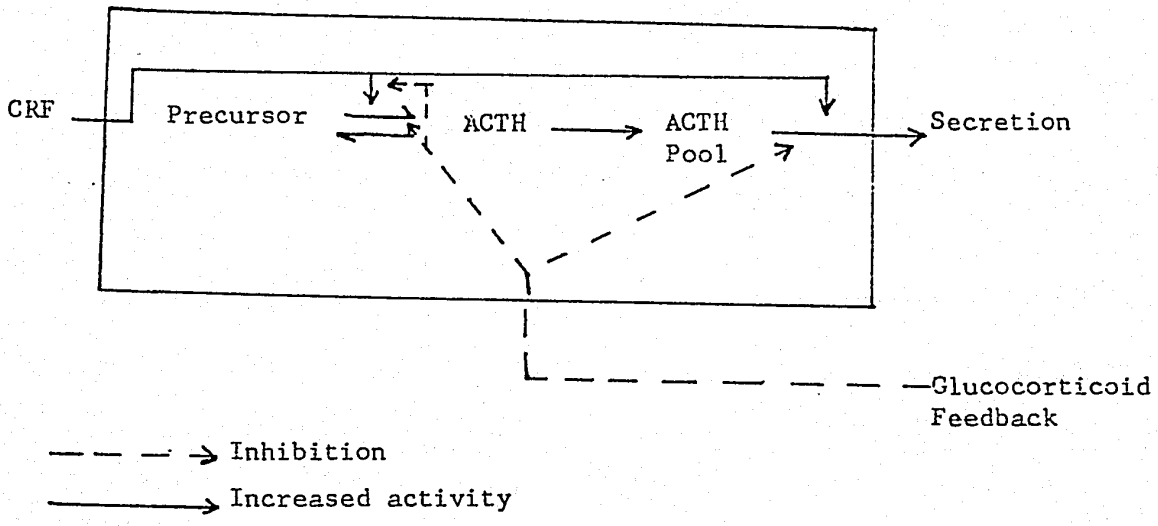
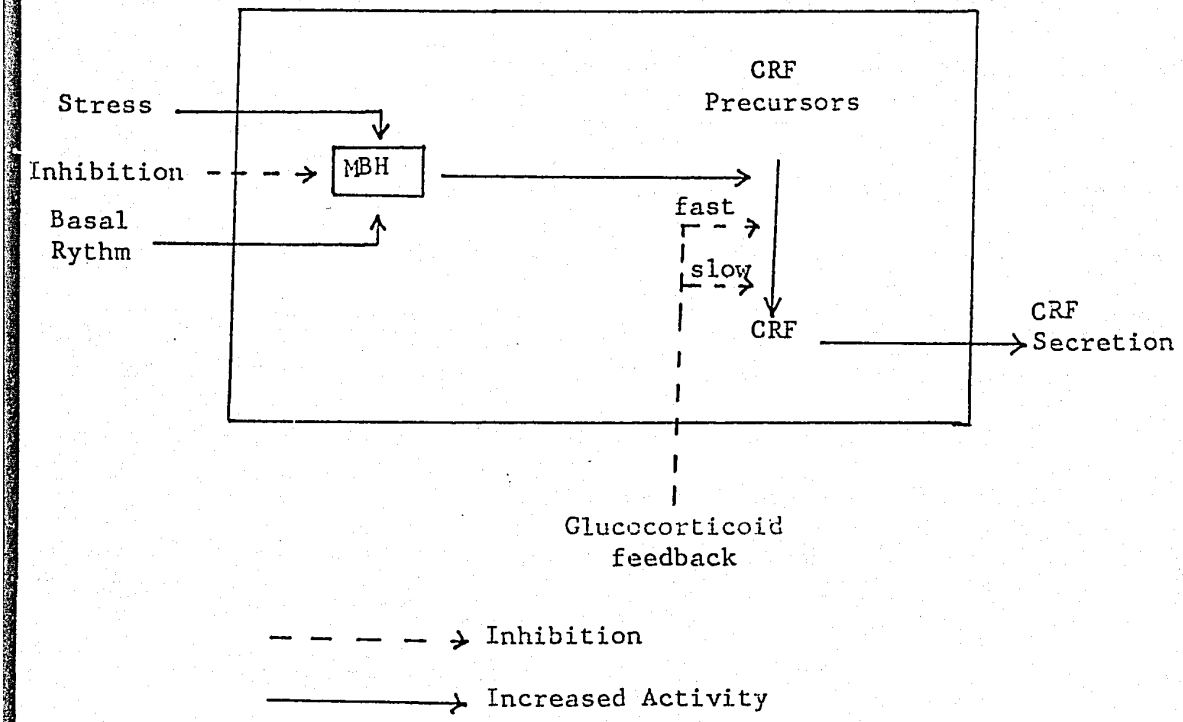


Figure 5

EFFECT OF BRAIN AND GLUCOCORTICOID ON HYPOTHALAMIC SYNTHESIS AND RELEASE OF CRF.



The major site of hormonal feedback, however, is at the level of the hypothalamus. Here, (see Figure 5) there appear to be three neural inputs into the MBH.

The fourth input is hormonal in nature and inhibiting in effect. The three neural inputs set a variable CRF secretion rate which can be altered by this glucocorticoid feedback. The hormonal feedback seems to be related to the unbound glucocorticoid in plasma (Kawai and Yates 1966). There exist sensitive areas in the hypothalamus and the pituitary. This feedback appears to be basically of two types. The first is a fast inhibitory feedback related to the rate of change of glucocorticoid as it rises and a slow or delayed feedback related to a cumulative effect of concentration alone (Dallman and Yates 1969).

4) INTRODUCTION TO THESIS RESEARCH

The research described in this thesis proposes a quantitative analysis of glucocorticoid feedback on ACTH secretion in dogs. The basis of the experiments involves the use of adrenalectomized dogs in which there is no negative feedback of glucocorticoid on ACTH release. In the absence of such feedback the pituitary releases tremendous amounts of ACTH. Such a system provides a good means of studying this feedback mechanism since feedback can be varied experimentally from no feedback (non-stress adrenalectomized dog) to those physiological concentrations of glucocorticoid sufficient to completely inhibit ACTH secretion from the pituitary.

Because currently no purified CRF exists and no sensitive assay for it, the response of the brain-pituitary system to this hormonal feedback will be measured in terms of ACTH (a sensitive bioassay for this

determination does exist, (Sayers et al 1971, Cowan et al 1971), which will be discussed later). Also, the infused glucocorticoid will be monitored and used as an index of feedback. When CRF is available it will be possible to get a more sensitive and direct measurement of the response of the brain and pituitary separately to this feedback.

The animals used in these experiments were dogs. Rats have been used by many investigators for their research on this axis but they were felt not to be suitable for this research. Dogs, lightly anesthetized, can achieve a relatively non-stress state: this is much more difficult in a rat which is much more excitable (it is important in these non-stress experiments, obviously, to have such a state). Also more blood can be withdrawn from dogs as samples than rats and dogs are higher up the phylogenetic scale than rats, which may make experimental results more comparable to man. The replacement therapy (to be discussed later) is very similar to that used on addisonian patients while a rat's therapy would only require access to a salt supplement.

The objectives of this research were: 1) to study the general nature of glucocorticoid feedback on ACTH secretion in adult adrenalectomized dogs: 2) to quantify such feedback and thereby model it; 3) to demonstrate physiological feedback in a stress situation in the adrenalectomized adult dog: and 4) to observe whether or not such feedback differs between a stress or non-stress situation or between stressful situations (anoxia versus histamine stress). Objectives 1) and 2) have been met, but 3) and 4) have not since these stress experiments were incomplete at the time of this writing. However, some of the preliminary results will be discussed in the appendices.

CHAPTER II

MATERIALS AND METHODS

1. ANIMAL CARE AND MAINTENANCE

The conditioned dogs used were 8 - 14 kg. in weight and of no specific breed. Only male dogs were used since this would avoid any potential problems which might arise due to the effect of the cyclic nature of female hormones on hypothalamic function (effects of estrogens in brain function are known to exist. This effect might well alter ACTH secretion rates (Kitay 1963a; Kitay 1963b: Kitay 1964). The dogs were bilaterally adrenalectomized (abdominal approach) and maintained until experiment time.

a) SUMMARY OF REPLACEMENT THERAPY

1. 0.3% saline ad lib.
2. balanced solid diet (mixed fat, carbohydrate and protein diet with essential vitamins, fatty acids etc.). High protein diets were only used when the animal did not respond well to the normal diet.
3. Doca (dissolved in peanut oil), (Sigma Chemical Co.) Each dog received 0.5 mg per day subcutaneously, except for the last 2 days where the dose was reduced to 0.25 mg, the last injection being given 24 hours before the experiment.

4a) Cortisol (in a solution of 5% ethanol, 95% saline), (Sigma Chemical Co.). Each dog received daily intramuscular injections of 5 mg of cortisol. The last injection was of 2.5 mg and was given 48 hours before the experiment. The first 3 dogs done received 20 mg cortisol for the first 2 days in 2 daily injections, thereafter this was reduced to 4 mg per day, in 2 daily injections except for the final 2 mg injection, 48 hours before the experiment. (These high doses for the first 2 days were discontinued since they tended to reduce the animal's ability to combat infection and heal wounds (especially the abdominal wound).

4b) 100 mg of cortisol was infused into each dog during the adrenalectomy.

5. As an index of successful therapy each dog was weighed

each morning and a stable weight with other normal physical behavior was taken to imply a stable physiological state.

(3) BASIS OF THERAPY

These are animals in which certain aspects of their physiology has been altered which must be taken into account in their replacement therapy. Because the animal lacks the adrenal medullary hormones, epinephrine and norepinephrine, he lacks a potential hyperglycemic agent and some potential circulatory effects (Keele and Neil, 1965). These however are not critical. The animal also lacks the mineralo and glucocorticoids formerly produced by its adrenal cortex. The absence of the mineralocorticoids is critical since their lack would lead to death by dehydration and effects attributable to dehydration (Swingle et al 1936) in a matter of days. The absence of glucocorticoid would imply lack of their hyperglycemic action (Goodman and Gilman 1965) which would not be too significant due to the short duration of dog maintenance.

The mineralocorticoid chosen was desoxycorticosterone acetate (doca). Although not the major mineralocorticoid found in dogs (that being aldosterone) and not as potent a mineralocorticoid as aldosterone (Swingle et al 1954). doca does have less of the glucocorticoid activity of aldosterone (i.e. doca has 1 - 2% of the glucocorticoid activity of cortisol the chief glucocorticoid of dogs) (Turner and Bagnara 1971). It was this lower glucocorticoid activity of doca which made it suitable for these feedback experiments. Even with such therapy, these animals loose significant amounts of salt, so they were allowed free access to 0.3% saline. This therapy with appropriate solid food has been shown to keep

adrenalectomized dogs in a reasonably stable physiological state for 2 to 3 years (Swingle et al 1954).

The absence of cortisol (the dog's major glucocorticoid) for a period of two weeks, although not critical for its life, might effect the sensitivity of the hypothalamus and adenohypophysis to the release of ACTH (Dallman et al 1974), which would certainly have a noticeable effect when cortisol was finally administered to the animal during the actual experiment. Therefore, so as to keep the sensitivity of this feedback loop operative, each animal received daily injections of cortisol (Dallman et al 1972) except for 2 days before each experiment.

2. EXPERIMENTAL DESIGN

a) GENERAL FEATURES OF ALL EXPERIMENTS

The dogs were lightly anesthetized with Nembutal (25 mg/kg) which was the same anesthetic used for ACTH MCR (metabolic clearance rate) determinations done previously (Cowan et al 1974). During any experiment the dogs were kept at this level of anesthesia by small injections of Nembutal (25 mg i.v.) when they showed muscle twitch activity. The animals were fasted for 24 hours prior to the start of any experiment but were allowed 0.3% saline ad lib. Where possible, two experiments were performed on each dog. One experiment type involved the feedback of glucocorticoid in a non-stress situation, and the other involved such feedback in a stress situation. There were two types of stress experiments: one involving histamine injections (i.v.) and the other stress associated with anoxia exposure for a short period of time.

Each experiment involved dual cannulation. Polyethyline cannulae were surgically inserted into two veins for each experiment. The cannulation done on a cephalic vein was used for cortisol infusion into the superior circulation. The other cannulation was done on a saphenous vein to be used for blood sampling from the inferior circulation. Infusions were made from a pre-calibrated 50 ml glass syringe by a Harvard infusion pump. The cannulae were removed and the dog was maintained until a second experiment was performed. At that time, cannulations were done on the other unused cephalic vein for infusion and a saphenous vein for blood sampling. The blood volume removed was replaced by an equal volume of physiological saline. The plasma from the blood samples (acidified and frozen to -70 degrees centigrade) was assayed for ACTH by the Sayers bioassay (Sayers et al 1971) somewhat modified (Cowan et al 1974). The plasma for cortisol analysis was assayed fluoremetrically (Silber et al 1958, modified by Swallow and Sayers 1969). These will be discussed at greater length below.

b) NON-STRESS EXPERIMENTS

These experiments were designed to observe ACTH secretion rates (discussed later) in adrenalectomized dogs, in the face of no glucocorticoid feedback (stable, non-stressed adrenalectomized dog), and subsequently during two periods of plateau concentration of cortisol in the physiological range and in the presence of cortisol remaining after one hour of cortisol clearance without replacement.

The cannulations and anesthetics were as above. A waiting period, of approximately 40 minutes after installation of cannulae and ¾ to 1 1/2 hours after anesthesia, was observed. 23 blood samples for ACTH analysis

and 25 for cortisol analysis were withdrawn from each dog over the time course of the experiment (290 minutes). About 5 1/2 ml of blood were removed for each of the 23 samples (3 ml going for cortisol analysis and 2 1/2 ml for ACTH analysis). 3 ml of blood were withdrawn for the 2 extra cortisol samples. In all cases the volume of withdrawn blood was replaced by physiological saline.

4 samples were taken over a 30-minute period to establish a stable non-stress ACTH plasma level. The dog was then exposed to a cortisol injection designed to quickly reach a low physiological plasma cortisol level which would be maintained by its accompanying infusion. 8 blood samples were taken during this first infusion period which lasted 90 minutes. This was followed by a second injection-infusion combination of cortisol roughly twice as great as the first (but still considerably less than an intact dog of his size could produce). Again 8 blood samples were withdrawn (and replaced with saline) over the 90-minute time-span of this infusion period. This second infusion was followed by a waiting period of 60 minutes during which 2 blood samples of 3 ml each were withdrawn for cortisol analysis. At the end of this 60 minute recovery period 3 samples were withdrawn over a 20-minute period.

The plasma from the blood samples for ACTH analysis was acidified with 15% its volume of 1N. HCL in saline. These samples were then frozen to -70 degrees centigrade for storage until they were analysed. (All glassware and instruments coming in contact with the ACTH was siliconized or non wettable (teflon) - tubes, vials, pipettes - (see ACTH assay). The plasma from the blood samples for cortisol analysis was withdrawn and

and stored at -70 degrees centigrade until analysis was carried out.

c) STRESS EXPERIMENTS

Note: A general outline of these experiments is included here, as they were carried out on some of the same dogs as the non-stress experiments. However, as the stress series has not been completed, the data and interim conclusions have been placed in the appendices.

i) Histamine

A 20-minute waiting period was allowed between cannulation and start of experiment (approximately 1 hour after anesthesia). Here the experiments were designed, such that, ACTH secretion rates could be observed in the face of 3 equal injections of histamine (equally spaced in time), one before, one during and one about 1 1/2 hours after a cortisol injection-infusion combination (physiological and certainly well below the maximum output of intact dog adrenal glands). 24 blood samples were withdrawn for ACTH analysis and 25 for cortisol analysis (again 3 ml for cortisol and 2 1/2 for ACTH) over the time course of the experiment (206 minutes).

2 samples were withdrawn over a 10-minute time interval before any injection of histamine to establish a pre-(baseline) period of ACTH secretion. This was followed by an injection of histamine, and withdrawal of 6 blood samples over a 16-minute period to establish the response, in terms of ACTH, to this injection. A cortisol injection-infusion combination was begun some time before the next injection (40 min. before). Again 2 samples set the pre-injection ACTH level and a histamine injection (equal to the first) was administered. 6 samples were withdrawn over the next 16 minutes. The cortisol infusion was turned off and a

94-minute waiting period ensued during which one 3 ml blood sample was withdrawn for cortisol analysis. After a pre-period interval was set with 2 blood samples, an injection of histamine and 6 samples set the response to this injection. The blood samples were treated as in the non-stress experiments. The histamine was dissolved in saline. The amount injected per dog was 61.7 µg/kg. These experiments will be discussed in appendix I (a) since the series is as yet incomplete.

ii) Anoxia:

These experiments followed the same design as the histamine stress experiments, but were longer experiments (246 minutes). They were also different in the type of stress used. Whereas the other experiments utilized histamine as a stressor, these experiments used a brief exposure to anoxia (95% N_2, 5% CO_2) (see table III) as a stressor. The experiments then, consisted of three equal time exposures to anoxia (equally spaced), one before, one during and one after a cortisol injection-infusion combination. A clear plastic bag, with a tube from the (N_2, CO_2) tank, which could be closed completely around the animal's head quickly, was used as means of exposing the animal to anoxia.

More will be said of this type of stress experiment in appendix I (b), which will include some results and more description on these as yet incompleted experiments.

d) NUMBERS OF ANIMALS

Seven adult male dogs between 8.25 and 13.1 kg. in weight were used for seven non-stress feedback experiments. Thus, far, five histamine stress experiments have been done on five dogs between 8.1 and 12.1 kg. in weight.

And three anoxia experiments have been done on two dogs (9.55 to 13.5 kg). These are summarized in Table I, and in tables found in the appendices. Since some dogs were used for both a non-stress and stress experiment the numbering of the dogs in the tables and figures was consistent with this.

e) EXCEPTIONS TO GENERAL EXP. PLAN

In the non-stress series of experiments dog # 1 constituted a pilot experiment. Instead of two plateau infusions of 90 min. duration each, as with all others, dog # 1 was subjected to three plateau infusions of 60 min. duration each. Also the injections of cortisol in this first dog were over-estimated and resulted in a declining cortisol plasma concentration instead of a plateau as in the other experiments.

In the histamine stress experiments, dog # 1 also was experimented on in a pilot of this experimental type. Again the injection to infusion ratio was higher than the other experiments of this series. Also while in the other experiments the three histamine injections are equally spaced throughout the experiment, in this first experiment injections 1 and 2 are 60 min. apart and 2 and 3 are 110 min. apart. The histamine stress experiment on dog # 7 was a longer experiment (246 minutes) than the others and had the three histamine injections 110 minutes apart.

3. ANALYSIS OF DATA

a) Calculations of secretion rates

The determination of ACTH in the plasma samples by the bioassay, gave ACTH concentrations (in mU/100ml) for those times at which they were sampled (an ACTH concentration vs time relation). Polynomial func-

tions were fitted to these bivariant data by computer and the resulting continuous representation of ACTH vs. time was then used to calculate the secretion rate function as follows:

$$\text{Secretion Rate} = MCR \times ACTH_t + \left(V \times \frac{d}{dt} ACTH_t\right)$$

where MCR= metabolic clearance rate for ACTH from plasma (ml/min. kg)
V= distribution volume for ACTH (ml/kg body weight)
$ACTH_t$= ACTH concentration at any time "t" (mU/100 ml)

This equation gives a secretion rate (mU/kg. min) for ACTH.

b) Substantiation for the Secretion Rate Calculation:

The MCR (volume cleared of a substance in a unit time - min) has been obtained for dogs (9 - 14 kg) by infusing physiological concentrations (Sydnor and Sayers 1954) of ACTH (porcine) into dogs and comparing this known amount to the resultant integral of plasma concentration (Cowan et al 1974). This calculation for MCR is a general expression of the Tait and Burstein (1964) relation. The

$$MCR = \frac{\text{amount of exogenous hormone administered}}{\int \text{plasma concentration of exogenous hormone}}$$

MCR calculated from these experiments (Cowan et al 1974) was found to be essentially constant (9.59 ± 0.24 ml/kg. min) for the dogs so tested.

The product of this MCR and the ACTH concentration is the rate of disappearance of ACTH from the blood. In a dynamic steady state the rate of removal (= MCR x ACTH concentration) would equal the rate of secretion (Normand and Fortier 1970). Even in the absence of a dynamic steady state for ACTH, the linear MCR allows a good approximation of secretion and removal rates (Cowan et al 1974). This relation for secretion rate, then, takes into account

$$\text{Secretion rate} = \text{rate of removal} + \left(V \times \frac{d}{dt} ACTH_t\right)$$

where V = measured distribution volume

the rate of removal and the rate of accumulation ($V \times \frac{d}{dt} ACTH_t$) of ACTH in the dog which may not necessarily be zero (i.e. absence of dynamic steady state).

The use of a single compartmental model, with a fixed "V" is, of course, a relatively crude first approximation, but may be adequate, given the rapid turnover and small distribution volume of ACTH in vivo. The approach, while theoretically unsophisticated, provides a useful basis for studying secretion rates of ACTH even in extreme departure from steady state (Cowan, 1975).

A Wang mini-computer (600-14-TP) was used to determine the ACTH concentration of samples (determined in duplicate) from the raw bioassay data giving ACTH concentrations (mU/100 ml plasma) for the sample times tested.

In fitting the continuous ACTH concentration versus time relations for these experiments, separate polynomials were fitted to individual experimental periods to parallel the natural divisions of the experiment. No attempts were made to fit continuous functions across experimental manipulations representing real discontinuities. In the non-stress experiments the experimental divisions are:

(I) pre-period

(II) first infusion period

(III) second infusion period

(IV) recovery period

Using the Wang mini-computer the data in each period were fitted with a continuous function representing ACTH concentration versus time. Periods (II) and (III) include the last sample of the previous period in their function fits as an initial boundary condition. Periods (I) and (IV) include only their respective sampling data (period(IV) is separated from the rest of the data by 60 minutes).

The functions so fitted were orthogonal polynomials solved by Forsythe's method, and the criteria for best fit are those described in Cowan and Hetenyi (1971b), corresponding closely to the selection of the best value of the F ratio. This conservative approach may cost some resolution but avoids excessive instability in the first derivative (Cowan and Hetenyi, 1971b).

These equations representing ACTH concentration versus time were then used to calculate secretion rates, again with the Wang mini-computer which entered the processed data in the above equation and evaluated the resulting function for secretion rates for ACTH at any selected closely spaced time interval, yielding a continuous representation of secretion rates (in mU/kg. min.) versus time (min.) for these experiments.

4. ACTH BIOASSAY AND PLASMA CORTISOL DETERMINATIONS

The assay (Sayers et al 1971), basically involves the corticosterone produced by a rat adrenal cell suspension incubated in the presence of ACTH (standard, plasma sample or blank). The adrenal cells were obtained from rats whose quartered glands were dispersed enzymatically by trypsin and mechanically by agitation. The cells were then, centrifuged, resuspended in resuspension medium, added to teflon beakers (with either

treated plasma sample, ACTH standard or blanks) and incubated. The corticosterone thus produced was extracted chemically with methylene chloride and the fluorescence elicited in H_2SO_4-ethanol was measured on an Aminco-Bowman spectrofluorimeter. By comparing the sample readings to known corticosterone standards, and known ACTH standards with their corresponding corticosterone reading, the ACTH concentration in the samples could be determined.

The cortisol samples were extracted chemically with methylene chloride and assayed fluorimetrically as above, using cortisol standards (Silber et al 1958).

The above Sayers (1971) bioassay was modified (Cowan et al 1974). The changes will be mentioned here.

CHANGES IN THE PREPARATION OF THE CELL SUSPENSION

Male retired breederrats (Sprague-Dawley) have been used in preference to smaller rats, as they provide a somewhat larger mass of adrenal tissue and the cells seem hardier. The rats were killed by decapitation, rather than by aortic section under ether anesthesia. The cleaning of the glands was carried out on siliconized glass surfaces maintained at 0°C. The Ca^{2+} concentration in the Krebs-Ringer bicarbonate buffer was 2.0 mM as opposed to 2.5 mM. The quartered glands were washed once before dispersion with oxygenated Krebs-Ringer bicarbonate buffer containing 200 mg% glucose. Dispersion was carried out in a Wheaton 100 ml spin-culture flask, rather than in a 50 ml Erlenmeyer flask with a glass paddle. As a result, the variations in the yield of the dispersion were greatly diminished. The amount of lima bean inhibitor (LBI) used in the cell

suspension was reduced to 0.4-0.5 mg/ml of cell suspension. The incubation of the aliquots of the cell suspension with the ACTH-containing samples in a Dubnoff shaker was unchanged, except that the agitation was reduced from 66 oscillations/min. to 40 oscillations/min.

CHANGES IN SAMPLE TREATMENT

Heparin in amounts up to 50 U/ml of sample was found to have no effect on the assay. Samples of blood were taken in plastic syringes and expelled into iced siliconized 2-ml glass tubes, containing 5-10 U of heparin, and centrifuged at 0°C for 10 min. To the plasma, removed to an iced siliconized vial, was added an amount of 1N HCl corresponding to 15% of its volume, and yielding a pH of 1.5-2.5. The samples were then frozen (-70°C). Later 100-µl aliquots were added directly to the assay system. This procedure, which has been extensively tested, yields recoveries of 95-103%. Poorer recoveries (63%±14%, mean±s.d.) were obtained with the QUSO extraction method described by Giordano and Sayers (1971), which was, therefore, not used in these studies.

To have comparable standards, the ACTH standard solutions have been made up in heparinized dog plasma in which the ACTH was previously destroyed by incubation a 45°C for 2 h at pH 7 and then acidification with HCl as above. This "deactivated" plasma showed no ACTH activity.

Samples treated in the above manner retain about 90% of their activity after storage at -70°C for 4 months.

The section above describes the adrenal cell suspension bioassay used with its modifications, more of its history and sensitivity with other ACTH assays will be described in appendix II. This makes a compari-

son of the assays currently available as well as some of their developmental history.

CHAPTER III
RESULTS

1. THE TURNOVER OF INFUSED CORTISOL IN THE ADRENALECTOMIZED DOGS.

The infusions of cortisol in seven male adrenalectomized dogs (8.3 to 13.1 kg) were primed, so as to rapidly achieve plateaux of plasma cortisol. As seen in table I, the injection-to-infusion ratios (InJ/InF) (which is the time that a given infusion rate or increase in infusion rate would require to deliver an amount of cortisol equal to the injection) were selected to produce as rapid an equilibrium as possible and corresponds to the ratio of distribution volume to metabolic clearance rate (V/MCR) for cortisol. The InJ/InF ratios near 25 (minutes) indicate fairly well matched injections to infusions and consequently the speedy achievement of new equilibria in plasma cortisol concentration to be held by the accompanying infusion rate. In dog # 1, the cortisol injections were overestimated and resulted in declining plasma cortisol levels over the first part of each of the three infusion periods in this dog.

In general, with the exception of dog # 1, the cortisol plateaux were achieved and maintained quite well. The mean plasma cortisol concentrations seen in table (II) have, in general, a low error associated with them; this feature being consistent over a significant range of physiological cortisol concentrations. The mean cortisol concentrations were determined on three plasma samples for the pre-infusion periods for each of the 7 dogs; five samples in each of the three infusions periods of dog # 1 and eight samples for each of the two infusion periods on the other 6 dogs; and three samples to establish the post-infusion cortisol means

of each of the 7 dogs. Those cortisol means which do show some significant variation are (1) the post-infusion periods based on three samples during a period of cortisol removal without replacement and (2) the three infusion periods of dog # 1 which were over-primed.

The mean cortisol values, shown in table (II), demonstrate the plasma concentrations before, during and after the cortisol infusions listed in table I. The pre-infusion period shows the absence of cortisol in an adrenalectomized dog 48 hours after its last maintenance injection (2.5 mg) of cortisol. The plasma cortisol concentrations of the first infusion period column in this table, are those low physiological concentrations resulting from primed infusions of cortisol over a 60-minute period in dog # 1 and a 90-minute period in the other dogs. The plasma concentrations in the second infusion period column are those slightly higher levels resulting from primed infusion rates nearly double the first infusion rates (see table I).

The post-infusion plasma cortisol concentrations are those reduced levels seen 90 to 110 minutes after the cessation of the last cortisol infusion in dog # 1, and 60 to 80 minutes in the other 6 experiments.

The known cortisol infusions and resultant plasma cortisol concentrations made possible the calculation of average metabolic clearance rates for the infusion periods in each experiment (cortisol infusion rate divided by the resulting mean plasma cortisol concentration and normalizing for body weight). As shown in table (III), a clearance of 12.79±1.43 ml/kg. min. (mean of 7 dogs) is associated with a mean plasma cortisol concentration of 4.27 ± 0.574 µg/100 ml plasma. A higher plasma cortisol concentration

(mean of 7 dogs) of 6.23±0.82 µg/100 ml has associated with it a clearance of 17.41± 2.47 ml/kg. min.

Thus there seems to be an increase in clearance associated with an increase in plasma cortisol concentration.

As a mean of 7 animals a 1.965 ± 0.001 fold increase in cortisol infusion rate causes a 1.482 ± 0.095 fold increase in plasma cortisol concentration and a 1.355 ± 0.077 fold increase in metabolic clearance of cortisol (since the dogs are adrenalectomized and have no endogenous cortisol, the infusion rate (cortisol added to the system) equals the product of the resultant plasma cortisol concentration (a steady level) and the metabolic clearance rate for cortisol).

The use of priming injections allowed the calculation of apparent distribution volumes for cortisol((the mass of the injected cortisol was divided by the cortisol concentration at the time of the injection (extrapolated from the cortisol concentration curve) and normalized for animal weight)). Where there were two consecutive infusions the injected cortisol for the second infusion period was divided by the cortisol concentration difference at the time of injection extrapolated from both cortisol concentration curves. With the exception of the second infusion periods for the second and third experiments in table (IV) in which no distribution volumes could be calculated due to poor statistical resolution between these infusion periods and their previous respective infusion periods, the other second infusion periods did give statistically satisfactory results. In the second infusion periods of dog's 2 and 3, the concentration differences were barely significant.

It is evident from table (IV) that there is no correlation between mean plasma cortisol concentrations and their calculated apparent distribution volumes. It is not possible to predict whether a given plasma cortisol concentration will give a high or low apparent distribution volume nor does prior exposure to a cortisol infusion affect subsequent distribution volumes associated with higher cortisol levels (see figure I).

Taken as an average of 7 dogs a mean cortisol concentration of 4.27 $\pm$ 0.574 µg/100 ml has an apparent distribution volume of 368.39 $\pm$ 76.51 ml/kg (36.8 $\pm$ 7.6%). And a second infusion period mean plasma cortisol concentration of 6.28 $\pm$ 1.053 µg/100 ml (mean of 5 dogs) has an apparent distribution volume of 434.31 $\pm$ 86.124 ml/kg (43.4 $\pm$ 8.6%). Thus the distribution volume for low physiological concentrations of cortisol appear to be of the order of 40 percent of the total volume of these dogs.

2. EFFECT OF INFUSED CORTISOL ON ENDOGENOUS ACTH CONCENTRATION AND SECRETION IN THE ADRENALECTOMIZED DOGS

Using the modified Sayers bioassay the ACTH concentration (mU/100 ml) versus time (minutes) results, shown in table (V) for the 7 dogs, were obtained. As mentioned above, polynomials were fitted to these data so as to give a continous representation of the data with respect to time. These fitted functions were then used in the calculation of ACTH secretion rates by the method described earlier. An example of such function fitting to ACTH concentration data is shown in figure (2) in which both the ACTH concentrations with their associated errors and the curves fitted to these data are shown for dog # 5. The fitted functions, here, do represent the data quite well.

Although the ACTH concentration data do show inhibition of ACTH release by the increased plasma cortisol, these declining ACTH concentrations in the face of continued cortisol exposure are merely a result of changes in ACTH secretion rates. Hence, ACTH secretion rates for these experiments were calculated to show this more direct effect of cortisol.

The ACTH secretion rate results from dog # 1 (figure 3) do show the suppression by cortisol seen throughout this series of experiments. Here, the pre-infusion period is seen to have a rising ACTH secretion rate (0.5 to 1.1 mU/kg. min.) over a 20-minute period of time. This represents the somewhat labile secretion rate 48 hours after the last cortisol injection, 85 minutes after the Nembutal administration and 40 minutes after the insertion of two venous cannulae. (This instability (non-steady state secretion level) in pre-infusion secretion rates seems to be a feature of this adrenalectomized state with no hormonal feedback).

During the first cortisol infusion (4.83 µg/100 ml cortisol), there was an almost immediate stopping of the pre-infusion rising secretion rate and a more gradual reduction in secretion to a plateau of 0.45 mU/kg. min. towards the end of the first period. The second infusion period (7.26 µg/100 ml cortisol) continues this downward trend with a rapid reduction in secretion rate to 0 mU/kg. min. three quarters of the way into this second period. The third infusion period (8.02 µg/100 ml cortisol)maintains the 0 mU/kg. min. secretion rate. The post-infusion period, beginning 90 minutes after the cessation of the third infusion period (1.33 µg/100 ml cortisol) shows a gradual increase in secretion rate to about 0.25 mU/kg. min. by the end of the 20-minute sampling period.

In all of the subsequent dogs the pre-infusion period secretion rates represent those seen 48 hours after the last cortisol maintenance injection, 45 to 80 minutes since anesthesia with Nembutal and 20 to 40 minutes since the insertion of two venous cannulae. In no case did these differences in elapsed times have any observable effect on results. As with dog # 1, there was also demonstration of a rising pre-infusion secretion rate in dogs 2, 5, 6, and 7 (figures 4, 7, 8, 9). There was a steady pre-infusion secretion rate in dog # 4 (figure 6). And dog # 3 (figure 5) showed a gradually decreasing pre-infusion secretion rate. This shows the unstable secretion rate pattern in these adrenalectomized dogs.

Although the 7 dogs differ in the absolute values and slopes of pre-infusion secretion rates, dogs 2 through 6, like dog # 1, show significant reduction in pre-infusion secretion rates during the first cortisol infusion period. Dog # 7, the largest of the 7 dogs, and having the lowest first infusion period mean cortisol level, showed no visible inhibitory effect on ACTH secretion whatsoever during this first infusion period.

The second infusion period (having roughly twice the cortisol infusion rate of the first infusion period) showed, in all 7 dogs, a significant decrease in secretion rate as compared to that at the end of the first period. The only difference among dogs was (a) whether there was a complete reduction in secretion rate to zero or to a new steady state and (b) how quickly this suppression took place. Dog # 2 showed rising secretion rates toward the end of the second period after an initial reduction

to 0 at the beginning of the period. This rise is a stress response probably due to insufficient anesthesia at that time. Although dog # 7, showed no suppression of ACTH secretion in period one, in period two (with a mean plasma cortisol level of 4.67 µg/100 ml) it did show some significant suppression.

The post-infusion period, which in dogs 2 through 7 was 60 through 80 minutes after cessation of the second cortisol infusion, showed a rising secretion rate in dogs 2 through 6. Dog # 7, having a post-infusion mean cortisol concentration of 4.18 µg/100 ml, only slightly less than that seen in its second infusion period, continued to show a reducing secretion rate in the post-infusion period.

When the secretion rate results from the seven experiments are analyzed as in table (VI), a better overall picture of the feedback of cortisol on ACTH secretion is developed. In this table the suppression of ACTH secretion by cortisol is calculated as a percent of pre-infusion ACTH secretion rates (where no cortisol was present). The lag time, referred to in this table, is that time (minutes) elapsed between the beginning of a cortisol infusion and the onset of significant suppression of ACTH secretion. The lag period is estimated on the basis of significant change in ACTH secretion rate and has an uncertainty of not more than 5 minutes. The period of changing secretion rate, is that time period between the end of the lag time and the onset of a new steady ACTH secretion rate (the time period during which effect of cortisol on the suppression of ACTH secretion is still increasing). Again, these end point times are determined as above. Since there was no steady state secretion

rate achieved in the last period (secretion rates were changing, usually rising), the mean of the continuous secretion rate function was compared to the pre-infusion period secretion rate to indicate a mean remaining suppression.

In the first infusion period plasma cortisol concentration (of 6 animals) of 4.59 ± 0.57 µg/100 ml, had an associated lag time of 22.33 ± 2.06 minutes, a period of changing secretion rate of 44.33 ± 3.5 minutes and a suppression of 65.05 ± 6.06 percent of the pre-period secretion rates. In the second infusion period a plasma cortisol concentration (mean of 6 animals) of 6.487 ± 0.92 µg/100 ml had an associated lag time of 15 ± 4.16 minutes, a period of changing secretion rate of 41.5 ± 10.95 minutes and a 95.3 ± 3.14 percent suppression of ACTH secretion rates as compared to pre-infusion levels. The post-infusion period had a 69.55 ± 12.62 percent suppression of pre-period ACTH secretion associated with a mean plasma cortisol concentration of 1.95 ± 0.43 µg/100 ml (mean of 6 animals).

It will be noted that the second infusion period mean lag time, the animals having had prior exposure to cortisol, is shorter than that for the first period. There is no significant difference between the periods of changing secretion rate for both the first and second infusion periods, although there is a higher error associated with the second infusion period changing secretion rates mean.

Even though the post-infusion mean plasma cortisol is the lowest of the three periods (4 periods in dog # 1) in which cortisol is present, it has a percent suppression value higher than that in the first period. However, this post-infusion period is a time of declining cortisol concentra-

tion after two infusion periods of cortisol lasting 180 minutes and a waiting period of 60 minutes of cortisol clearance without replacement (in dog # 1, three periods of 180 minutes total and a waiting period of 90 minutes).

There is a very evident inter-animal variation in suppression response to a given concentration of cortisol.

The seventh dog was not grouped with the other six in table (VI). It was the largest of the seven dogs and had the lowest first infusion period mean plasma cortisol concentration (2.38 µg/100 ml). It showed no suppression whatsoever in this first infusion period. Suppression began during the second infusion period (4.67 µg/100 ml mean cortisol) and continued over the 60-minute waiting period into the post-infusion period. Here the cortisol concentration had decreased only slightly (4.18 µg/100 ml) from the second infusion period level. This animal seemed to show a delayed response to cortisol feedback but one which continued for an extended period of time, and exhibited unusual cortisol turnover characteristics.

Figure (10) demonstrates a composite mean picture of the suppression of ACTH secretion by cortisol, using mean elements calculated from six dogs. It shows the lag time between the commencement of the plasma cortisol plateau concentration and the abrupt onset of suppression down to a new steady state ACTH secretion level. It shows the subsequent lag time associated with the second but slightly higher physiological plasma cortisol concentration and a less abrupt suppression of ACTH secretion rate to a second new steady state level. The post-infusion period shows a suppression of secretion rate slightly more than that in the first period but considerably less than that seen in the second infusion period.

CHAPTER IV

DISCUSSION

1. CONCENTRATIONS AND TURNOVER OF CORTISOL

The resting cortisol concentrations seen in human plasma are variously reported as 5 to 16 µg/100 ml (5 at midnight, 16 between 6 and 8 am) (Peterson 1959) and 1 to 13 µg/100 ml according to Richards and Praitt (1957) along with numerous other reports in the same range. Although there seems to be some disparity in "resting" cortisol levels (time of sampling, some slight "stress" of sampling etc.), the upper boundary of "resting cortisol levels" is probably below 16 µg/100 ml. The maximum cortisol levels seen in humans have been observed to be 60 µg/100 ml (Peterson 1959) or 70 µg/100 ml (Yates and Urquhart 1962), although this value of Yates includes both cortisol and corticosterone (0.3 to 5 µg/100 ml). Since the cortisol values for humans and dogs are comparable (Yates and Urquhart 1962), it is clear that the plasma cortisol concentration seen in these experimental dogs (see table II) which varied between 2.74 and 8 µg/100 ml never exceeded the accepted resting levels for dogs and certainly never approached the maximum levels. Hence the cortisol concentrations seen in these experiments were certainly physiological.

In order to assess what the cortisol infusion rates should be in these experiments, something of the normal cortisol secretion rates in these animals had to be known. Secretion rates from human data yielded maximum continuous values of 110 µg/kg.hr (Nugent et al 1963) and 90 µg/kg.hr (Peterson 1959), which correspond respectively to 1.83 and 1.5

µg/kg.min secretion rates. These are considerably higher than the resting secretion rates measured more recently in humans of 0.01 to 0.17 µg/kg.min (Flood et al 1961) and 0.14 to 0.26 µg/kg.min (Van der Straeton et al 1963). The resting cortisol secretion rates reported for dogs of 0.2 to 1.25 µg/kg.min (Yates and Urquhart 1962) and more recently 0.883 µg/kg.min (McCormick et al 1974) are seen to be higher than human resting secretion rate levels. In the 7 dogs of the series, the 15 cortisol infusions vary from 0.31 to 1.88 µg/kg.min. These correspond to the cortisol secretion rates for human and animal subjects which have been associated with ACTH resting concentrations of less than 1 mU/100 ml (Syndor and Sayers 1954) and more recently with measured ACTH levels of 0.25 mU/100 ml (Gray and Bacharach 1967).

To quickly achieve plateau plasma concentrations, the cortisol infusions were primed. Human data reveal a total cortisol mass of 10 to 15 mg (Peterson 1959). Arbitrarily a mass of 150 µg/kg was chosen and used to determine priming doses in dog # 1. Although this pilot experiment was overprimed, it did yield an apparent cortisol distribution volume of 50% V (V= total available volume of dog) and an MCR of approximately 20 ml/kg.min which were used as guidelines in subsequent experiments. These data were used as follows:

Infusion= MCR x Cxwt

where, Infusion= Infusion rate (µg/min)
MCR= clearance from dog # 1 (ml/kg.min)
C= desired concentration of cortisol in plasma
wt= weight of dog (kg)

This equation yielded the approximate concentration which a given infusion rate would achieve and which the priming dose was designed to reach quickly. This method proved its validity as it did provide infusions which were well matched to the priming injections (see table I) and it eliminated many of the assumptions which had been made to get the estimations used in the pilot experiment.

This series of experiments yielded average metabolic clearance rates of 12.79 ml/kg.min for a mean plasma cortisol concentration of 4.27 µg/100 ml and a 17.4 ml/kg.min clearance rate for a mean cortisol concentration of 6.23 µg/100 ml (see table III) (both cortisol levels are mid-range for a normal resting animal). These data compare favourably with the 17.7 ml/kg.min metabolic clearance rate obtained by McCormick et al (1974) for adrenalectomized adult dogs. An increase in clearance with increased plasma cortisol is suggested by McCormick et al (1974) confirmed by Yates (1967b) and Trait (1963) and suggested for corticosterone by Dallman et al (1969). This is in contradiction to Nugent et al (1963) who found that the rate of removal of cortisol was not related to concentration. Furthermore, McCormick et al determined that since the clearance of cortisol by individual organ systems (kidney, liver, GI tract and spleen) was linear (cortisol input into an organ vs. output) then the total metabolic clearance of the entire animal must be a linear process. This agrees with Peterson (1959) who found the clearance of cortisol linear between 50 and 400 mg of injected cortisol in humans based on fairly uniform half-lives (96-110 min) which is pharmacological (resulted in mean plasma cortisol concentrations of 70 to near 600 µg/100 ml) and therefore probably not comparable to the results of McCormick

which were physiological. Our data here are insufficient to definitively support or disagree with any claim of linearity in specific organs but they do agree with an increase in overall clearance associated with an increase in cortisol concentration in agreement with the findings of Rotsztejn et al (1975). This relationship between clearance and concentration physiologically makes sense, since an increasing clearance with an increasing concentration or a decreasing clearance in face of a decreasing concentration helps stabilize the level of cortisol in blood.

From the cortisol data, a mean distribution volume for cortisol of 40% of the total volume (V) was calculated. This is slightly less than was calculated from the pilot experiment and used as an estimator for subsequent experiments but considerably more than is obtained for humans. A pool mass of 10 to 15 mg cortisol (Peterson 1959) yields a distribution volume of 15 to 22% V. Since the 50% V distribution volume worked reasonably well in calculation for cortisol priming doses, although on occasion there was a slight decreasing plasma cortisol concentration after injection (indicating overpriming), the calculated mean V seems reasonable.

In summary then, comparing human and dog data in cortisol handling, it becomes apparent that although they have comparable plasma cortisol concentrations, humans have lower metabolic clearance rates, lower cortisol secretion rates and lower distribution volumes than dogs.

2. GLUCOCORTICOID FEEDBACK ON ACTH SECRETION.

In table (VI) there is evidence of a relationship between the concentration of cortisol present in the plasma and the amount of ACTH suppression (compared to pre-cortisol infusion ACTH levels) associated with

this cortisol. In fact, viewing the results of the first 6 dogs (see Table VI), if the ratio of the amount of suppression (percent of pre-period levels (65.05%)to its mean plasma cortisol concentration (4.59 µg/100 ml) for the first infusion period is compared to the ratio of suppression (95.3%) to the mean cortisol concentration (6.487 µg/100 ml) of the second infusion period, an almost linear relationship* is observed ($\frac{S_1}{C_1}$ = 14.17, $\frac{S_2}{C_2}$ = 14.691). This feature does not hold for the post-infusion period because while the two infusion periods represented platcau concentrations of cortisol, the post-infusion period represented declining cortisol concentrations with delayed feedback effects of earlier (higher) concentrations. These data serve to reaffirm the negative feedback of glucocorticoids on ACTH release (Yates and Urquhart 1962; Yates et al 1974; Fortier 1963 and Venning 1955). It is highly significant that such suppression was possible with such low physiological concentrations of cortisol.

Although these experiments do not, nor were designed to, show where this feedback takes place, they do show the presence of a finely regulated system in which low concentrations of cortisol significantly suppress ACTH secretion (often to 100%) and where cessation of cortisol infusion (cortisol clearance without replacement) for a relatively short period of time reduces this suppression.

a) Site of glucocorticoid feedback-review.

However, because neither the precise location of such feedback nor the relative contribution of any subunit to such feedback suppression is known, some of the current literature on glucocorticoid feedback at the pituitary and hypothalamic levels, with some of the characteristics of such feedback, will be reviewed here.

* This linearity assumes the minimum concentration of cortisol required to cause near complete suppression of ACTH secretion. This is substantiated by the assymtotic decline in ACTH secretion seen in the face of the low cortisol concentrations used.

(i) Inhibition at the pituitary level.

The "long" (Zniewald et al 1972) glucocorticoid feedback on ACTH release has been studied quite extensively (this type of feedback implies that the controlling messages are provided by target organ hormones which feedback on the pituitary and/or hypothalamus). Cortisol, infused into the hypophyseal area of uni-laterally adrenalectomized rats, inhibited the ACTH-dependent compensatory adrenal hypertrophy (Rose and Nelson 1956). Dexamethasone studies also support a pituitary site of feedback. ACTH pituitary content and release, respectively, have been inhibited by dexamethasone pituitary implants (Chowers et al 1967) and by pituitary injection of dexamethasone (Russell et al 1969). Also, dexamethasone pretreatment of unanesthetized dogs abolished the ACTH releasing ability of microinjected exogenous CRF (corticotropin releasing hormones) (Gonalez-Luque et al 1970). Dexamethasone was also shown by de Wied (1964) to inhibit ACTH release by Vasopressin in rats with lesioned hypothalamic median eminences (CRF is a term which describes all substances which have the ability to release ACTH, including the endogenous releasing factor CRH).

Further, pituitary cells in a monolayer culture (Fleischer and Rawls 1970) and incubated pituitary glands showed corticosteroid inhibition of ACTH release stimulated by CRF's and ions (Pollock and Labella 1966; Fleischer and Vale 1968; Kraicer et al 1969). Vernikos-Danellis (1964) showed suppression by exogenous steroids of the release of ACTH from the pituitary by CRF's. With their endogenous CRH source missing (brain removal or destruction), systemic injection of CRH and other CRFs into such animals, which usually cause an increase in ACTH release, fails to cause

this when they were injected with corticosteroids (de Wied 1964; Dunn and Critchlow 1969). In these animals corticosteroids could still cause adrenal atrophy (Ganong and Hume 1955) implying that ACTH secretion was still present which could be decreased by these corticosteroids. In a further study using dexamethasone, Kendall and Allen (1968) found that dexamethasone decreased plasma corticosteroid concentrations in hypophysectomized rats with anterior pituitaries transplanted under their renal capsules.

These data establish that the release of ACTH induced by either exogenous or endogenous CRFs or by CRH can be inhibited locally at the pituitary by corticosteroids in such experimental conditions. This raises the possibility that the anterior pituitary may be an important site of physiological negative feedback. Henkin et al (1968) found that the anterior pituitary of the rat contained both cortisol and corticosterone at tissue concentrations 800 times higher than those present in plasma. However, other locations in the brain were not checked for concentrating ability and a higher steroid concentration in one part of the brain over others does not necessarily imply higher activity (inhibition).

(ii) Glucocorticoid Inhibition of CRH.

Notwithstanding the possibility of inhibition of CRH by CRH (Motta et al 1969) and the inhibition of CRH by ACTH (Hodges and Vernikos 1959; Vernikos-Danellis and Trigg 1967) there is also substantial evidence for negative glucocorticoid feedback on the hypothalamus or other areas of the brain.

Labelled corticosteroids, administered systemically, are taken up by various regions of the brain, especially the hypothalamus, hippocampus and septum (McEwen et al 1970: Walker et al 1971). This uptake appeared to be by neurons (as opposed to glial cells) as shown by McEwen. Corticosteroids have also been shown to affect brain excitability (Feldman and Davidson (1966a), morphology (Aus Der Kuehlen and Ockenfels 1969) and can either increase or decrease neuronal firing, particularily in the hypothalamus and midbrain (Steiner et al 1969). It seems fairly certain that uptake occurs in neurones and this seems the most likely way in which corticosteroids can affect adrenal cortical function by varying the synthesis or release of CRH (although due to the short duration of most experiments only the release of CRH would be affected significantly).

Although, when corticosteroids are introduced into various parts of the brain there is the possibility that they may spread to the pituitary from the brain, there have been too many well-controlled experiments for this to be the only underlying reason for feedback inhibition at the brain level. Corticosteroids introduced into the midbrain, hypothalamus and septum have repeatedly been shown to inhibit the adrenal cortical system (Corbin et al 1965: Russell et al 1969; Chowers et al 1967). Corticosteroids can increase, have no effect, or decrease firing rates of various neurones in the brain (Feldman and Dafney 1966b; Ruf 1967). These findings suggest that corticosteroids might act in several ways on the CRH releasing system: they could cause inhibition by inhibiting CRH stimulatory neurones or by stimulating CRH inhibiting neurones (Yates et al 1974).

There are other lines of evidence indicating corticosteroid inhibition of CRH. McHugh and Smith (1967) showed that systemic administration of cortisol in monkeys was more effective in inhibiting ACTH release following amygdala stimulation rather than stimulation of the hypothalamus (suggesting a negative feedback present somewhere between the amygdala and the hypothalamus). Yates et al (1971b) showed that graded (progressively increasing) doses of dexamethasone continue to cause progressive inhibition of the adrenocortical system to a CRH releasing stimulus, without affecting the response of CRH itself at the pituitary. This was interpreted (and supported by Vernikos-Danellis 1964) as indicating separate central nervous system and pituitary sites for inhibition of the adrenal cortical system by corticosteroids and that the pituitary sites are saturated at lower doses of corticosteroids than brain sites. Takebe et al (1971), then, showed a decrease in hypothalamic CRH content after steroid administration. Chowers et al (1967) also found a decrease in hypothalamic CRH after hypothalamic implantation of dexamethasone. They also found an increase in CRH after implantation of dexamethasone into the anterior pituitary. They concluded that steroids could suppress the synthesis of CRH by an action on nervous tissue (if the only effect of steroids was on the pituitary, no change in CRH content would have been expected after implantation of dexamethasone).

Taken as a whole this evidence points to corticosteroid sites of inhibition of ACTH release at the pituitary and inhibition of CRH at several points in the brain, their number and location undetermined (Yates

et al 1974).

3. Properties of Glucocorticoid Modulation of Adrenocortical System.

It has been well established that pretreatment with corticosteroids can inhibit the secretion of ACTH induced by stress. This inhibitory action of corticosteroids has been observed to occur about two hours after administration of supraphysiological doses of steroids (Smelik 1963; Hodges 1953). Physiological doses of corticosteroid have been reported by some workers to inhibit ACTH secretion immediately after administration (Sayers and Sayers 1947; Dallman and Yates 1969). In fact, Dallman and Yates (later confirmed by Jones et al 1972) reported a rapid rate- sensitive inhibition and a delayed concentration- sensitive corticosteroid feedback. This involves the release of endogenous corticosterone by injected ACTH any time during a corticosterone infusion into rats. However, if histamine is injected as levels of corticosterone are rising sharply (infusion) no release of ACTH is seen from this stressor. Histamine is ineffective until plasma steroid levels have stopped rising and are in a steady elevated level. Thereafter, it will activate the system for one to two hours after the attainment of this level. After two hours a new period of inhibition appears in which histamine will not activate the system. The presence of rapid corticosteroid inhibition has also been confirmed by Dallman (1972).

The experiments by Dallman and Yates (1969), Jones et al (1970) and Jones et al (1974) were all performed on rats. The mean resting plasma corticosterone concentration seen in rats is about 8 µg/100 ml (Dallman and Yates 1969). The diurnal variation in corticosterone levels is dif-

ferent from dogs since rats are nocturnal animals and consequently have a night-time peak as opposed to a mid-morning peak in dogs. The maximum corticosterone levels reported for the rat are slightly less than 100 µg/100 ml (Dallman and Yates 1969). These features of the rat, unlike the dog, describe an animal easily stressed and not conducive to non-stress studies. However, the feedback studies by these three researchers used stressed rats (injections of histamine, Dallman and Yates, 1969; sham bilateral adrenalectomy, Jones et al 1972; and ether vapour or sham surgery, Jones et al 1974).

Our experiments do not show much evidence of this fast feedback of which Dallman and Yates (1969) and Jones et al (1972) speak. If this were so, we would see a sharp discontinuity between pre-infusion and first infusion ACTH secretion rates and perhaps between consecutive infusions. We do not see this. Instead, we see a lag time of about 20 minutes during which some sort of feedback activity is undoubtedly occuring but which is not noticeable. This is followed by a period of about three quarters of an hour during which significant suppression occurs and ACTH levels are reduced to a new steady level. What is seen here is a delayed feedback of the order of 1 hour as opposed to 2 hours as reported by Dallman and Yates (1969) and Jones et al (1972). This is, however, comparable to Jones et al (1974) who found a delayed feedback of 1 hour which was related to the dose of corticosteroid administered.

Our experiments were performed on non-stressed adrenalectomized anesthetized dogs.(It has been shown that pentobarbital does have some inhibitory effect in ACTH release (Greer and Rockie 1968; Critchlow 1972).

This is in contrast to Rotsztejn et al (1975) who found no inhibition of ACTH in the rat.) The corticosteroid infusions were primed so as to achieve very rapid plateau concentrations. The work by Dallman and by Jones involved rising steroid levels which demonstrate a rapid inhibitory effect (Paton 1961). Our experiments at first glance would simply demonstrate feedback in the presence of fairly stable plasma steroid levels, which they (Dallman, Jones) equated with a slow feedback response.

The fast feedback seen a few minutes after commencement of the exogenous glucocorticoid exposure in the experiments of Dallman and Yates (1969) and Jones et al (1972), during the period of rising plasma glucocorticoid levels, ought to be present in our feedback experiments in dogs. The cortisol infusions in our experiments were primed and theoretically the rate of rising glucocorticoid levels would be in excess of the critical 1.3 µg/100 ml/min rate which Jones et al (1972) associate with fast feedback. However, the initial and final plasma glucocorticoid concentrations of the rat and dog studies are quite different. The plasma cortisol levels in our non-stress dog experiments varied from 0 (before any cortisol infusion) to a plateau concentration (mean of 6 dogs) of 4.59 µg/100 ml during the first infusion and a plateau concentration (mean of 6 dogs) of 6.49 µg/100 ml during the second infusion. In Dallman and Yates' paper an infusion of corticosterone raised the plasma corticosterone levels from 8 µg/100 ml to 47 µg/100 ml over a 17 minute-period. In the paper by Jones et al (1972), an *infusion of corticosterone raised the plasma corticosterone concentration from 8 to 38 µg/100 ml over a 10 minute-period. Both of these researchers raised the levels of glucocorticoid significantly

* administered intraperitoneally

higher than the resting levels for rats (as stated above the plasma cortisol levels for the dog experiments were low physiological). Also, in both rat experiments, resting levels of glucocorticoid were present before the addition of exogenous glucocorticoid. So, perhaps this fast feedback is not just a feature associated with rising plasma glucocorticoid levels, but also a function of the beginning and ending concentrations of this rise. Perhaps, then, these dog experiments did not conform to a fast feedback situation because of the low beginning (Zero) and low plateau plasma glucocorticoid levels, although the rate of rise of concentration resulting from the priming was probably consistent with the criteria of critical rate of glucocorticoid rise.

Although, there is some sort of a delayed feedback in our experiments of 1 hour (a 20-minute delay period + a 40-minute period of duration of action), it is considerably less than the 2-hour delayed feedback reported by Dallman and Yates (1969) and Jones et al (1972). As well as any interspecies differences (glucocorticoid receptor binding characteristic differences) and the much higher levels of glucocorticoids used in these rat experiments, a third possible reason for this discrepancy may lie in the methods of measuring ACTH used in these different studies. In our series of experiments plasma ACTH levels were measured directly, yielding ACTH secretion rates which could be related to plasma levels of glucocorticoid. The results of Dallman and Yates, Jones et al (1972) and Jones et al (1974) were obtained by measuring ACTH indirectly, that is, in terms of changes in plasma concentrations of corticosterone resulting from changes in plasma levels of ACTH. This latter technique of measurement has inherent

time delays since a change in ACTH secretion resulting from the exogenously administered glucocorticoid would have to change the circulating levels of ACTH, which would alter the adrenal secretion of corticosterone, which would manifest itself in changes in plasma levels of corticosterone and which finally would be measured and used as an index of the initial changes in ACTH which caused this cascade. This time delay before a significant change in the plasma levels of corticosterone would occur in the rat, is probably of the order of 20-30 minutes (Jones et al 1972). Therefore, a combination of inter-species variability, the fact that these animals produce and respond to different glucocorticoids, the higher levels of glucocorticoids used in the rat experiments, and the delays associated with measuring the changes in ACTH in terms of changes in glucocorticoid levels, may account for the discrepancies between the values obtained for delayed feedback in these studies. Also, it may be suggested that physiological delayed feedback (using low physiological levels of glucocorticoid) is not as slow as previously thought.

There is another interpretation of the discrepancy between our delayed feedback results and those obtained by Dallman and Yates and by Jones et al. Although no clear fast feedback was obtained in our experiments (low initial and plateau cortisol concentrations), the sustained low plateau concentration of cortisol may have resulted in an activation of both fast and delayed feedback mechanisms, resulting in a duration of feedback somewhere between those times cited for fast and delayed feedback. More information on the mechanics and characteristics of these types of feedback must be obtained before these discrepancies can be resolved.

One further aspect in the comparison of the experiments done on rats and dogs, which must be emphasized, is the fact that while our dog experiments involved feedback in a non-stress situation, the rat experiments determined glucocorticoid feedback in the presence of stress. Whether or not stress can alter hormonal feedback significantly is another interesting variability in this comparison.

The delayed feedback obtained by Jones et al (1974) is similar to ours. However, they used subcutaneous injection of corticosterone (in saline or oil, 20 minutes and 120 minutes before the administration of stress) as a source of glucocorticoid feedback in rats. Although they claimed that this resulted in physiological plasma levels of corticosterone, because of the route of glucocorticoid administration and the uncertain time of exposure of the animal to glucocorticoid prior to stress, it is difficult to compare these results to those obtained by i.v. infusion of glucocorticoid.

The suppression of ACTH secretion seen in dog # 7 (Table V) is of interest. The low first infusion period plasma cortisol concentrations showed no suppression of ACTH secretion. However, only a slight increase in cortisol concentration (2.74 to 3.7 µg/100 ml) in the second infusion period caused a significant suppression of ACTH which continued into the post-infusion period. It would seem that the cortisol reached a critical level where suppression was evident and below which it was not. But, when inhibition did occur it continued for at least 170 minutes to the end of the post-infusion period.

SUMMARY

1. Physiological levels of cortisol were effective in inhibiting ACTH secretion rates in resting adrenalectomized adult dogs. Numerical relationships between them were established.

2. A linear relationship may exist between plateaux plasma concentrations of cortisol and the suppression caused by them in these animals.

3. A delayed maximum feedback of the order of 1 hour, by the infused cortisol is seen in these experiments. The onset of any feedback effects were delayed about 20 minutes.

4. The fast feedback and the 2-hour delayed feedback found by others, using much larger doses of glucocorticoid, were not seen. Possible explanations are given.

5. The infusions of cortisol resulting in physiological plasma concentrations of cortisol had the following characteristics in these experiments: metabolic clearance rates of 12.79 and 17.4 ml/kg. min associated, respectively, with mean plasma cortisol concentrations of 4.27 and 6.23 μg/100 ml (increasing clearance with increasing concentration) and a distribution volume of 40% V.

6. An as yet, incomplete series of experiments shows preliminary results indicating inhibition of stress-induced secretion of ACTH by physiological plasma concentrations of cortisol in adrenalectomized adult dogs.

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APPENDIX I

STRESS EXPERIMENTS

Since the series of experiments involving glucocorticoid feedback on ACTH release in a stress situation is incomplete, the interim results will be discussed in Appendix I, ((a) Histamine, (b) Anoxia).

(a) Histamine:

These experiments were initially prompted by a paper written by Critchlow (1972) in which he claimed to be able to differentiate stress and non-stress ACTH secretion on the basis of physiological glucocorticoid feedback; that is, physiological levels of glucocorticoid could inhibit non-stress ACTH secretion, but not stress-induced ACTH secretion in the rat. However, since he was using plasma levels of corticosterone as an index of ACTH secretion (never directly measuring ACTH) and since he seemed to assume that the adrenal glands would respond proportionately to ACTH regardless of the concentration of ACTH (he assumed no maximum secretory capacity of the adrenals), his results were put in some doubt. Therefore, we undertook experiments to determine if secretion of ACTH resulting from stress could be inhibited by physiological levels of glucocorticoids in dogs. Since adrenalectomized dogs were used, glucocorticoid feedback could be varied from zero to any concentration of cortisol, by infusion.

The chosen stressor, (which Yates et al (1974) describes as any stimulus, internal or external, that ultimately causes increases in secretion rates of CRH, ACTH and corticosteroids to produce a rise in plasma corticosteroid concentration above the levels found at that time of day in

resting subjects on the same sleep-wake cycle) was histamine (BDH Chemicals Ltd., England). It is a potent experimental stressor, easily quantifiable, (as to dose) and easily administered.

The experimental design, which includes three histamine injections, one before, one during and one after a cortisol infusion is described in more detail in the methods section. The dose of histamine (in physiological saline) administered intravenously; was approximately 62 µg/kg body weight. The blood sampling, ACTH and steroid analysis, and data analysis are the same as the non-stress experiments.

Results and Discussion:

Thus far, only five histamine-stress experiments have been performed on five adult male adrenalectomized dogs (see Table A I). The infusion rates and amounts of injected cortisol with the relative matching of injection to infusion $\left(\frac{INJ}{INF}\right)$ are also shown in table A I. Here, an $\frac{INJ}{INF}$ ratio near 25 minutes indicates a well matched injection and infusion (i.e. not an over or under primed infusion). From this table, it is seen that the infusion of dog # 1 (a pilot experiment) was over-primed. The plasma cortisol concentrations resulting from these infusions were physiological. As seen in table A III, the cortisol concentrations, of the one infusion period in each dog, vary from 8.86 to 12.34 µg/100 ml. And, the cortisol concentrations immediately before and during the third histamine injection period (the cortisol having been cleared for 64 to 84 minutes after cessation of the cortisol infusion) varied from 1.56 to 5.5 µg/100 ml, these concentrations lie within the resting cortisol values for dogs quoted by Peterson (1959) and Richards and Praitt (1957) 5 to 16 µg/100 ml and 1

to 13 µg/100 ml respectively), and were nowhere near the maximum cortisol levels found in plasma reported by Peterson (1959) and Yates and Urquhart (1962) (60 µg/100 ml and 70 µg/100 ml respectively).

To further assess the manner in which the cortisol was handled in dogs half-lives (T 1/2) though they assume an unlikely linearity (see Table AV) were estimated for the five experiments. The half-times in these five animals varied widely from about 47 minutes (dog # 2) to 95 minutes (dog # 7). These values compared reasonably to the generally accepted T 1/2 for cortisol of 60-120 minutes as reported by many authors for humans (Gray and Bacharach 1967) less than the 1.5 to 2 hour T 1/2 quoted by Peterson (1959) (although the cortisol levels were pharmacological) and generally higher than the 50-minute T 1/2 quoted by McCormick et al (1974) for the handling of physiological levels of cortisol by adrenalectomized dogs. The cortisol concentrations seen in our experiments were physiological and the half-lives higher than those seen in non-stress acutely adrenalectomized dogs (our animals took part in stress experiments and had been maintained for two weeks or more after adrenalectomy).

Table AVII shows the effects of this cortisol on the histamine-induced ACTH levels in these five dogs. In dogs 1, 2 and 4, we see the following response: A first histamine injection pre-period ACTH level of 5 to 8 mU/100 ml then a first histamine injection ACTH response 5 to 12 times the first pre-period ACTH level (40 to 80 mU/100 ml ACTH). This represents the ACTH response to histamine in the absence of cortisol. A cortisol infusion is begun about 40 minutes before the next equal histamine

injection. In all three dogs the second pre-period ACTH levels were lower than the first (5 mU/100 ml). The second histamine injection increases the ACTH levels 2 to 5 times the first pre-period ACTH level (12 to 35 mU/100 ml ACTH). These are the ACTH responses in the presence of the plasma cortisol concentrations seen in Table A III. The infusion was turned off and a 64 to 84-minute waiting period ensued until the next pre-injection period. Here, the pre-injection period ACTH levels are the same as or lower than the respective first pre-period levels. The third histamine injection (equal to the respective previous two) caused a significant increase in ACTH in each dog, over the second histamine injection ACTH response (a 2 to 12 fold increase over the first pre-period levels, 14 to 106 mU/100 ml ACTH response).

In general, what is seen, is a significant ACTH response to the first histamine injection, in the absence of cortisol, a significantly reduced (about 1/3 of the first injection response) second histamine injection ACTH response, in the presence of physiological levels of cortisol and ACTH response to the third histamine injection significantly greater than the second injection response. This third injection period has cortisol levels significantly lower than those seen in the second injection period but more than the first period which has no cortisol present.

Dogs 3 and 7 do not show this type response. Although, there is significant response to the first histamine injection (no cortisol) and a reduced response to the second injection, which is in the presence of cortisol, the third injection period ACTH response is less than that seen in the respective second injection period of each experiment. However, if we

look at the absolute cortisol concentrations in the infusion periods of these two experiments, they are the highest of the five animals (12.3 µg/100 ml for dog # 3 and 10.9 for dog # 7). Also the respective cortisol concentrations of 5.5 and 5.3 µg/100 ml for dogs 3 and 7, seen during the third injection period, are the highest of the five dogs. This is born out by the half-times of cortisol in these animals (69.5 and 94.6 minutes for dogs 3 and 7, respectively), which are the highest seen in the series and which indicate slower clearances than seen in the other dogs. The half-time nearest to that of dog # 3 (69.5 minutes) are those for dogs 1 and 4 (65.1 and 65 respectively) which did show recovery to the histamine injection in the third period. So, there may be a critical half-time clearance between 65 to 70 minutes below which recovery is seen in the third period and above which it is not. It seems conceivable, then, that the exposure of an animal to higher (physiological) cortisol concentrations, especially in an animal which has a slower clearance of cortisol, could result in significant inhibition of ACTH in the 3rd histamine injection period, which would simply be a continuation of that seen in the second injection period (as in dogs 1 and 4).

Therefore, unlike Critchlow (1972) who felt physiological levels of glucocorticoid could not inhibit stress-induced ACTH release, we have shown exactly this in adrenalectomized dogs, in which physiological levels of cortisol did inhibit histamine-induced ACTH release and that there was a demonstratable recovery from this inhibition if the levels of cortisol were not excessive.

APPENDIX I (b)

These experiments are of the same design as the histamine-stress experiments (as discussed in the methods section) except the injection of histamine is replaced by a brief exposure of anoxia (95% N_2, 5% CO_2). This is a natural and a very potent stressor. The time of exposure varied from animal to animal (but was the same in any one animal) since we allowed each animal to begin its phase of deep and rapid breathing (in the face of anoxia - the CO_2 maintaining a respiratory drive) before removing the anoxia source; and this time naturally varied from animal to animal. These were simply, another series of stress experiments, in which the source of stress was varied.

The experimental design, method of ACTH and steroid analysis, as well as data analysis have already been discussed.

Results and Discussion:

Only three anoxia-stress experiments have been completed thus far, on two adult male adrenalectomized dogs (see Table A II). Table A II also shows, the infusion rates, amounts of injected cortisol and the matching of injection to infusion. The INJ/INF ratios are all near 25 minutes, which constitutes well-matched infusions and injections. The plasma cortisol concentrations (see Table A IV) resulting from these primed infusions were physiological (Peterson 1959, Richards and Praitt 1957) and well below the maximum cortisol levels seen in plasma (Yates and Urquhart 1962; Peterson 1959). More specifically, the cortisol infusion rates were reduced in this series of experiments as compared to the histamine-stress series, but the length of infusion was increased to 106 minutes as opposed to 56 minutes,

resulting in low physiological levels of cortisol (2.04 to 3.42 µg/100 ml in the infusion periods and 0.25 to 0.37 µg/100 ml in the third anoxia period, 84 minutes after cessation of cortisol infusion). (The infusions were at a low rate but sustained for a longer period of time resulting in low plasma cortisol concentrations in the second anoxia period and almost zero levels in the third period).

The half-lives for cortisol (see table AVI) were estimated for these three experiments and found to be consistently near 54 minutes. This compares favourably with the T 1/2 of 50 minutes obtained by McCormick et al. (1974) in non-stressed, acutely adrenalectomized dogs.

The effect of this cortisol on anoxia-induced ACTH release, is shown in table AVIII. Since only three experiments exist, at present, the results of each will be discussed separately. In dog # 8, the first anoxia pre-period ACTH levels are in a decreasing phase (2.9 to 0.6 mU/100 ml) over the 10-minute sampling period. The response to the first anoxia exposure is a rapid increase in ACTH to 23 mU/100 ml ACTH four minutes after the beginning of the anoxia exposure, and a rapid decline to about 5mU/100 ml by the end of the sampling period. This is the response in the face of zero cortisol plasma levels. Then, the primed infusion was begun. The second anoxia pre-period ACTH values were much lower than the first pre-period levels (0.1 to 0.5 mU/100 ml). The response to the second anoxia exposure (equal in duration, to the first) was roughly 1/3 that of the first anoxia period ACTH response, (7.8 mU/100 ml ACTH). This was in the face of a mean plasma cortisol concentration of 3.42 µg/100 ml. The third anoxia pre-period ACTH levels were between the first and second pre-period values (1 mU/

100 ml ACTH). The third anoxia exposure, in the face of 0.37 µg/100 ml cortisol, showed an increase in ACTH significantly above the second anoxia period ACTH level. Here, then, as seen in the histamine stress experiments there is evidence of the ability of cortisol to inhibit the stress response to anoxia (in the second anoxia exposure) and the significant recovery of the ACTH stress response as the cortisol levels decreased (in the third stress period).

The response of dog # 9 in experiment 9(b) is similar to the response of dog # 8, in that the second stress period does show inhibition, (but not as much as dog # 8). However, the cortisol concentration in this period is quite low (2.14 µg/100 ml). Perhaps, this is the reason that only marginal inhibition is seen to stress in this period. The third anoxia period stress response does not show recovery. Instead, it shows a continuation of the marginal inhibition seen in the second anoxia period response (the second anoxia period response is about 80% of the first stress period response and the third is about 64% of the first). Again, this response does not conform to a simple drift model. It is possible that the inhibition of stress caused by this range of cortisol concentration is near its lower sensitivity and depends heavily on the long-term feedback spoken of by Dallman and Yates (1969), which she finds to be of the order of two hours.

Experiment 9a, performed on dog # 9, does not follow the general scheme of inhibition seen previously. The response to the first anoxia exposure is lower than the second response, which, in turn, is lower than the third anoxia stress response. Certain of our dogs had very slow res-

piratory rates (5-6 breaths/minute) which was not consistent throughout a given experiment. This meant that an equivalent-timed anoxia exposure at a lower breathing rate may not give the same effective degree of stress as would result at a higher breathing rate (i.e. a quicker exchange of gas with some O_2 in it from the animal for anoxic gas would hasten the onset of a stress response). This animal was probably not as stressed in the first period as it was in the subsequent two. However, it is of interest that the third period response was so much greater than the second period response (second about 58% of the third) while the second response is only slightly greater than the first (first is 80% of the second). There may be some justification for believing that the second period response was inhibited (compared to the third response, assuming equivalent stress in the second and third periods).

This anoxia experiment series, although incomplete, together with the histamine-stress experiments, does show some inhibition of stress induced ACTH secretion by low physiological levels of glucocorticoid. The anoxia used in these experiments, although it presents a more natural stress than the histamine, is more difficult to quantify in terms of stress-dosage than histamine (as seen in dog 9a). In future it may be necessary to increase the length of time that the animal is exposed to anoxia to make certain that the exposure is stressful and not border-line as seen in dog # 9, experiment (a), (also it is seen in comparing tables AVII and AVIII that the stress response to the anoxia used thus far is, in absolute terms, less than obtained with the low doses of histamine used).

A further feature of these stress experiments, both histamine and anoxia, is the use of anesthesia. It has been pointed out in the discussion of the non-stress experiments that Nembutal has been shown to have a depressant effect on ACTH secretion by itself (Greer and Rockie 1968; Critchlow 1972) although more recently it has been shown to have no effect on plasma levels of ACTH in the rat (Rotsztejn et al 1975). This inhibition by Nembutal should it exist in these dogs, would be even more important for stress experiments, in which the response to the stress, in terms of ACTH secretion, is very rapid and typically of short duration. Any inhibition in such a system would have a marked effect, especially on the peak response.

TABLES - STRESS EXPERIMENTS

TABLE AI

Summary of Primed Infusions of Cortisol and
Histamine Injections in Histamine Stressed Adrenalectomized Adult Dogs

Dog number	Weight (kg)	Cortisol Infusion Period			Intravenous (1)Histamine Injection (µg/kg)
		Infusion Rate (µg/min)	Injection (µg)	INJ / INF (min)	
1	8.1	15.5	1350	87	61.7
2	9.4	15.5	380	24.5	63.8
3	11.55	21.7	542	25	60.9
4	10.3	21.7	542.5	25	59.9
7	12.1	15.5	389	25	66.3

Note (1) Histamine injection were the same for the 3 injections in each dog.

TABLE AII

Summary of Primed Infusions of Cortisol and Duration of
Anoxia, in Anoxia Stressed Adrenalectomized Adult Dogs

Dog number	Weight (kg)	Cortisol Infusion Period			Duration of Anoxia		
		Infusion Rate (µg/min)	Injection (µg)	INJ / INF (min)	First (min)	Second (min)	Third (min)
8	9.55	11.04	276	25	0.77	0.77	0.77
9a (1)	13.95	11.04	276	25	1.15	1.15	1.15
9b	13.5	11.04	276	25	0.75	0.75	0.75

Note (1) Two anoxia experiments were performed on dog number 9.

Note (2) Anoxia comprised the exposure of the animal to a gas mixture of 95% N_2 and 5% CO_2.

TABLE AIII

Mean Plasma Cortisol Concentrations(1) Before, During and After Cortisol Infusion Into Histamine-Stressed Adrenalectomized Adult Dogs

Dog #	Cortisol Before Infusion (2) (µg/100 ml)	Cortisol During Infusion (3) (µg/100 ml)	Cortisol After Infusion (µg/100 ml)	Time Elapsed Since (4) Cessation of Infusion (min)
1	zero	9.75 ± 0.86	2.49 ± 0.42	84
2	zero	9.07 ± 0.72	1.56 ± 0.52	64
3	zero	12.34 ± 0.38	5.5 ± 0.32	64
4	zero	8.86 ± 0.29	3.61 ± 0.29	64
7	zero	10.9 ± 0.619	5.31 ± 0.567	84

Note (1) Mean ± S.E.M. of 2 pre-injection values + 6 values over the 16 minutes after the injection.

Note (2) Adrenalectomized dogs 48 hours after last cortisol injection.

Note (3) Infusions lasted 56 minutes except dog # 1 (59 minutes).

Note (4) Represents time elapsed between cessation of cortisol infusion and beginning of sampling for third histamine injection pre-period.

TABLE AIV

(1)

Mean Plasma Cortisol Concentrations as Seen Before, During and After Cortisol Infusion Into Anoxia-Stressed Adrenalectomized Adult Dogs.

Dog #	Cortisol Before Infusion (2) (µg/100 ml)	Cortisol During Infusion (3) (µg/100 ml)	Cortisol After Infusion (µg/100 ml)	Time Elapsed Since Cessation of Infusion (min) (4)
8	zero	3.42 ± 0.28	0.37 ± 0.12	84
9a (5)	zero	3.42 ± 0.38	0.35 ± 0.23	84
9b	zero	2.14 ± 0.4	0.25 ± 0.17	84

Note (1) Mean ± S.E.M. of 2 pre-injection values + 6 values over the 16 minutes after exposure to anoxia.

Note (2) Adrenalectomized dogs 48 hours after last cortisol injection.

Note (3) Infusions lasted 106 minutes.

Note (4) Represents time elapsed between cessation of cortisol infusion and beginning of sampling for third anoxia pre-period.

Note (5) Two experiments on dog number 9.

TABLE AV

Half-lives of Cortisol in Histamine-stressed Adrenalectomized Adult Dogs.

Dog #	Cortisol Concentration Change (µg/100 ml)	Period of Time Over which Cortisol Decreasing (min)	Half-life (T 1/2) (min) (1)
1	(9.75 - 2.49)=7.26 ± 1.28	97	65.1
2	(9.07 - 1.56)=7.51 ± 1.24	77	46.5
3	(12.34 - 5.5)=6.84 ± 0.7	77	69.5
4	(8.86 - 3.61)=5.25 ± 0.58	77	65
7	(10.9 - 5.31)=5.59 ± 1.19	97	94.6

Note (1) Half-lives calculated from end of cortisol plateau to middle of third histamine injection sampling period (mean of 8 samples)

TABLE AVI

Half-lives of Cortisol in Anoxia-stressed Adrenalectomized Adult Dogs

Dog #	Cortisol Concentration Change (µg/100 ml)	Period of Time Over Which Cortisol Decreasing (min)	Half-life (T 1/2) (min) (1)
8	(3.42 - 0.37)=3.05 ± 0.4	97	54
9a	(3.42 - 0.35)=3.07 ± 0.61	97	54
9b	(2.14 - 0.25)=1.89 ± 0.57	97	54.9

Note (1) Half-life values calculated from end of cortisol plateau to middle of third anoxia period (mean of 8 samples)

TABLE AVII

Histamine-stress (1) ACTH concentration vs time data for dogs 1, 2, 3, 4 and 7

(a) Dog # 1

Sample #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Experimental time (min)	0	9.5	12	14	16	18	22	26	60	69.5	72	74	76	78	82	86	170	179.5	182	184	186	188	192	196
ACTH (mU/100 ml)		7.8	19.4	78.9	55.1	56.4	37.6	45.1	9.1	5.6	12.9	30.6	33.7	35.2	17.5	15.4	9.1	8.5	41.5	106.4	84	92	38	28.1

(b) Dogs 2, 3 and 4

Experimental time (min)	0	9.5	12	14	16	18	22	26	90	99.5	102	104	106	108	112	116	180	189.5	192	194	196	198	202	206
Dog # ACTH (mU/100ml)																								
2	5	5	273	623	36.6	28.2	26	17.2	4.7	2.4	6.7	22.9	15.1	13.1	7.4	5.7	3.7	4.1	9	28.5	224	21.7	11	7.2
3	9.3	8.9	14.2	115.6	102.4	102.4	79.9	68.4	45.1	13.1	17.9	53.5	42	28	36.2	19.8	1.7	1.9	2.9	15	14.9	11.4	8.4	6
4	8	6.3	7	36.3	40.1	40.8	39.3	23.7	6.6	4.4	4.9	12.1	11.7	7.5	7	6.1	0.9	4	3.7	14.3	12.7	12.1	7.5	6.2

(c) Dog # 7

Experimental time (min)	0	9.5	12	14	16	18	22	26	110	119.5	122	124	126	128	132	136	220	229.5	232	234	236	238	242	246
ACTH (mU/100 ml)	7.3	6.9	8.5	24	40	42	45.3	39.6	7	5.9	5.1	11.7	16.3	16	10.4	6.4	0.5	0	0.7	5.5	6.6	4.7	3.2	2.

Note (1) Histamine injections at 10, 70 and 180 minutes in dog # 1; 10, 100 and 190 minutes in dogs 2, 3 and 4; and 10, 120 and 230 minutes in dog # 7.

TABLE AVIII

Anoxia-stress (1) ACTH Concentration vs time data for dogs 8 and 9 (2)

Sample #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Experimental time (min)	0	9.5	12	14	16	18	22	26	110	119.5	122	124	126	128	132	136	220	229.5	232	234	236	238	242	246
Dog # 8 ACTH (mU/100 ml)	2.9	0.6	7.8	2.3	14.8	13.1	7.1	4.9	0.1	0.5	0.4	4.5	3.9	7.8	0.7	4.7	0.9	1.1	2	10	3.8	6.6	3	3.6
9 (a)	1.3	1.5	1.7	4.3	3.5	2.2	1.9	1.9	0.6	0.9	1.2	5.7	4.2	4.5	1.8	0.7	0.8	1.8	2.4	9.7	6.1	4.7	3	2.8
9 (b)	2.1	2.6	2.8	4	6.4	5.6	3.6	4.2	1.7	2.5	2.4	5.2	5.2	4.4	0.8	1.4	1.6	1.9	1.7	4	2	0.4	1.7	1.6

Note (1) Anoxia exposures at 10, 120 and 230 minutes
Note (2) Two experiments done on dog # 9

APPENDIX II

METHODS OF ACTH DETERMINATION

Since the ACTH values obtained in this series of experiments were obtained through a bioassay technique, something of the accuracy of this technique as well as some of its developmental history will be sited. Also, since other methods of ACTH determination are available, some of the more notable methods will be mentioned, again, with some reference to their beginnings.

The first method with any real ability to quantify ACTH was by Simpson et al (1943). Its basis was the beginning of repair of the adrenals of hypophysectomized rats. The adrenals of 20-day old female rats were allowed to regress for 14 days after hypophysectomy, then i.p. injections of ACTH were given and an increase in the number and distribution of lipid droplets as compared to the droplets in the adrenal cortex of a rat hypophysectomized for 18 days without injected ACTH, was taken as an index of whether a trophic effect was present or not. The lowest significant response was obtained for 0.01 to 0.025 mg ACTH administered over 4 days.

Since this technique, assay methodology has evolved in three different directions (Girard et al 1965):

(1) ascorbic acid assay in rat adrenal
(2) Immunological assays
(3) the measured action of ACTH on steroidogenesis, (cell suspension, whole adrenals, in vito, in vivo).

(a) Ascorbic Acid Assays:

The ascorbic acid assay of Sayers (Sayers et al 1948) is based on the depletion seen in adrenal ascorbic acid 30-120 minutes after ACTH administration in hypophysectomized rats. Since both adrenals respond equally to ACTH, one is removed to serve as control, then some operation is performed (i.e. ACTH administration) and the other adrenal is removed and assayed for ascorbic acid by the colorimetric technique of Roe and Kuether (1943). The difference in the ascorbic content is the depletion due to that operation performed. The lower end of sensitivity of this assay is approximately 0.15 mU ACTH and the depletion is logarithmically linear to ACTH between 0.15 and 2.5 mU/100 gm body weight. This assay was later improved technically by Syndor and Sayers (1954) such that the ACTH sensitivity was to 0.5 mU/100 ml plasma and used by Brodish and Long (1956) to study the effect on adrenal ascorbic acid depletion in hypophysectomized recipient rats infused with blood from stressed donor rats.

Munson et al (1948) seeing only slight variability in adrenal ascorbic acid concentration in rats, altered the Sayer technique by not removing an adrenal before any experimental operation and used both adrenals as experimental indicators of depletion to be compared to control adrenals from other rats. This was further modified by removing only one of the two test adrenals (Munson 1958a; Rerup 1958) since again there was no significant variation between the two adrenals subjected to the same procedure.

Later, the ascorbic acid assay was further simplified by replacing hypophysectomy by corticosterone blockade of ACTH secretion in the test animals (Hodges and Vernikos 1959). Munson and Toepel (1958b) further

improved the sensitivity of the assay to 0.01 to 0.02 mU ACTH/100 gm body weight by injection of ACTH directly into the adrenal vein.

The significance of this release of ascorbic acid following stimulation by ACTH is still unknown. In general, these ascorbic acid assays required large volumes of blood for analysis, involved fairly complicated surgical technique and were imprecise at low ACTH doses (5-50 μU ACTH).

(b) Immunological Techniques:

Since immuno-sensitive techniques were proving valuable in determination of many hormones (i.e. insulin), attempts were made to quantify ACTH using these methods. One of the earlier studies (McCarry et al 1962) involving hemagglutination of ACTH with ACTH anti-serum demonstrated normal human resting ACTH levels of 3-4 mU/100 ml which were higher than bioassay results. Next, a radio-immunoassay was used by Yallow et al (1964) in which the binding of I-labelled ACTH and unlabelled ACTH to a specific anti-body indicated the concentration of ACTH present. This technique had a sensitivity of 0.06 to 0.125 mU/100 ml required only 100-200 μl of sample for analysis (and could detect 0.1 μU ACTH) and gave normal human plasma ACTH levels of 0.6 mU/100 ml which agreed with readings obtained with bioassay techniques. The sensitivity of this technique was later improved to 1 pg/ml or 0.014 mU ACTH/100 ml by Berson (1968). (500 μl of plasma was typically used for the assay but 100 μl of plasma represented the lower sensitive volumes of this method).

Although, this system of ACTH concentration analysis seemed fairly precise and required much less sample than the bioassay techniques the consistently higher immunological-determined ACTH levels over bioassay deter-

mination led researchers like Matsuyama et al (1971a) to believe in the existence of immunologically active ACTH fragments which were not biologically active. These findings were confirmed later by Matsuyama et al (1972). Also, he determined(Matsuyama et al 1971b) consistently that immunoassay gave levels 2.2 times as great as bioassay results in non-stressed rats and 1.2 times in stressed rats.

One feature of this technique as pointed out by Matsukura et al (1969), was the fact that the antisera to that time were not as sensitive for ACTH as those developed for other hormones. This was of importance due to the low ACTH levels found in normal subjects. This, however, has improved with more sensitive antisera and improved ACTH concentrating methods.

(c) ACTH Bioassays:

Although the ascorbic acid assays are no longer in use, the immunological techniques are. A third method of ACTH analysis, still in use, involves the direct action of ACTH on adrenal steroidogenesis. This last type of assay places ACTH in direct contact with the adrenal gland (or cells) as opposed to injection of ACTH or ACTH-containing blood into the general circulation. One of these assays, the in vitro assay of Saffran and Schally (1955), involves the corticosterone produced (and measured spectrophotometrically) by incubated rat adrenal quarters in the presence of ACTH. The sensitivity of this technique is between 2 and 4 mU/100 mg adrenal weight. (Approximately 0.1 ml of sample or standard used for analysis). About this same time an in vivo bioassay was developed by Nelson and Hume (1955) in which adrenal venous samples from an hypophy-

sectomized dog, were analysed for cortisol by the photometric method of Nelson and Samuels (1952). ACTH standards or blood samples could be injected (femoral vein) into such a preparation and the adrenal response (production of 17-OH, corticosteroids) was sensitive to 1-2 mU ACTH (required 5 ml adrenal blood diluted v/v with distilled water for the Nelson and Samuels assay). Silber et al(1958) then developed a fluorometric technique which allowed as little as 0.2 ml of plasma to be assayed for glucocorticoids and which permitted measuring as low as 0.1 mU ACTH in the rat using the above techniques. Later, Lipscomb and Nelson (1962) using the Silber fluorometric technique and hypophysectomized rats, developed a "jugular technique" in which adrenal responses were measured after intra-jugular injection of ACTH and a "retrograde technique", in which ACTH was injected directly into the adrenal vein. The blood samples required for analysis were 0.6 ml and the sensitivities respectively, for the jugular and retrograde techniques were 0.05 mU ACTH and 0.001 mU ACTH. These techniques though sensitive were rather imprecise.

The next improvement in bioassays came with the rat adrenal cell suspension of Swallow and Sayers (1969) in which the corticosterone produced due to ACTH stimulation of equal volumes of adrenal cells was measured spectrofluorometrically (Silber et al 1958). With improvements to the technique by Sayers (1971), Giordano and Sayers (1971), Cowan et al (1971a), and Cowan et al (1974) the assay uses only 50-100 µl of plasma and is sensitive to less than 1µU ACTH (0.5 µU in this laboratory).

TABLES - NON-STRESS EXPERIMENTS

TABLE I

Summary of Primed[1] Infusions of Cortisol in Resting Adrenalectomized Adult Male Mongrel Dogs.

Dog #	Weight (kg)	First Cortisol Infusion			Second Cortisol Infusion			Third Cortisol Infusion		
		Infusion Rate (μg/min)	Injection (μg)	INJ/INF (min) (2)	Infusion Rate (μg/min)	Injection (μg)	INJ/INF(2-1) (min) (3) (2)	Infusion Rate (μg/min)	Injection (μg)	INJ/INF(3-2) (min) (3)
1	8.25	5.63	490	87.0	11.04	470	86.9	15.5	389	87.0
2	9.25	5.63	142.6	25.0	11.04	133	24.6			
3	11.6	7.89	197.3	25.0	15.5	190.2	25.0			
4	10.4	7.89	196	25.0	15.5	191	25.1			
5	12.5	4.01	100	25.0	7.89	97	25.0			
6	11.3	4.01	98	24.4	7.89	109.5	28.2			
7	13.1	4.01	100.6	25.0	7.89	95	24.5			

Note (1) each cortisol infusion was primed with an injected amount of cortisol.

Note (2) the injection to infusion ratio (time (min) that a given infusion rate or infusion rate increase would require to deliver an amount of cortisol equal to the injection) is selected to produce as rapid an equilibrium as possible and corresponds to $\frac{V \text{ (distribution volume)}}{MCR \text{ (metabolic clearance rate)}}$ in the pilot experiment (no. 1) this was over estimated.

Note (3) Inf (2-1) = Inf 2-Inf 1 and Inf (3-2) = Inf 3 - Inf 2.

TABLE II

Plasma Cortisol Concentrations During Plateaux Resulting From Cortisol Infusion In Resting Adrenalectomized Adult Dogs.

Experiment Number	Pre-Infusion (µg/100 ml)(1) Mean ± S.E.M.	First Infusion (µg/100 ml) Mean ± S.E.M.	Second Infusion (µg/100 ml) Mean ± S.E.M.	Third Infusion (µg/100 ml) Mean ± S.E.M.	Post-Infusion (µg/100 ml) Mean ± S.E.M.
1	zero	4.83 ± 0.976	7.26 ± 0.9	8.02 ± 0.313	1.33 ± 0.222
2	zero	6.28 ± 0.478	7.82 ± 0.469		2.45 ± 0.58
3	zero	3.46 ± 0.464	4.36 ± 0.317		2.39 ± 0.431
4	zero	5.98 ± 0.452	9.73 ± 0.572		3.48 ± 0.995
5	zero	4.24 ± 0.872	6.05 ± 0.981		1.6 ± 0.0358
6	zero	2.74 ± 0.293	3.7 ± 0.423		0.45 ± 0.068
7	zero	2.38 ± 0.393	4.67 ± 0.356		4.18 ± 0.935

Note (1) adrenalectomized dogs 48 hours after last cortisol injection.

Note (2) the post-infusion period consists of those cortisol concentrations seen 90-110 minutes after the cessation of the last infusion in dog number 1 and 60-80 minutes in the other 6 experiments.

TABLE III

Average Metabolic Clearance Rates[1] (ml/kg.min) of Plasma Cortisol in Resting Adrenalectomized Adult Dogs

Experiment #	Dog weight (kg)	First Infusion		Second Infusion		Third Infusion	
		Mean Cortisol concentration (µg/100 ml)	Clearance (ml/kg.min)	Mean Cortisol concentration (µg/100 ml)	Clearance (ml/kg.min)	Mean Cortisol concentration (µg/100 ml)	Clearance (ml/kg.min)
1	8.25	4.83	14.13	1.26	18.43	8.02	23.43
2	9.25	6.28	9.69	7.82	15.26		
3	11.6	3.46	19.66	4.36	30.65		
4	10.4	5.98	12.69	9.73	15.32		
5	12.5	4.24	7.56	6.05	10.43		
6	11.3	2.74	12.95	3.7	18.87		
7	13.1	2.38	12.86	4.67	12.9		
Mean ± S.E.M.		4.27 0.574	12.79 1.43	6.23 0.82	17.41 2.47		

Note (1) Clearance rates were obtained by dividing the cortisol infusion rate by the resulting mean plateau concentration of cortisol in plasma, and normalized for body weight.

TABLE IV

Apparent Cortisol Distribution Volumes(1) for Cortisol Infusion in Resting Adrenalectomized Adult Dogs.

Experiment Number	First Infusion		Second Infusion		Third Infusion	
	Mean Cortisol concentration (µg/100 ml)	Distribution volume (ml/kg)	Mean Cortisol concentration (µg/100 ml)	Distribution volume (ml/kg)	Mean cortisol concentration (µg/100 ml)	Distribution volume (ml/kg)
1	4.83	750.87	7.26	660.9	8.02	550.96
2	6.28	225.69	(7.82)	(2)		
3	3.46	348.04	(4.36)	(2)		
4	5.98	392.04	9.73	410.55		
5	4.24	188.68	6.05	151.86		
6	2.74	188.53	3.7	556.26		
7	2.38	484.9	4.67	392.		
Mean ±S.E.M.	4.27 0.574	368.39 76.51	6.28 1.053	434.31 86.124		

Note (1) calculated by dividing the mass of cortisol injected by the cortisol concentration at the time of injection (extrapolated from the cortisol concentration curve) and normalizing for the animal weight.

Note (2) no second infusion distribution volume data in experiments 2 and 3 are available due to poor statistical resolution between these consecutive cortisol infusion periods.

TABLE V

ACTH CONCENTRATIONS IN SAMPLES OF VENOUS BLOOD IN RESTING ADULT MALE DOGS SUBJECTED TO CORTISOL FEEDBACK

(a) Dog number 1[1]

Sample Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Time (min)	0	10	20	25	35	50	65	80	85	95	110	125	140	145	155	170	185	200	290	300	310
ACTH (mU/100 ml)	3.44	5.46	9.78	11.73	13.17	9.78	7.42	4.61	3.31	1.98	1.62	0.55	0	0	0.23	0	0.30	0.94	2.4	2.23	2.67

(b) Dogs 2 to 7[2]

Sample Number	Dog number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Time (min)		0	10	20	30	35	45	60	75	90	100	110	120	125	135	150	165	180	190	200	210	220	280	290
ACTH (mU/100 ml)	2	3.74	6.51	9.75	7.19	7.13	9.46	7.71	5.26	4.03	4.0	2.82	3.05	1.09	2.99	0	3.34	2.65	3.51	3.60	3.51	0.16	0.2	2.01
	3	5.98	6.07	5.2	5.32	6.61	6.15	5.69	3.8	1.1	0.52	0.43	0.43	0.31	0.38	0.01	0.38	0	0	0	0	0	0	0.27
	4	11.39	12.71	10.35	10.24	10.24	10.35	8.4	5.95	3.34	2	1.62	2.34	2.15	4.05	3.19	2.7	1.48	0	2.93	0.51	2.93	3.42	3.74
	5	3.28	4.0	5.29	6.67	6.93	8.22	8.45	5.41	4.74	3.57	3.51	2.26	2.89	4.23	1.45	1.63	1.09	0.59	0.38	0.43	0.32	0.72	1.55
	6	28.35	25.53	25.01	26.68	25.01	29.56	31.34	33.29	22.43	18.23	17.19	13.97	12.02	12.36	10.12	8.91	6.73	5.89	4.6	6.21	12.71	16.39	26.45
	7	2.0	7.71	7.73	8.24	8.88	9.55	7.62	8.37	10.47	10.52	10.18	10.06	9.75	9.72	8.88	7.71	6.76	7.16	5.98	6.24	5.84	4.95	3.77

Note (1) three 60-minute cortisol infusions: 20 to 80 min, 80 to 140 min, 140 to 200 min.

Note (2) two 90-minute cortisol infusions: 30 to 120 min, 120 to 210 min.

TABLE VI

Feedback Suppression[1] of ACTH Secretion by Cortisol during and after Cortisol Infusion in Resting Adrenalectomized Adult Dogs.

pe-ment #	First Infusion				Second Infusion				Third Infusion				Post-Infusion	
	Lag time[2] (min)	Period of[3] changing sec. rate (min)	Mean Cortisol µg/100ml	% Supp.	Lag time (min)	Period of changing sec. rate (min)	Mean Cortisol (µg/100ml)	% Supp.	Lag time (min)	Period of changing sec.rate (min)	Mean Cortisol (µg/100ml)	% Supp.	Mean Cortisol (µg/100ml)	% Supp.[4]
1	21	49	4.83	43.47	25	20	7.26	100	0	0	8.02	100	1.33	69.47
2	20	45	6.28	70.15	0	7	7.82	100					2.45	91.18
3	25	30	3.46	86.64	20	37	4.36	100					2.39	96.76
4	15	40	5.98	71.46	25	40	9.73	89.81					3.48	64.53
5	23	47	4.24	62.2	13	72	6.05	100					1.6	83.6
6	30	55	2.74	56.38	7	73	3.7	81.99					0.45	11.73
AN	22.33	44.33	4.59	65.05	15	41.5	6.487	95.3					1.95	69.55
.E.M.	2.06	3.5	0.57	6.01	4.16	10.95	0.92	3.14					0.43	12.62
(5) 7	0	0	2.38	0	0	90	4.67	28.94					4.18	48.19

Note (1) Suppression was calculated as a percent compared to the pre-infusion ACTH secretion rate.

Note (2) Lag time refers to the time elapsed between the beginning of a cortisol infusion and the onset of significant suppression of ACTH secretion.

Note (3) Period of changing secretion rate is that time period between the end of the lag time and the onset of a new steady ACTH see rate.

Note (4) Since there was no steady state secretion rate achieved in the post-infusion period, the mean of the continuous secretion rate function was compared to the pre-period ACTH secretion rate to get a suppression.

Note (5) Dog # 7 excluded from mean calculation since it showed no feedback suppression to first cortisol infusion (which had the lowest cortisol plateau concentration) which made the response to the second infusion difficult to compare to the other experiments.

Apparent Distribution Volumes Of Cortisol vs Mean Plasma Cortisol Concentration

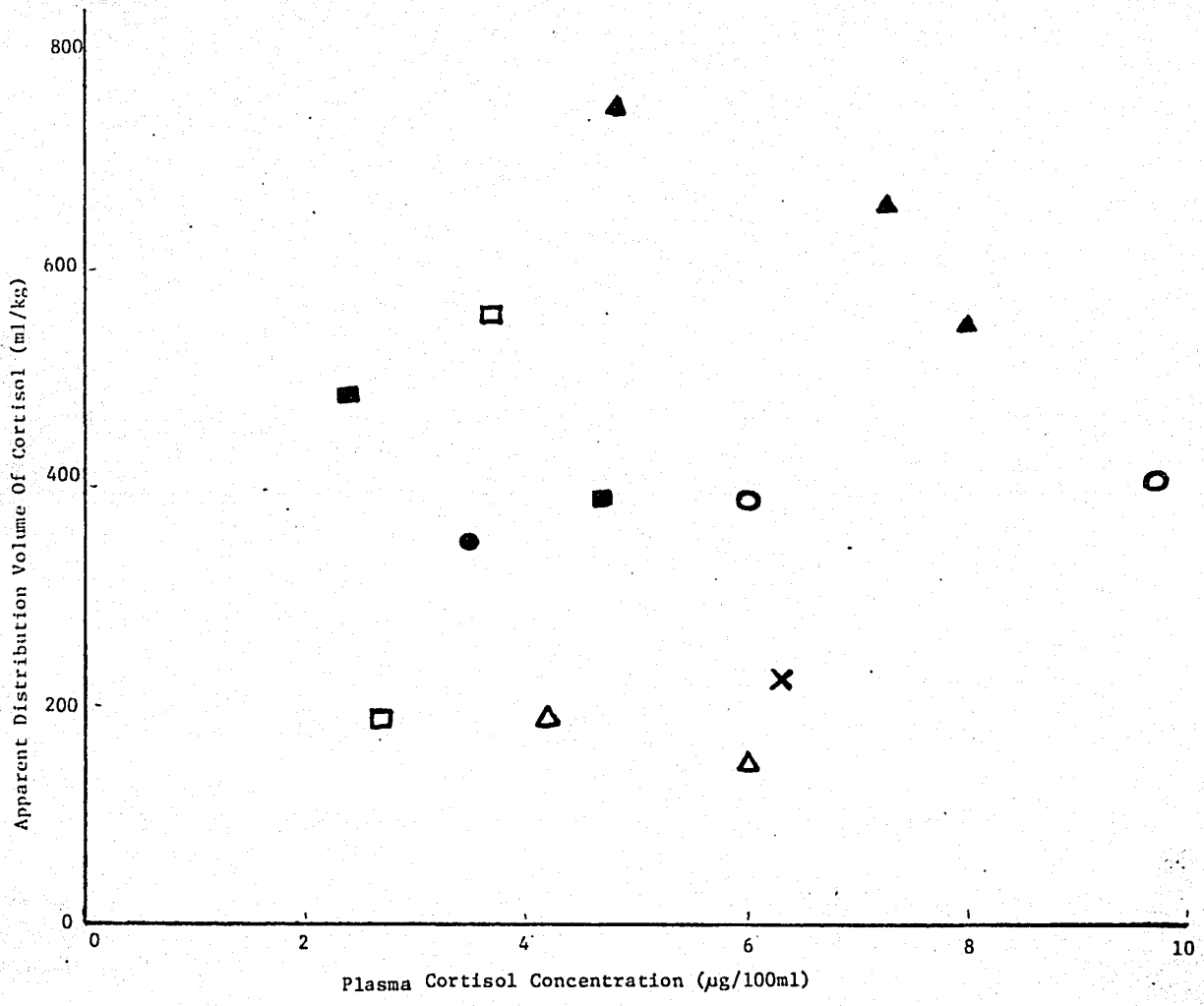

Figure 1

This shows the lack of correlation between mean plasma cortisol concentration (µg/100ml) and apparent distribution volumes (ml/kg) for dog no. 1 (▲), dog no. 2 (×), dog no. 3 (●), dog no. 4 (○), dog no. 5 (△), dog no. 6 (□), and dog no. 7 (■).

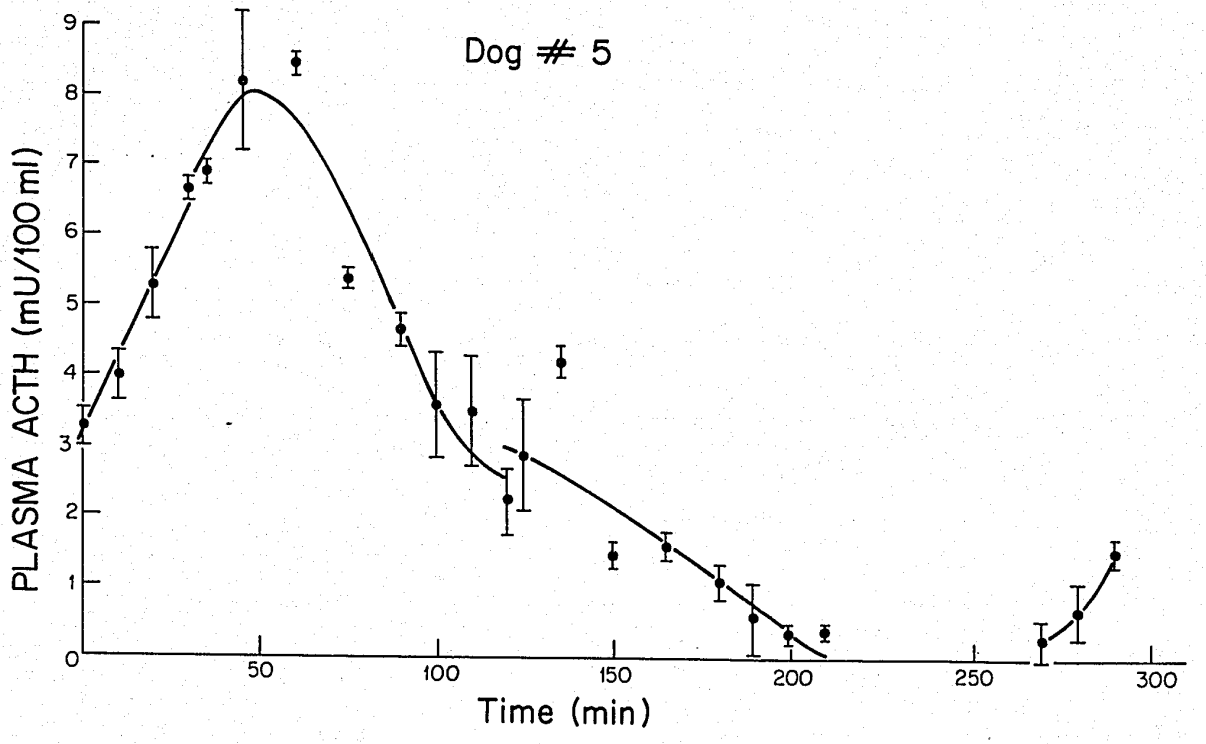

Figure 2.

A typical ACTH concentration (I) (mean of duplicate samples ± SEM) with polynomial functions fitted to individual experimental periods (solid line) as seen in dog no. 5.

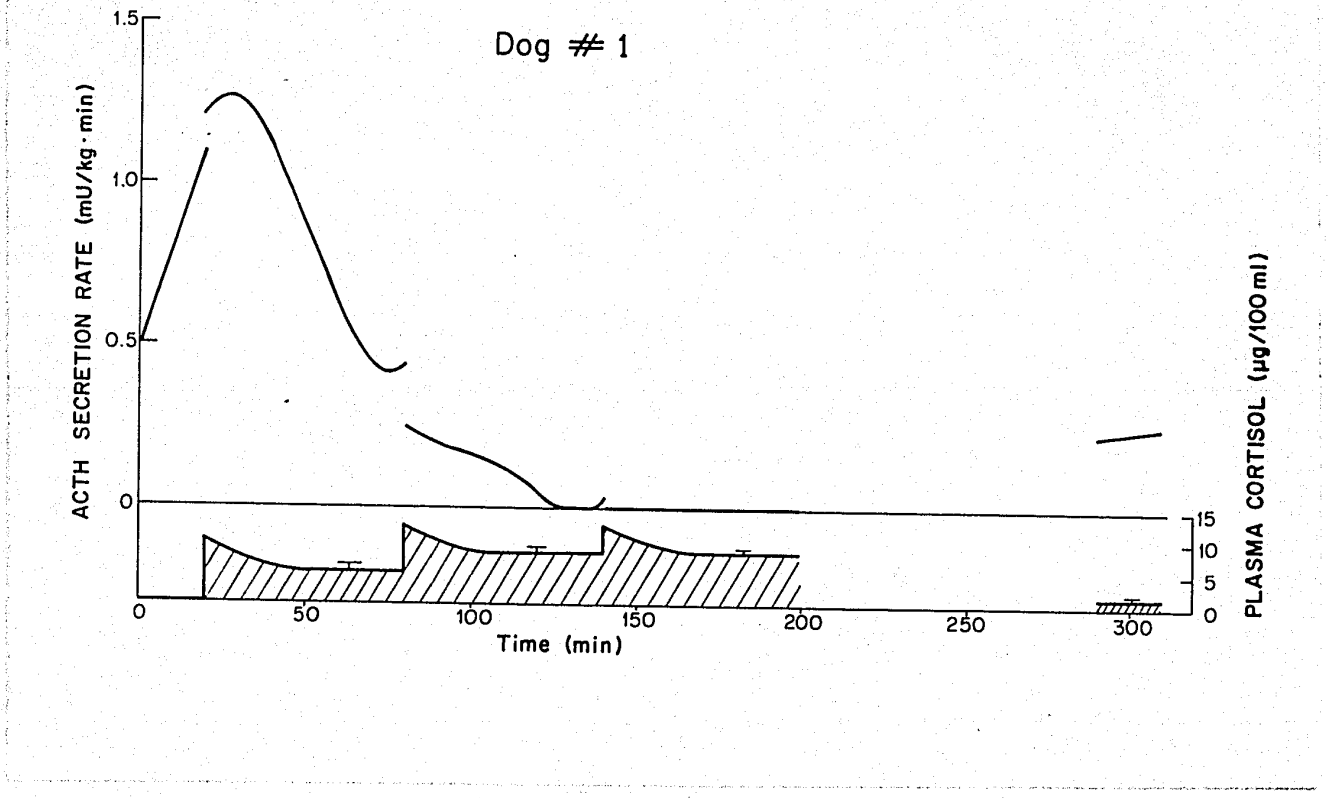

Figure 3

ACTH secretion rates (solid line) and associated plasma cortisol concentration (shaded area) as seen in dog no. 1. Three cortisol infusions, which were over-primed, were administered in this pilot experiment.

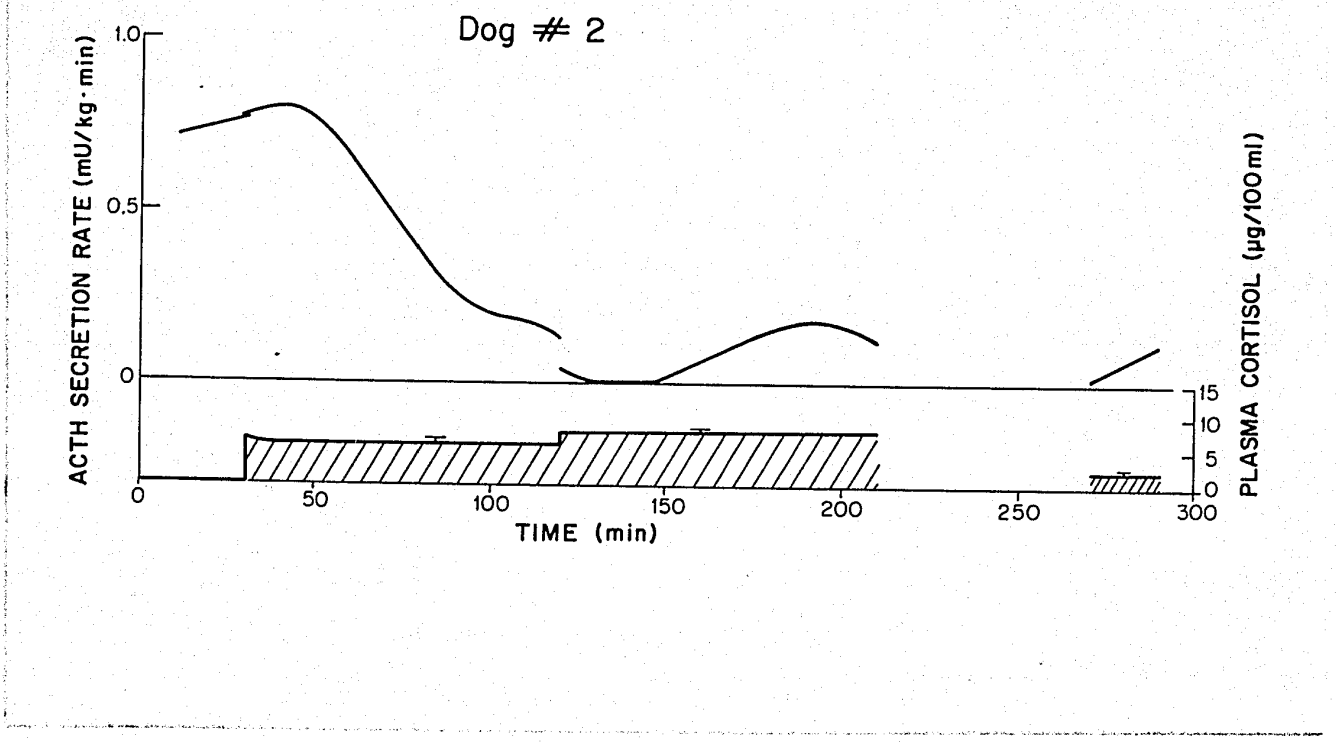

Figure 4

ACTH secretion rates (solid line) and associated plasma cortisol concentrations (shaded area) resulting from the two cortisol infusion periods in dog no. 2.

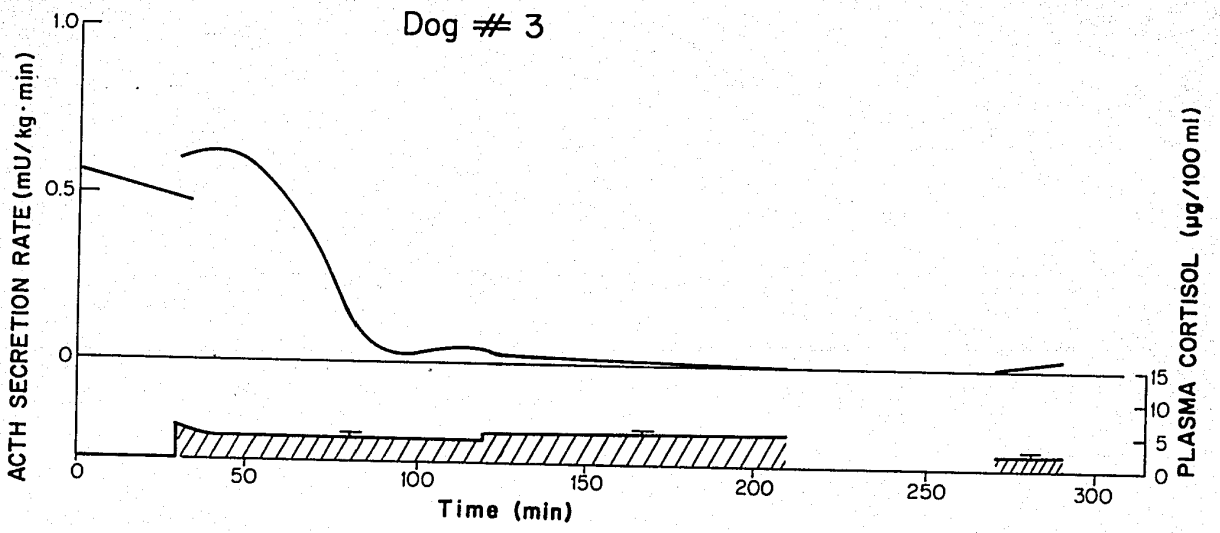

Figure 5

ACTH secretion rates (solid line) and associated plasma cortisol concentrations (shaded area) resulting from the two infusion periods in dog no. 3.

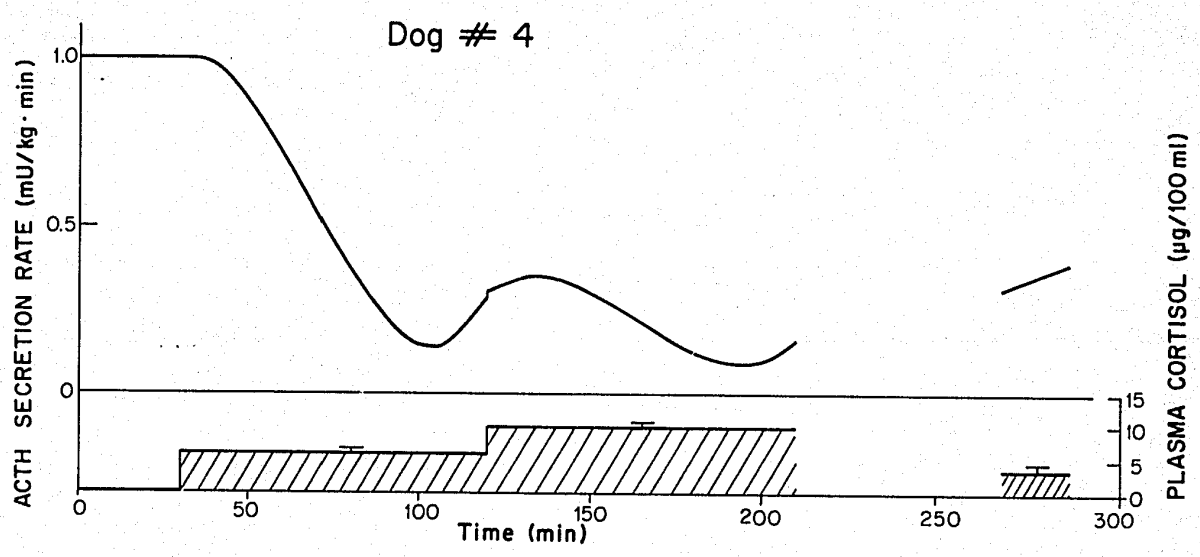

Figure 6

ACTH secretion rates (solid line) and associated plasma cortisol concentrations (shaded area) resulting from the two infusion periods in dog no. 4.

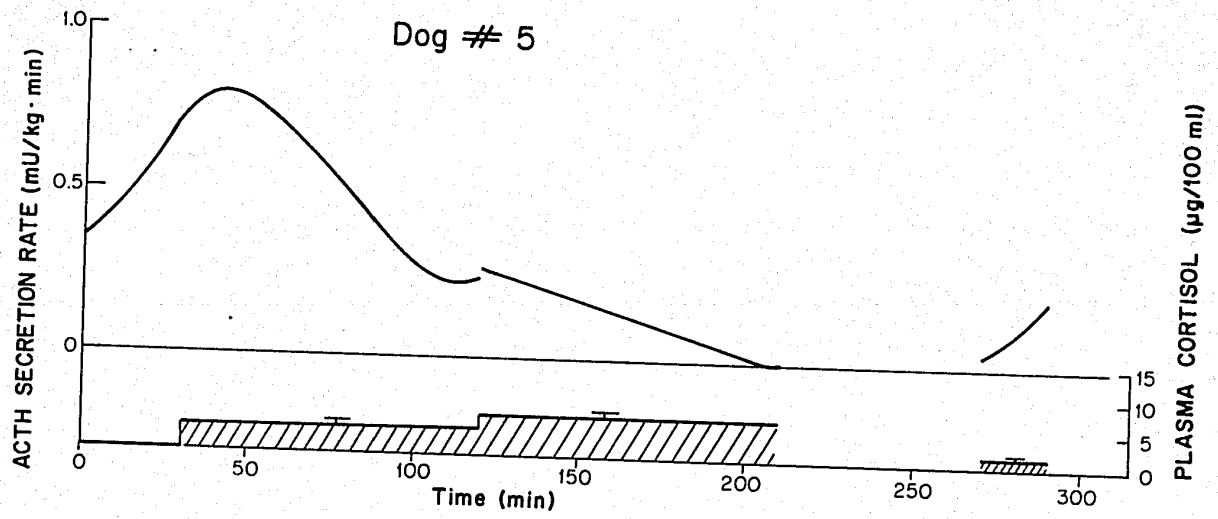

Figure 7

ACTH secretion rates (solid line) and associated plasma cortisol concentrations (shaded area) resulting from the two infusion periods in dog no. 5.

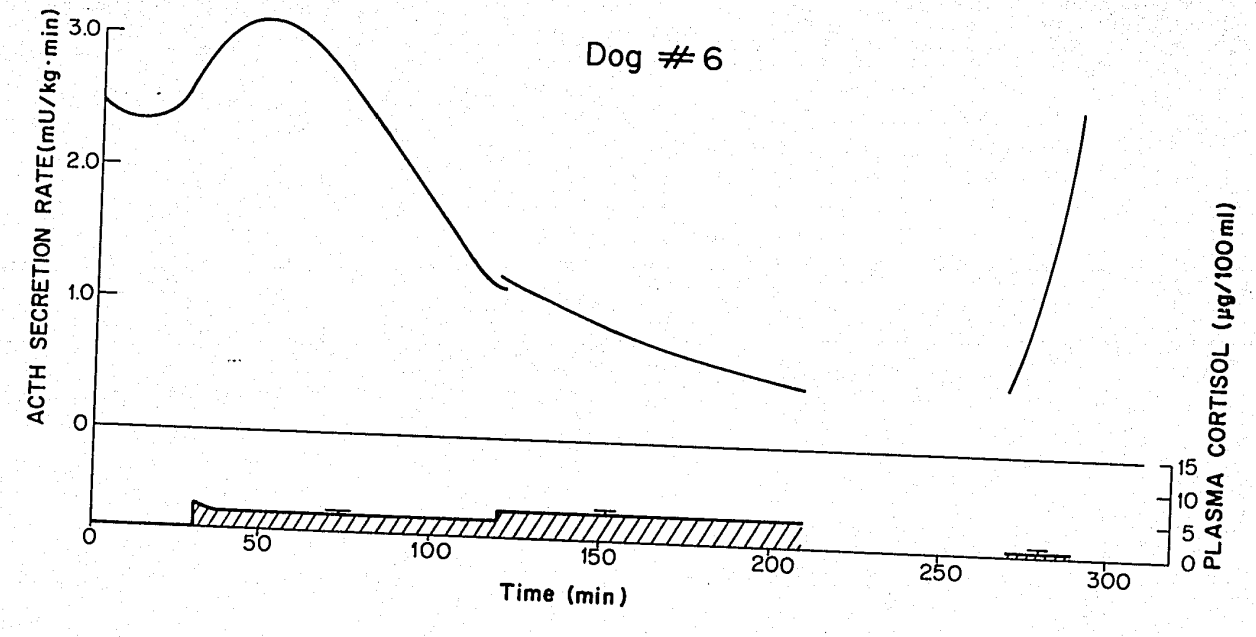

Figure 8

ACTH secretion rates (solid line) and associated plasma cortisol concentrations (shaded area) resulting from the two infusion periods in dog no. 6.

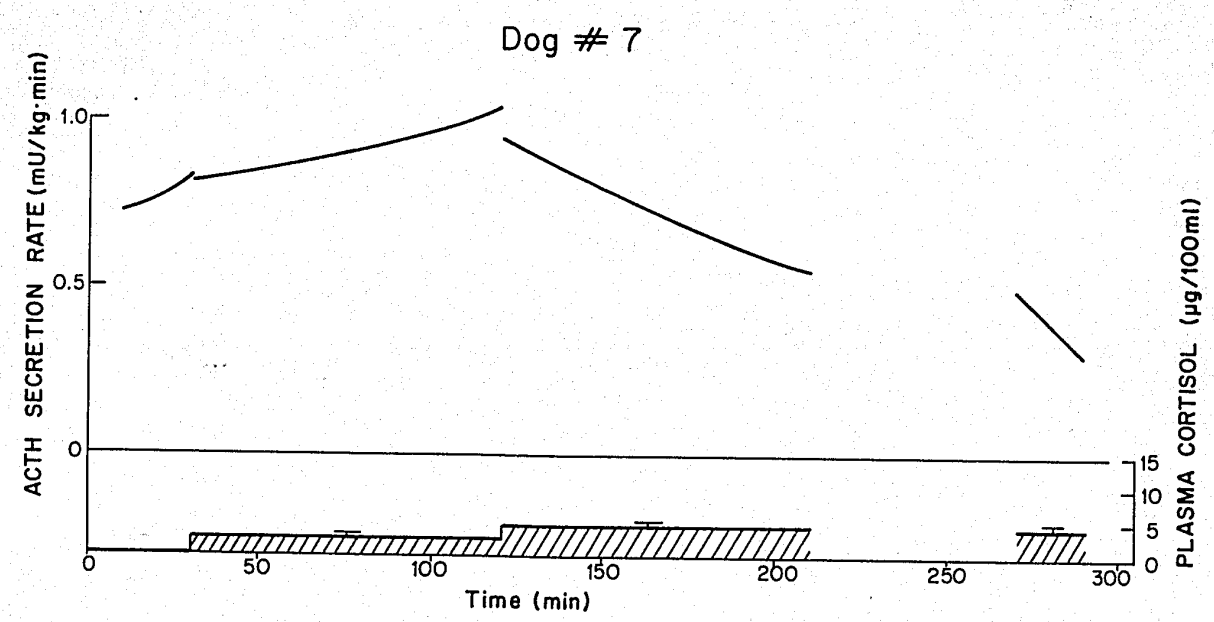

Figure 9

ACTH secretion rates (solid line) and associated plasma cortisol concentrations (shaded area) resulting from the two infusion periods in dog no. 7.

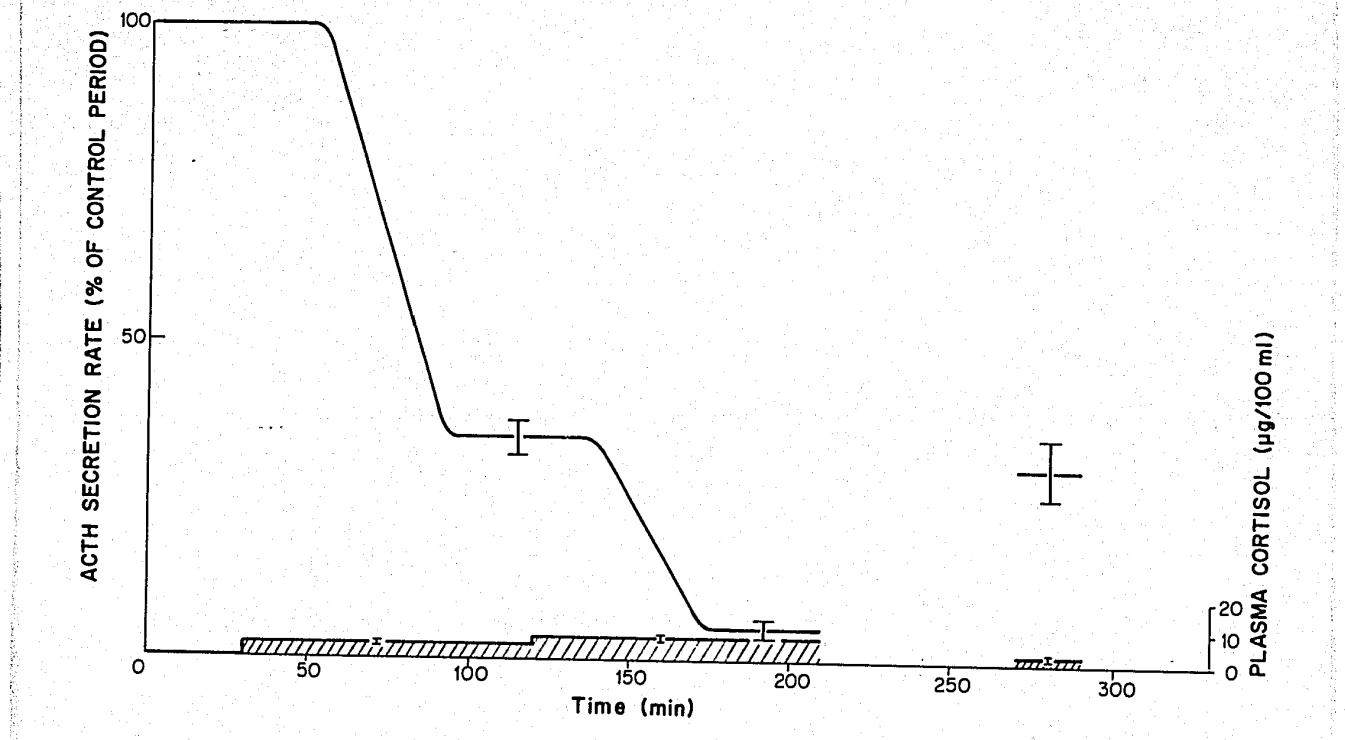

Figure 10

A mean composite picture of the suppression of ACTH secretion rates (solid line) (expressed as a percentage of control period rates) in the face of the mean plasma cortisol concentrations (shaded area) for dogs 1 through 6. Dog no. 7, the largest of the seven dogs, was not included in this figure because it showed no suppression of ACTH secretion rates during the first infusion period, during which it had the lowest mean plasma cortisol concentration of the seven dogs.