DEPLETION OF PITUITARY CORTICOTROPIN BY VARIOUS STRESS STIMULI

by

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THESIS

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INTRODUCTION

Rapid progress has been made in the understanding of the pathways of homeostatic responses. It is now known that any disturbance in the steady state of the body's internal environment, be it abrupt or tedious, exogenous or endogenous to the organism, will evoke in it rapid and complex responses, mediated through its endocrine and nervous systems and tending to counteract the effects of the disturbing stimulus (1,2).

I. THE STRESS STIMULI

Selye has defined stress as being "a condition in which - due to function or damage - extensive regions of the body deviate from their normal resting state" (3). Correlative to the theory of the General Adaptative Syndrome (3), he showed that a great variety of stressors will cause the alarm reaction (3), and he described the gross and histological changes which follow the application of these stimuli: involution of the thymo-lymphatic apparatus, ulceration, enlargement of the adrenal cortex, increased secretion of adrenal cortex hormones, and depletion of the adrenal cortical content of cholesterol and ascorbic acid (3). Selye (3) lists numerous stressors, i.e., stimuli which cause systemic stress: to name a few, trauma, hemorrhage, burns, temperature, radiation, fatigue, nervous stimuli (including emotional stress produced by immobilization, rage, fear, audiogenic stimulation), infection and pharmacologic intoxication.

With the elucidation of the primary role of the adrenal cortex-pituitary axis in homeostatic mechanisms, stress could be defined as any condition that increased the rate of release of adrenocorticotropic hormone from the adenohypophysis (4,5,6). On the basis of this definition and subsequent to experiments designed to elucidate the pathways of anterior pituitary activation in stress, Fortier (6) divided the stress stimuli into two groups: (a) neurotropic stressors, such as strong audiogenic stimulation, intense light, immobilization, "those that elicit ACTH discharge by an action solely through the central nervous system" and (b) systemic stressors, such as trauma, adrenaline and histamine injections, "which act by producing chemical or metabolic changes (with or without) an action through the central nervous system" (7).

For the purpose of the considerations in this thesis, this definition and division of stress stimuli have been accepted.

II. THE ENDOCRINE RESPONSE TO STRESS

The endocrine system, unlike the nervous system, does not possess anatomical continuity, nor does it have much histological similarity. However, its constituent parts are identical in one respect: each endocrine gland exerts important controlling effects on tissues far removed from itself, through potent chemical substances that travel via the circulation.

Of the endocrine glands, the adrenal cortex and the pituitary play a predominant role in the maintenance of homeostasis (4).

1. The adrenal cortex and stress.

That the adrenal cortex was fundamentally involved in the organism's response to stress stimuli has been demonstrated often and by numerous indicators.

a) Stress induces gross anatomical and morphological changes in the adrenal cortex.

The following changes occur in the adrenocortical gland of the normal animal subjected to numerous and various stressors: the gland becomes very hyperemic (8,9) and its whole vascular pattern may become severely altered (10). To the naked eye, the gland then appears reddish-brown, enlarged and somewhat transparent. The cortical cells hypertrophy (11) (to enlarge the corticoid production plant)(3), and in cases of severe and prolonged stimuli, some of these cells may undergo partial or complete cytolysis (12,13). Enlargement of the gland occurs 6 to 24 hours after initiation of a severe stress (14,15), apparently due to the laying down of new protoplasm soon after stimulation (14). As shown by the fat stains Sudan and Scharlach red (which are claimed to stain fatty acid esters and cholesterol esters)(16,17), stress depletes the intensity of lipid staining of the adrenal cortex (8,9,18, 19). Sayers et al. (18) have reviewed the literature on this subject; and it is accepted that a "very close parallelism exists between the density of sudanophilic material and the concentration of total cholesterol in the adrenals, including circumstances in which these substances are

stored in excess, depleted or present in normal quantities" (4).

b) Stress induces changes in the concentration of cholesterol and ascorbic acid in the adrenal cortex.

Stress readily induces measurable changes in the concentration of certain chemical substances in the adrenal cortex. The cholesterol and ascorbic acid content of the gland diminish during stress, the ascorbic acid somewhat more rapidly than the cholesterol (18,20-26).

The total cholesterol content of the adrenal cortex has been shown to be depleted by the administration of ACTH and has been correlated with the occurence of metabolic changes characteristic of adrenocortical hyperactivity (18,24,27). In the absence of the anterior pituitary, the adrenal cholesterol is inert to stress and is usually fixed at a concentration higher than normal (28,29). The change in adrenal cholesterol esters or in total adrenal cholesterol has been used as a measure of the response to stress (24,30,31). The theoretical basis for this method seems sound, since cholesterol has been shown to be a precursor of adrenocortical steroids (32-34) which are secreted in larger amounts in stress and under the influence of ACTH (4, and see below).

Sayers and Sayers (24) have reviewed the numerous experimental conditions in which alterations in the concentration of adrenocortical ascorbic acid (and total cholesterol) occur: trauma (13,22), hemorrhage (24,35), burns (36,37), cold (13), x-irradiation (38), intense sound and light (39,40) and many others (24) are effective agents in this

respect. In hypophysectomized animals, stress was shown to be ineffective in depleting the adrenal ascorbic acid content (28); but it was demonstrated that the administration of ACTH, with the consequent adrenocortical activity, caused a rapid and significant drop in the ascorbic acid content of the rat and guinea pig adrenal (41). Although the role of ascorbic acid in the metabolic and secretory processes of the adrenal cortex was and still is unknown (4,42), Sayers and his colleagues adapted the depletion of adrenocortical ascorbic acid to a bio-assay for ACTH (43). This assay method has been used quite extensively and has permitted many advances in the field of pituitary-adrenal research (see Annual Reviews of Physiology, 1950 to 1957 and Volumes VI to XII, Recent Progress in Hormone Research).

c) Stress causes an increase in the secretion of adrenocortical steroid hormones.

It has been known for a long time that adrenalectomized animals are very much less resistant to stress situations than normal animals. As early as 1914, Elliot (44) found that adrenalectomized rats would survive for longer periods if kept in heated cages. Cannon (2) pointed out the intimate relationships of the adrenals to homeostatic regulation; Wyman and Suden (45) found that cortical transplants in adrenalectomized rats restored to normal their ability to maintain a normal body temperature when exposed to moderate cold, while rats with cortical insufficiency were unable to do so. This was confirmed by Tyslowitz

and Astwood (46). The diminished homeostatic capacity of adrenalectomized animals has also been shown with the stress stimuli of heat (47), prolonged muscular exercise (14), work, histamine, bacterial infections (47), etc.

In 1931, Hartman and his co-workers (48) observed that the administration of an adrenal cortex extract enabled adrenalectomized animals to maintain their body temperature almost as well as normal rats when placed in a cold environment. Such adrenal cortex extracts and, as they became available, purified and synthetic steroid compounds, were shown to possess life-maintaining properties, and to increase work performance and body weight gain of adrenalectomized animals (14,49). These studies enforced the conclusions that the adrenal cortex and its hormonal secretion were vital to homeostatic mechanisms and that the adrenal cortex must increase its secretory activity at such times.

These conclusions have been amply confirmed by the experimental data of many workers. Venning, Hoffman and Browne (50) demonstrated an increased excretion of biocorticoids in the urine of humans subjected to surgical trauma. In her now classical studies, Vogt (51) directly extracted corticoids from the adrenal vein blood of mammals (dog, cat, goat, rabbit, pig) subjected to the stresses of ether anesthesia and surgical procedures: as judged by the mean survival time corticoid assay of Selye and Schenker (52), "considerable cortical activity was invariably found in the venous blood collected from the suprarenals". With improved methods of corticoid extraction and assay (32), it was demonstrated conclusively that the application of stress stimuli invariably causes

an increased secretion of corticoids by the adrenal cortex (4).

2. The pituitary gland and stress

a) The anterior pituitary gland.

The anterior pituitary gland enjoys, anatomically and functionally, a first-order importance in the endocrine system; well protected by bone structures, it has often been referred to as "the conductor of the endocrine orchestra" (53). Harris (54) recently has reviewed succinctly the morphology and function of the adenohypophysis and its relationships to the nervous system.

i) The anterior pituitary controls the activity of the adrenal cortex in stress by its adrenocorticotrophic hormone (ACTH).

The regulation of the adrenal cortex by the anterior pituitary has been recognized for three decades, since P.E. Smith (55,56) demonstrated that hypophysectomy causes the adrenal cortex to atrophy. Soon thereafter, the hypophysectomized animal, similarly to the adrenalectomized animal, was shown to be extremely sensitive to a variety of non-specific stimuli (46,57,59), and it was further demonstrated that adrenocortical extracts increased the resistance of hypophysectomized rats to cold (46,57). In the absence of the pituitary, stress failed to decrease the concentrations of sudanophilic substance, cholesterol esters and ascorbic acid of the adrenal cortex (14,28). It was thus firmly established that the anterior pituitary played a fundamental role in the response of the organism to stress.

The experiments of Smith (55,56) had suggested that the pituitary control over the adrenal cortex was hormonal, since the implantation of pituitary tissue or the administration of anterior pituitary extracts could prevent the adrenal cortex atrophy in the hypophysectomized animals. Collip, Anderson and Thomson (60) and other workers (61,62) prepared anterior pituitary extracts which were capable of arresting many of the dysfunctions caused by removal of the pituitary gland. The existence of the anterior pituitary adrenocorticotrophic hormone became firmly established when two groups of workers, Li, Evans and Simpson (63) and Sayers, White and Long (64) independently prepared from sheep (Li) and from hog (Sayers) anterior pituitaries, a protein which acted as a homogeneous substance and showed specific adrenocortico trophic activity. The substance restored the weight and the histology of the adrenal cortex in the hypophysectomized rat to a status indistinguishable from normal (24); it caused the same dynamic change in the sudanophilic substance of the cortex as did stress (24); it evoked, on injection, depletion of the adrenal cholesterol esters and ascorbic acid (4,24,43). The purified hormone also could induce the metabolic changes characteristic of an increased steroid production, such as lymphocytopenia and eosinopenia (65,66) and increased urinary excretion of corticoids and 17-ketosteroids (32,65) even in the hypophysectomized animal. There could be no doubt, after such experiments, that the anterior pituitary controls the secretory activity of the adrenal cortex by its ACTH.

ii) Stress causes an increased release of ACTH from the anterior pituitary.

The conclusion that the accelerated discharge of ACTH from the pituitary gland is an essential link in the series of responses initiated by the application of stress stimuli was reached by indirect methods of measurement. The lack of a specific and sensitive method of assay did not allow any direct measurement of ACTH levels in the blood of the stressed animal, nor was it possible to demonstrate a decrease in the glandular content of the hormone. With development of the adrenal ascorbic acid depletion assay (43) and with improved extraction and purification techniques, a few such studies were undertaken.

Burns, Merkin, Sayers and Sayers (67) were the first to quantify the ACTH in mammalian anterior pituitaries. With an extraction technique claimed quantitative, and the adrenal ascorbic assay method, they established that the rat pituitary contained about 100 milliunits of ACTH; severe stress could reduce this amount to approximately one-half, within one hour after the application of the stress (68,69). Adrenalectomy was shown to seriously deplete the ACTH content of the adenohypophysis, although the glandular concentration started to rise within 24 hours and reached a level much above the normal in a few weeks (70). This was confirmed by Gemzell (71), who also demonstrated that the addition of a second stress soon after the first (adrenaline after adrenalectomy) caused a further drop in the gland's ACTH stores. Sydnor and Sayers (72) however, found only slight, statistically not significant, pituitary

ACTH depletion after various stresses and but relatively slight change after adrenalectomy. Saffran and Schally (73) and Birmingham and her co-workers (42) have questioned the normal resting values of ACTH in the rat anterior pituitary since, with the *in vitro* bioassay of Saffram and Schally (74) and extraction with HCL or glacial acetic acid (75), they found concentrations 4 to 10 times those reported by Sayers and his group. Fortier (76), extracting with HCL and assaying by the *in vitro* method (74), has carefully followed the successive changes in pituitary ACTH concentration induced by adrenalectomy and surgical trauma. He corroborated the polyphasic response of the pituitary ACTH level to adrenalectomy noted by Gemzell *et al.* (70); after splenectomy he observed an initial 40% fall in pituitary ACTH which persisted for 12 hours, then rose to a maximum level of 144% after 4 days. It is to be noted that the amount of ACTH obtained by Fortier (76) from normal rat pituitaries is identical to that reported by Saffran and Schally (73), using the same extraction and assay methods.

iii) Stress causes an increased titer of ACTH in the body fluids.

There have been many attempts to extract ACTH from the blood of normal and/or stressed animals and man (77-81). The evidence presented in these early attempts was not consistent, due to the use of insensitive assay methods and radical extraction procedures (70). In 1950, Gemzell *et al.* (70), assaying acid-acetone extracts of blood by the adrenal ascorbic acid method, reported that after adrenalectomy, the ACTH content

in the body fluids increased at least 30 times. Soon thereafter, Sydnor and Sayers (82) claimed the development of a quantitative and specific extraction technique for blood ACTH, based on oxycellulose adsorption of the hormone (75). With this method and the Sayers assay, ACTH activity could not be detected in the blood of normal, non-stressed human subjects, but it was judged present in that of nontreated and cortisone-treated Addisonians (83). In the blood of the intact, non-stressed rat, the concentration of ACTH was too low to be detected by the techniques employed (72); the application of stress stimuli (ether, ether plus scald, ether plus exsanguination) within two minutes increased the blood hormone level to detectable levels (72). Using a cross-circulation technique, in which the adrenal ascorbic acid response of a hypophysectomized rat receiving 20 ml. of blood from the stressed intact donor indicated the latter's relative ACTH blood level (84), Brodish and Long (85) reported that the stresses of unilateral or bilateral adrenalectomy, laparåtomy or stimulation of the sciatic nerve resulted in a greatly increased blood ACTH level. Sayers (86), using a somewhat similar technique, also reported that the application of stress causes an increased ACTH titer in the blood, as judged by the adrenal ascorbic acid assay.

In summary: stress causes the anterior pituitary to release, in relatively large amounts, its adrenocorticotrophic hormone, whose primary function is to stimulate an increased secretion of corticoid hormones by the adrenal cortex.

b. The posterior pituitary gland.

The posterior pituitary (neural lobe, infundibular process) is only a part of the neurohypophysis, which consists of three parts - the median eminence of the tuber cinereum, the infundibular stem and the infundibular process (87). Scharrer (88) considers the posterior pituitary as only a storehouse for the hormones which the neurohypophysis secretes, viz., vasopressin or the antidiuretic hormone (ADH) and oxytocin.

i) Stress causes an increased secretion of ADH from the neurohypophysis.

Exposure of the animal or man to systemic or neurotropic stimuli causes an inhibition of water diuresis (89). Exercise and epinephrine (90), surgical trauma and histamine (91), hypertonic solutions of sucrose or of sodium chloride (89), hemorrhage (92), flashing lights (93), noise and exposure to strange surroundings (94) all result in a depletion of ADH in the neurohypophysis and in a rapid increase in the antidiuretic activity of the serum (94,97). Painful stimuli also rapidly deplete the hypothalamic and neurohypophysial neurosecretory material (98,99), which is thought to be either the carrier of the active oxytocic and antidiuretic principles (100,101) or the hormones themselves (102).

The physiological significance to the release of the ADH during stress remains ill-understood, although it has been postulated by several workers (94,98,103,104) that the antidiuretic hormone (vasopressin) is the neurohumoral agent which controls the release of ACTH from the

anterior pituitary. Others have advanced the hypothesis that this postulated neurohumoral agent is distinct from, but chemically closely related to the known neurohypophysial hormones (105); and it has been suggested that the neurohypophysial neurosecretory system is a mechanism which reacts indiscriminately to a stimulus, for example pain, by a release of neurosecretory substance at all its outlets with the result that both the ACTH-releasing factor and the posterior lobe hormones are released at the same time (88).

ii) Neurotropic stress causes the release of ACTH from the posterior pituitary.

The presence of ACTH in the posterior lobe of the pituitary has been well substantiated (106-109), although the source of the corticotropic hormone in this tissue remains unknown. Mialhe-Voloss (107) was unable to find corticotropic activity in beef hypothalamic extracts. Post-mortem diffusion of the ACTH can be ruled out (109). Rochefort and Saffran (109) have suggested that the neurohypophysis may manufacture - and release - a type of ACTH of its own, while Nowell and Jones (110) have implied a possible movement of ACTH from the anterior lobe to the posterior lobe of the pituitary during stress. The presence of small vascular links between the two lobes (111) would be in favor of this suggestion. The problem is worthy of serious investigation.

It has been suggested that neurotropic stresses cause the

discharge of ACTH from the posterior lobe of the pituitary without affecting the anterior lobe stores of the hormone (112).

III. THE NERVOUS SYSTEM AND STRESS

The participation of the nervous system in homeostatic mechanisms has received repeated attention. The participation of the sympathetic nervous system has been rather well elucidated (2,113); and that of the central nervous system, in the last decade, has become a focus of investigation for numerous workers (114).

a. Stress activates the sympathetic nervous system.

Ramey and Goldstein (113), in a recent monograph discussing the relationships of the adrenal cortex and the sympathetic nervous system, listed the numerous stimuli which have been shown capable of activating the sympathetic-adrenal medullary system, as judged by signs of adrenaline release. Among others, they enumerate hemorrhage, cold, heat, fever, infections, fear, pain, burns and dehydration. Similar stresses have been shown, by refined bio-assay techniques and chemical analyses, to cause increased blood and urinary levels of adrenaline and nor-adrenaline (115). They have also been shown to produce an almost immediate discharge of chromaffin granules from the adrenal medulla (9); this is the morphologic expression of the discharge of adrenaline and nor-adrenaline from the gland (3). Cannon (2) has carefully explained the importance of the sympathetic-medullary system in "emergency" reactions to stresses. Adrenalectomy produces a much greater stress sensitivity (3,4) and complete

sympathectomy greatly decreases the resistance of dogs to the stresses of histamine, acetylcholine or intravenous injections of peptones (116). Total sympathectomy may inhibit many of the manifestations of shock because the preferential blood-shift to the heart and brain no longer occurs and the other organs are therefore not so severely deprived (117). In general, it may be said that the sympathectomized animal shows "inadequate responses to stress with regard to the maintenance of blood pressure, blood sugar, blood temperature, capacity to maintain anoxia and work capacity" (113).

b. Stress activates the central nervous system.

i) The central nervous system is involved in the reactions of the organism to stress.

During an intense and prolonged stress, the central nervous system may show morphologic and functional derangements, such as edema and congestion of the brain, petechial hemorrhages especially in the meninges, degenerative changes in the ganglion cells (118), tension and excitement and finally, intense depression and shock (3). Selye (3) points out that neurotropic stimuli are among the most potent instigators of the "alarm reaction". Stress, of a mental or physical nature, has been repeatedly considered to be one of the main precipitating factors of both psychoneurotic and psychotic states (119). Emotional stress, nervous tension and anxiety are well known to cause serious derangements in the electroencephalogram patterns of humans (120,121). However, there

is a notable lack of integration in this field of central nervous system-stress research: in the last decade, much attention has been focused on the participation of one particular part of the central nervous system, the hypothalamus, in the pituitary-adrenocortical response to stress stimuli.

ii) The hypothalamus is involved in the release of ACTH from the anterior pituitary.

Remote control electrical stimulation of the hypothalamus in normal, unanesthetized rabbits resulted in an lymphopenia that was similar in time relation and magnitude to that following an emotional stress stimulus or intravenous injection of an appropriate dose of ACTH (121). Similar results were obtained with dogs, cats and monkeys, using eosinopenia as the indicator of increased ACTH secretion (122-124). Since only with stimulation of the posterior hypothalamus (posterior tuber cinereum, mamillary body) could significant results be obtained, it was concluded that the posterior hypothalamus was in some way functionally related to the secretion of ACTH (54).

The effects of hypothalamic lesions on the mechanism of ACTH secretion in response to stress stimuli have been studied quite extensively. De Groot and Harris (121) found that bilateral electrolytic lesions placed in the mamillary body or posterior part of the tuber cinereum reduced or abolished the lymphopenia produced by emotional stress. Lesions in various parts of the pituitary gland had no effect on the lymphopenic response to

such stress, with the exceptions of those lesions which destroyed the anterior pole of the gland (zona tuberalis; that part of the gland which receives the hypothalamo-hypophysial portal system. This system of blood vessels originates in the median eminence from a multitude of capillary loops, or tufts, which are in intimate contact with the nerve fibres of the hypothalamic nuclei; these capillaries form portal trunks which descend mainly on the anterior or ventral surface of the pituitary stalk and enter the anterior pituitary at its zona tuberalis. The portal vessels then break up and form prominent sinusoids throughout the gland (54,125,126). Blood has been shown to flow from the median eminence to the pituitary gland through this system)(127). Numerous other workers (128-131) have also reported that hypothalamic lesions diminish or abolish ACTH secretion in response to stress, as evidenced by the criteria of adrenal ascorbic acid depletion or rise in blood corticoids. Lesions in the median eminence were also very effective in reducing or abolishing the pituitary response to stress (131). The general conclusion from these experimental data is that the hypothalamus is involved in the transmission of the stimulus of stress to the anterior pituitary gland to initiate increased ACTH secretion.

Other experimental data which support the concept that the central nervous system and the hypothalamus are involved in anterior pituitary activation during stress are those of Briggs and Munson (132), Gordon (133), Hume (134), and Fortier, Harris and McDonald (7). Briggs and Munson (132) showed that the central nervous system depressant drug, morphine, combined to pentobarbital anesthesia, will suppress the release

of ACTH upon the application of stress stimuli, as judged by the fall in adrenal ascorbic acid. Gordon (133) and Hume (134) found that the denervation of a limb was found to abolish the effect of operative trauma in causing ACTH discharge, although very severe scalds or burning of the denervated limb could still result in anterior pituitary activation. Fortier, Harris and McDonald (7) showed that the insertion of a waxed-paper plate between the cut ends of sectioned pituitary stalks in rabbits "reduced or abolished the lymphopenic response to restraint and exposure to cold, but exerted little effect on the response to injection of adrenaline or laparatomy or on the adrenal ascorbic acid depletion following unilateral adrenalectomy". These findings also support the view that stress stimuli may be divided into those that effect ACTH secretion solely by an action through the central nervous system, and those that act also by affecting the composition of the blood in the systemic circulation. Also of interest is the work of de Groot (135) who studied the effect of various types of stress at varying periods after cutting the pituitary stalk in mice. The lymphopenic response was abolished after the operation, but was slowly re-established between the 6th and 15th day after operation. By histological study, it was revealed that the hypophyseal portal vessels regenerate across the site of stalk section and that the magnitude of the lymphopenic response could be correlated with the degree of vascular regeneration. Waxed-paper plates inserted between the cut ends of the stalk prevented the return of responsivity. These experiments profer some support to the hypothesis of hypothalamic involvement in homeostatic

mechanisms and they also underline the importance of the hypothalamo-hypophysial portal system in the transfer of the stimulus from the hypothalamus to the pituitary.

iii) Parts of the central nervous system other than the hypothalamus are involved in the organism's response to stress.

The fact that neurotropic stresses, including such emotional states as anxiety, worry, excitement, cause an increased secretion of ACTH suggested that other parts of the central nervous system were in some way concerned with the endocrine homeostatic response.

The experiments of Porter (124), in 1954, presented direct evidence that the cerebral cortex probably played some role in the complex mechanisms leading to increased ACTH secretion. Porter made the following observations on cyclopropane-anesthetized monkeys: stress stimuli (adrenaline, histamine, surgical trauma) which increased the electrical activity of the tuberal and mamillary regions of the hypothalamus, also increased that of the anterior nucleus of the thalamus and the anterior cingulate gyrus. The increased activity of these regions was dependent on the presence of an intact hypothalamus. Electrical stimulation (a) of the orbital surface of the frontal lobe and (b) of the hypothalamus resulted in a marked eosinopenia, while electrical stimulation of the hippocampal region, in particular the uncus, inhibited stress-induced eosinopenia. This investigator concluded that the hypothalamus alone is essential for the manifestation of stress-induced responses and that "facilitatory and

inhibitory influences of neural origin appear to be integrated at the hypothalamic level". Gloor (136) has presented an excellent functional concept of the relations of the higher cerebral centers to the hypothalamus, and he suggests that these centers may act "in correlating appropriate homeostatic and adaptative mechanisms to neocortically directed activities as... voluntary physical work or intense intellectual concentration and so on". Anderson et al. (137) postulated the existence of a mesencephalic hypothalamico-pituitary activating system, since transection of the mid-brain, but not that of the spinal cord at a lower cervical level, interfered with the ACTH releasing mechanisms during stress.

c. Summary

The participation of the nervous system in homeostatic responses to stress is indubitable. The sympathetic-medullary system is involved, probably, in regulating the respiratory, circulatory and in some part, metabolic functions of the organism; the central nervous system integrates the various stimuli and is involved in the control of the release of ACTH from the pituitary gland.

IV. MECHANISM OF CONTROL OF ACTH SECRETION IN STRESS

Stress causes a release of ACTH from the anterior pituitary gland. Because of the almost complete lack of nerve fibres to this gland (126,138), it is most unlikely that the ACTH release in reaction to stress is mediated by nervous control. The alternative left for consideration is

a humoral or neurohumoral (substance produced by neurosecretory cells) type of control or a combination of both. Four theories, each advocating a different method and agent of control, have been proposed to explain the ACTH release controlling mechanism: (a) adrenaline, released from the adrenal medulla and reaching the pituitary via the blood; (b) the blood levels of adrenocortical hormones; (c) a hypothalamic factor, secreted by the neurosecretory cells and reaching the anterior pituitary by the hypothalamo-hypophysial portal system; (d) vasopressin, released from the posterior pituitary.

1. Adrenaline controls the release of ACTH

This theory was held primarily by Long and his co-workers (22,139). Since stressors always caused the secretion of adrenaline from the adrenal medulla and since the infusion of small physiological doses of adrenaline resulted in ACTH secretion, as judged by a fall in adrenal ascorbic acid and adrenal cholesterol (140,141), these workers suggested that the medullary adrenaline was the humoral agent controlling ACTH secretion by the anterior pituitary. Supporting evidence was gained when it was shown that a) adrenaline did not cause an increased corticoid output when added to blood perfusing the adrenal gland (142); b) the injection of adrenaline did not cause the depletion of ascorbic acid and cholesterol in hypophysectomized animals (143); c) injection of adrenaline into an eye containing a pituitary transplant will effect an ACTH release, as judged by eosinopenia and fall in adrenal ascorbic acid (144). However, it was demonstrated that

the intravenous infusion of adrenaline in normal young men did not increase the 17-hydroxycorticoids levels in the blood during or after the infusion (145). When adrenals are enucleated, the cortices will return to normal size but the medullae will not regenerate; after regeneration, stress will still cause an eosinopenia, although at a somewhat slower rate (6). In the complete absence of the adrenals in an animal maintained on a constant dose of corticoids, eosinopenia can still occur if the stress applied is strong enough (146). Moreover, the intravenous injection of histamine effects a more rapid eosinopenia than the injection of adrenaline and lastly, histamine, like adrenaline, is effective in causing eosinopenia and adrenal ascorbic acid depletion when applied to the eye containing a pituitary transplant, suggesting therefore that the effect of these agents is non-specific (6).

2. The level of corticoids in the blood controls the release of ACTH

Sayers (4,24) was the exponent and defender of this theory. He suggested that the level of corticoids in the blood controls the release of ACTH from the pituitary since, in stress, the utilization of adrenocortical hormones by the peripheral tissues is probably increased, thus sharply reducing their quantities in the venous blood; this should stimulate the pituitary to discharge ACTH. Conversely, a high corticoid level in the blood should inhibit the ACTH secretion. It was well demonstrated that pretreatment of an animal with adrenal cortex extracts or crystalline cortical steroids increases the resistance to stress and blocks the release of ACTH from the pituitary, as judged by the lack of adrenal ascorbic acid depletion during the stimulus (14,21,23,24).

Although it is generally admitted that the "feedback" mechanism on the pituitary plays a part in the fine adjustments of ACTH secretion, the possibility that it is the fundamental mechanism of control of ACTH secretion in stress has met with serious objections. Sayers and his group (72) themselves have questioned it: in adrenalectomized animals, they found elevated blood ACTH levels. When stress is applied to adrenalectomized animals, their blood ACTH titre rises to even higher levels (72). Other objections: Pituitary transplants to sites distant from the area of the hypophysial portal vessels do not maintain adrenal weight and normal adrenal cortex responsiveness (148,149); there are no arteriovenous differences in the concentration of 17-hydroxycorticoids in blood samples from normal, diseased or traumatized limbs (54); there is an immediate rise in blood corticoids after stress, without any sign of a preliminary fall (54,150) and strong stress still can cause ACTH discharge in normal animals pretreated with large amounts of corticoids (23,151).

3. A hypothalamic factor, released in the hypothalamo-hypophysial portal vessels, controls the secretion of ACTH.

The evidence that the hypothalamus constitutes a major pathway for excitation of ACTH release from the pituitary has been reviewed above. The link between the hypothalamus and the anterior pituitary was postulated to be effected by "a chemical transmitter liberated into the hypophysial portal vessels, which in turn carry the substance to the pars distalis" to exert a specific influence over the activity of the gland (121).

In 1954, Slusher and Roberts (152) reported the preparation from bovine hypothalamic extracts of a non-saponifiable lipid, not cholesterol, which appeared capable of stimulating pituitary ACTH release as judged by its ability to cause eosinopenia or a decline in adrenal ascorbic acid after intraperitoneal administration to intact rats. No further reports have been published on this substance, except that de Wied (153) demonstrated that chloropromazine and morphine abolish its ascorbic acid depleting activity, suggesting that the activity of this extract depends on the function of the hypothalamic centers.

In 1953, Guillemin and Fortier (154) rejected histamine as the possible neurohormone; in 1955, Guillemin (155), utilizing pharmacodynamic blocking of the hypothalamo-pituitary axis, reported that adrenaline, nor-adrenaline and acetylcholine also were not identical with the neurohormone. Soon after, Swingle and his colleagues (156) demonstrated a histamine-like component of commercial Pitressin, active in releasing ACTH in the Saffran and Schally in vitro system (73), the incubation medium from the stimulated pituitaries inducing a significant fall in the adrenal ascorbic acid of hypophysectomized rats. However, Schally and Saffran (157) could not obtain ACTH release with histamine in their system. A subsequent publication from Swingle et al. (158) withdrew their claim that histamine or the histamine-like component of Pitressin was the ACTH-releasing agent. In 1955, Saffran and Schally (73) reported that, although little activator substance could be found in

hypothalamic tissue or hypothalamic extracts (5), neurohypophysial tissue plus noradrenaline increased the release of ACTH from incubated anterior pituitary tissue. Very soon after this report, Saffran, Schally and Benfey (159) announced the isolation from posterior pituitary preparations of a corticotrophin-releasing factor (CRF), distinct from oxytocin and vasopressin. CRF was also shown to be present in hypothalamic extracts (105); it has been purified and found to possess the chemical and chromatographic properties of a peptide, distinct from oxytocin and vasopressin (105). The amino acids obtained after acid hydrolysis of CRF also support the claim that it is a relatively small peptide (105). Guillemin and his co-workers (160-165) have also isolated from hypothalamic tissue and posterior pituitary extracts, an ACTH "hypophysiotropic" factor capable of causing an ACTH release in the Saffran and Schally in vitro test system. These investigators claim that their substance "appears to be a small peptide, different from vasopressin, oxytocin, ACTH, histamine, acetylcholine, adrenalin, nor-adrenalin and 5-hydroxytryptamine by its pharmacologic and chromatographic characteristics" (164).

It must be added, however, that ACTH-releasing activity (in the in vitro test system) has been found in extracts of brain cortex (5,73,164) and in substance P of gut origin (164). Few reports on the in vivo activity of CRF or of the "hypophysiotropic factor" have appeared. However, CRF has been shown to produce eosinopenia in a stalk-sectioned dog (159); in two endocrinologically normal cancer patients, CRF caused an increase in the blood corticoids (Porter-Silber reaction) but no increase in the urinary

corticoid levels (166). The ACTH-hypophysiotropic factor (fraction D, Guillemin) has recently been shown to cause, upon prolonged infusion of rather large quantities into humans, increases in the levels of free 17-hydroxycorticoids in the blood (165).

The independent isolation by two laboratories, from hypothalamic and neurohypophysial extracts, of a substance of small molecular weight and capable of stimulating ACTH release in a test system claimed sensitive and specific (167), strongly supports the theory of neurohumoral control of ACTH release.

4. Vasopressin, released from the neurohypophysis in stress, controls the release of ACTH.

This theory rests on the demonstrated fact that stresses, known to activate the pituitary-adrenal cortex axis, will simultaneously cause a depletion of the neurosecretory material in the hypophysis (98,99), a prolonged antidiuresis and an increased level of antidiuretic activity in the blood (89,94). Since the increases in blood ACTH (72) and blood antidiuretic activity (94) occur very rapidly, both being significantly elevated within two minutes after subjecting the animals to stress, it was suggested that the antidiuretic hormone (vasopressin) is the neurohormone responsible for ACTH discharge (94,98,103,104). This hypothesis was strongly supported by the demonstration that the administration of Pitressin to normal animals produced a release of ACTH from the anterior pituitary, as judged by eosinopenia and the fall in adrenal ascorbic acid content (103,104,168). This effect was not obtained in hypophysectomized

animals (168) and McCann (104,169) claimed that vasopressin was capable of eliciting the signs of ACTH discharge in animals whose pituitary-adrenal response to stress had been blocked by Nembutal-morphine anesthesia, administration of large doses of hydrocortisone or hypothalamic lesions. Recently, McCann and Fruit (170) showed that commercial and synthetic vasopressin preparations were equipotent in evoking ACTH release (judged by adrenal ascorbic acid depletion) in rats bearing effective hypothalamic lesions. McDonald and Weise (171) infused Pitressin in humans and obtained a significant eosinopenia and rise in plasma 17-hydroxycorticoids. They repeated the experiment with highly purified arginine-vasopressin and highly purified and synthetic lysine-vasopressin and obtained, with all these substances, increases in plasma concentrations of free 17-hydroxy-corticoids (172,173). They attributed these increases to the ACTH-releasing activity of vasopressin; however, it should be emphasized that the infusion of these vasopressor preparations was accompanied by various side-effects, such as nausea, abdominal cramping and peripheral vasoconstriction which may have well constituted a potent stress stimulus. Significantly, in their last publication, McDonald and his co-workers (174) could not offer any evidence that endogenous vasopressin release acted as an ACTH-releasing mechanism.

Objections have come from other workers. Guillemin and Hearn (160) attributed the "ACTH hypophysiotropic activity of commercial Pitressin to a contaminant of vasopressin of probable hypothalamic origin", and Schally and Saffran (157) could not detect any acceleration in the release of ACTH

by isolated rat pituitaries incubated with purified lysine-vasopressin. Highly purified arginine-vasopressin also had no effect in the tissue culture technique of Guillemin and Hearn (160). The hypothesis must also answer to the objection offered by the demonstration that the administration of large doses of water causes simultaneously the inhibition of antidiuretic hormone release and the signs indicative of pituitary-adrenal response to stress (175). Briggs and Munson (132) could not find adrenal ascorbic acid depletion in Nembutal-morphine treated rats injected with physiological doses of vasopressin. Greer and Erwin (176) have shown that in rats with lesions of the median eminence and marked diabetes insipidus, amphenone still could induce adrenal hypertrophy; they suggested that these results ruled out the possibility that vasopressin plays a significant role in the control of ACTH release. Finally, Schally, Saffran and Zimmerman (105) demonstrated that purified CRF has the chemical and chromatographic properties of a peptide distinct from vasopressin.

5. Summary

It is generally admitted that the hypothalamus plays an important role in the control of ACTH release from the anterior pituitary. This role appears to consist of the elaboration of a specific neurohormone, distinct from vasopressin, probably stored in the neurohypophysis (177) and, at times of stress, released in the hypothalamo-hypophysial portal system to reach the anterior pituitary and effect an increased secretion of ACTH.

EXPERIMENTAL

I. MATERIALS AND METHODS

1. Animals.

Young adult male rats (170-220 gm. body weight) of the Sprague-Dawley strain were used throughout these studies. The animals were acclimatized to the constant temperature animal room before use for experimental or assay purposes. Before being transported to the laboratory, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, Abbott), 4 mg. per 100 gm. body weight.

2. Basic methods.

a) Methods of extraction of ACTH from tissues.

i) Extraction with glacial acetic acid-dilute HCL.

The tissue was weighed on a micro-torsion balance, placed into 40-100 times its weight of glacial acetic acid in a thick wall test tube, and ground as completely as possible by means of a glass rod and a small amount of pulverized sea sand. A further volume of approximately 1 ml. per 10 mg. tissue of acetic acid was added to the tube. The mixture was then heated for 30 minutes, with occasional stirring, in a water bath kept at 75°C. The tubes were removed from the bath and the rods rinsed with 0.5 ml. of glacial acetic acid. The tubes were then stoppered with

Parafilm and stored in the deep-freeze. The day before assay, the tubes were thawed and placed in a vacuum dessicator over NaOH pellets at room temperature. In the morning, the dry residues in the tubes were extracted for 2 hours with 0.01 N HCL, the volume depending on the amount of tissue extracted and the assumed ACTH potency. Aliquots of the HCl extracts were then diluted with Krebs-Ringer-bicarbonate-glucose (KRBG) medium (178, 74) for assay.

ii) Extraction with acid-acetone (179).

The tissue was weighed and placed into a conical centrifuge tube containing 1.5 ml. of acid-acetone (70 parts absolute acetone, 30 parts distilled water and 2.5 parts concentrated HCl). The tissue was ground with the help of a glass rod. The resulting mixture was centrifuged for 5 minutes at 2000 r.p.m. or more. The supernatant was then added to 9 ml. of absolute acetone in another centrifuge tube. After a few minutes to allow precipitation, the tube was spun for 10 minutes at 2000 r.p.m. or more, bringing down a white precipitate. The extraction and precipitation were repeated 3 times, using the same tubes. The precipitate was then dried in a vacuum dessicator over NaOH pellets at room temperature, On the morning of the assay, the precipitate was dissolved in 0.1 N HCl and aliquots of the solution were diluted with KRBG medium for assay.

b) Determination and expression of ACTH potency in extracts.

In our laboratory, the extracts were assayed for their ACTH

potency by the Saffran and Schally *in vitro* technique (74), against the U.S.P. Corticotropin Reference Standard (1.14 I.U./mg.) and are expressed as milliunits of ACTH per mg. (mU. ACTH/mg.) of fresh tissue. Percentage values were obtained by comparative assays of one extract against another, using one of the extracts as 100% standard. The values are expressed as percent (%) control.

c) Treatment of tissues.

Rat anterior pituitaries, rat posterior pituitaries, rat hypothalami and the anteromedial zones (109) of beef and hog anterior pituitaries were extracted for ACTH by the glacial acetic acid-dilute HCl method. Rat anterior and posterior pituitary lobes in two experiments were also extracted for ACTH by the acid-acetone method.

Rats were decapitated with a guillotine or with large scissors, the top of the skull was removed with scissors and the brain was carefully scooped out, exposing the pituitary gland. The gland was freed from adhering membranes with fine forceps and the posterior lobe was carefully separated from the anterior lobe. The lobes were placed separately in ice-cold KRBG medium. Where necessary, several anterior or posterior lobes were pooled. The glands were then drained on a glass plate, rapidly weighed and placed into the extraction tubes.

The hypothalamic tissue from 8-10 rats was obtained by dissection from the scooped-out brains, pooled in medium, drained, weighed and placed into glacial acetic acid for extraction.

Beef and hog pituitaries were obtained frozen from the slaughterhouse. At the laboratory, they were thawed and freed of their membranes. With scalpel and scissors, the anteromedial zones of the anterior lobes were dissected out (109), weighed and dropped into glacial acetic acid.

d) Purification of ACTH by adsorption on oxycellulose.

Partial purification of ACTH was carried out by oxycellulose treatment, as prescribed by Astwood, Raben and Payne (75). The standard ACTH was dissolved in 0.1 M acetic acid, and aliquots of the centrifuged glacial acetic acid extracts of rat anterior pituitary were diluted to 0.1 acetic acid molarity. Oxycellulose was added to the solutions and the mixtures stirred in the refrigerator for a minimum of 24 hours. The ACTH was eluted from the oxycellulose with 0.1 N HCl, in the cold. Aliquots of the HCl eluates were diluted with KRBG medium for assay.

e) Determination of protein concentration.

The concentration of protein in the extracts was measured with the Folin Phenol reagent by the method of Lowry et al. (180).

f) Extraction and assay of ascorbic acid from adrenal tissue.

The adrenal glands were removed from the decapitated bodies of the animals as soon as possible and placed, in pairs or singly, on identified filter papers slightly moistened with KRBG medium. The adrenals were freed

of adhering fat with fine scissors and dropped into centrifuge tubes containing 4% trichloroacetic acid. After grinding with pulverized sea sand, the extracted ascorbic acid was determined by a modification (181) of the method of Roe and Kuether (182).

3. Materials.

The CRF preparations were purified from Protopituitrin by serial paper chromatography by Dr. A.V. Schally in our laboratories (167). CRF 39-74 standard was obtained at the second m-cresol stage in the paper chromatographic purification steps and was said to be concentrated 160-180 times over the starting material; CRF 39-74 purified was obtained at the final propanol-water stage in the chromatographic purification and was attributed a purification factor of 4600-5000 over the starting material. The preparations were diluted with 0.5% acetic acid or saline for injection.

Pitressin, the Parke-Davis preparation of vasopressin with an activity of 10 Units of pressor activity per ml., was diluted with 0.5% acetic acid for injection. Synthetic arginine vasopressin, possessing a pressor activity of 386 international units per mg., was obtained through the graciousness of Dr. V. du Vigneaud; aliquots of the preparation were diluted with saline for injection.

II. PROCEDURES AND RESULTS

A. Investigations on the extraction and assay methods.

1. Comparison of extraction techniques.

The efficiency of the methods of ACTH extraction from fresh tissue was determined by comparison of the two techniques. One of the halves of each of 10-12 anterior pituitary glands were pooled and extracted by the glacial acetic acid-dilute HCl method; the remaining halves were pooled and extracted by the acid-acetone method. The extracts were assayed against each other, using the glacial acetic acid extract as the 100% standard. Similarly two groups of 6 posterior pituitary lobes were extracted by the two methods and assayed one against the other.

The results are given in Table 1. No significant difference was obtained between the potencies of the extracts: the range of the acid-acetone extracts, as percent of the glacial acetic acid- dilute HCl standards, was 91.5 to 131 percent.

Table 1

Comparative ACTH potencies of rat pituitary extracts

Experiment Number	Tissue	Potency of acid acetone extract as percentage of glacial acetic acid-dilute HCl extract			
		%	Limits	Lambda	Geometric mean
1	Rat Anterior Pituitary	131.0	80-216	0.11	120
		100.0	34-293	0.24	
2	Rat Anterior Pituitary	111.0	89-139	0.05	102
		91.5	79-106	0.04	
3	Rat Posterior Pituitary	102.0	65-161	0.10	

These experiments were carried out in collaboration with Dr. A. Ghilain, a World Health Organization visiting Fellow from Brussels, Belgium.

2. Comparison of assay methods.

The potencies of ACTH extracts obtained by the *in vitro* technique were compared with those obtained for identical extracts by the Sayers adrenal ascorbic acid depletion method. In each of the first two experiments, 5 rat anterior pituitary glands were halved and the halves were evenly divided into two tubes. Each sample of tissue was extracted by the glacial acetic acid-dilute HCl method, dried and sealed in an ampule. One ampule was then sent to the cooperating laboratory to be assayed for ACTH by the Sayers' method, the other was kept in the laboratory at room temperature, for assay by the *in vitro* technique on the same predetermined day. In the third experiment, the tissue from 10 rat anterior pituitary glands and that of the anteromedial zones of hog and beef anterior pituitary glands were extracted with glacial acetic-acid-dilute HCl. Two equal aliquots of each HCl extract were separately placed into clean ampules and sealed. One ampule from each pair was sent for assay by the Sayers' method, the other was kept in the laboratory at room temperature to be assayed *in vitro* on the same day.

The results of these experiments are shown in Table 2. With the exception of the hog extract, a significantly higher ACTH potency was obtained for all the extracts with the *in vitro* technique. The ratios "*in vitro* assay ACTH potency: ascorbic acid assay ACTH potency" are (a) for rat anterior pituitary 13:1, 7:1, 9:1; (b) for hog tissue, 1.35:1; (c) for beef tissue, 2.4:1.

Table 2

Comparison of ACTH assay methods

Extract	In vitro assay	Sayers A.A.A. assay	Laboratory
	mU. ACTH/mg. (5% confidence limits)		
Rat ant. pituitary	133 (100-179)	ca. 9.7	Rerup
	136 (116-161)	20.5 (11-34)	Rerup
	177 (134-225)	20.4 (16-26)	Leeman and Munson
Anteromedial zone of hog ant. pit.	179 (134-228)	132.0 (93-187)	Leeman and Munson
Anteromedial zone of beef ant. pit.	77 (64-91)	32.0 (23-44)	Leeman and Munson

The ascorbic acid depletion assays on the extracts were carried out by Dr. C. Rerup, University of Lund, Lund, Sweden and Drs. S. Leeman and P. Munson, Harvard University, Boston, Massachusetts, U.S.A.

3. Purification of U.S.P. Reference Standard ACTH and rat anterior pituitary ACTH extracts.

The 0.1 N HCl eluates of oxycellulose treated U.S.P. ACTH and rat anterior pituitary ACTH extracts were assayed for their ACTH potency against untreated U.S.P. Corticotropin Reference Standard. The potency of the untreated portion of the rat pituitary glacial acetic acid extract was likewise determined. The percent recoveries were calculated for each eluate and are shown in Table 3 for U.S.P. ACTH and in Table 4 for rat anterior pituitary tissue extracts. When the ratio of mg. oxycellulose: ml. acetic acid ACTH solution was about 1.6, the percentage recovery of the ACTH activities was satisfactory. The recovery of the rat pituitary ACTH is very similar to that obtained for the U.S.P. Reference Standard.

Table 3

Oxycellulose treatment of U.S.P. Corticotropin Reference Standard

U.S.P.	ACTH	Volume of solution	Oxycellulose added	ACTH recovered	Recovery
mg.	mU.	ml.	mg.	mU.	%
28.0	31,920	100	60	16,650	51.9
				18,420	57.7
				Mean*	54.8
37.8	43,092	60	100	31,500	73.1
				35,375	82.0
				Mean*	76.2

* Geometric mean of two assays

Table 4

Oxycellulose treatment of rat anterior pituitary ACTH extract

Rat ant. pit. tissue	Volume of solution	Oxycellulose added	Untreated extract	Oxycellulose eluate	Recovery
mg.	ml.	mg.	mU. ACTH / ml.		%
193.4	1200	500	3260	1156	36
116.3	400	650	3996	3348	70

B. Changes in the ACTH content of the posterior and anterior lobes of the rat pituitary with various stresses.

1. Sound stress.

Rats, obtained as a group of approximately the same age and of the same body weight (200 ± 10 gm.) were audiogenically stimulated by the ringing of a loud bell suspended above their cage. At 5, 15, 30, 60 and 120 minutes of stimulation, groups of 3 animals were sacrificed and their posterior and anterior pituitary lobes pooled, extracted by the glacial acetic acid-dilute HCl technique and assayed against U.S.P. Corticotropin Reference Standard. The experiment was carried out twice; the results are presented in Tables 5 and 6 and graphically depicted in Figures 1 and 2.

Five minutes after the beginning of the stimulus, the ACTH in the posterior pituitary gland had begun to fall; after 30 minutes, the hormone content in this lobe had fallen to a level significantly below that of the starting (control) level. The ACTH concentration of the posterior lobe did not return to pre-stress levels until 2 hours after the beginning of the stimulus. The anterior lobe stores were not affected until the stress had been applied for at least 30 minutes; after 1 hour of stress application, the anterior lobe ACTH stores were at a level significantly below that of the controls. At 120 minutes, the anterior lobe ACTH concentration had returned or was returning to normal, pre-stress levels.

Table 5

Effects of sound stress on rat pituitary ACTH

Minutes of stress	Posterior Pituitary	Anterior Pituitary
	mU. ACTH/mg. (5% confidence limits)	
0	25.6 (11-61)	146 (127-168)
15	13.4 (9-21)	149 (127-174)
30	7.6 (5-12)	150 (91-247)
60	7.3 (4-12)	99 (62-158)
120	49.6 (26-96)	147 (112-193)

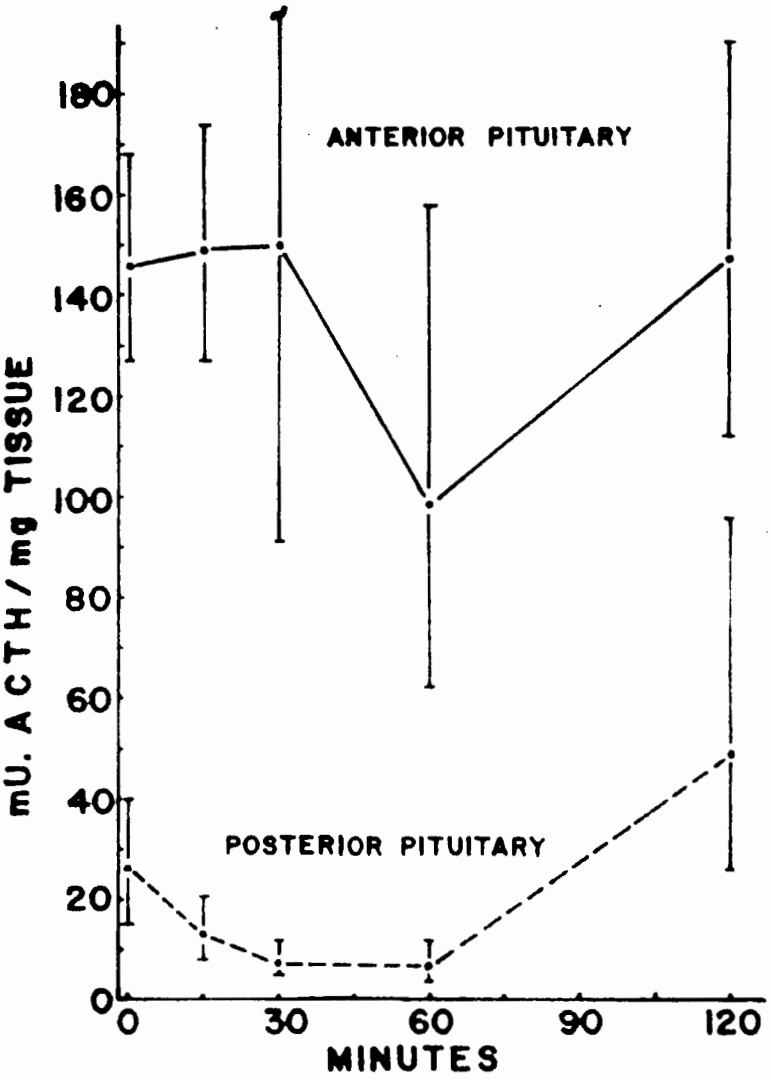

Figure 1: Changes in ACTH concentration of the rat pituitary lobes during sound stress.

Table 6

Effects of sound stress on rat pituitary ACTH

Minutes of stress	Posterior Pituitary	Anterior Pituitary
	mU. ACTH/mg. (5% confidence limits)	
0	22.4 * (18-28)	150.5 * (121-186)
5	18.1 * (9-36)	-
15	9.6 * (7-13)	153.0 (92-254)
30	9.3 * (7-12)	134.5 * (111-163)
60	11.9 * (9-16)	46.8 * (34-64)
120	24.8 * (19-32)	118.0 (74-186)

* Geometric mean of two assays

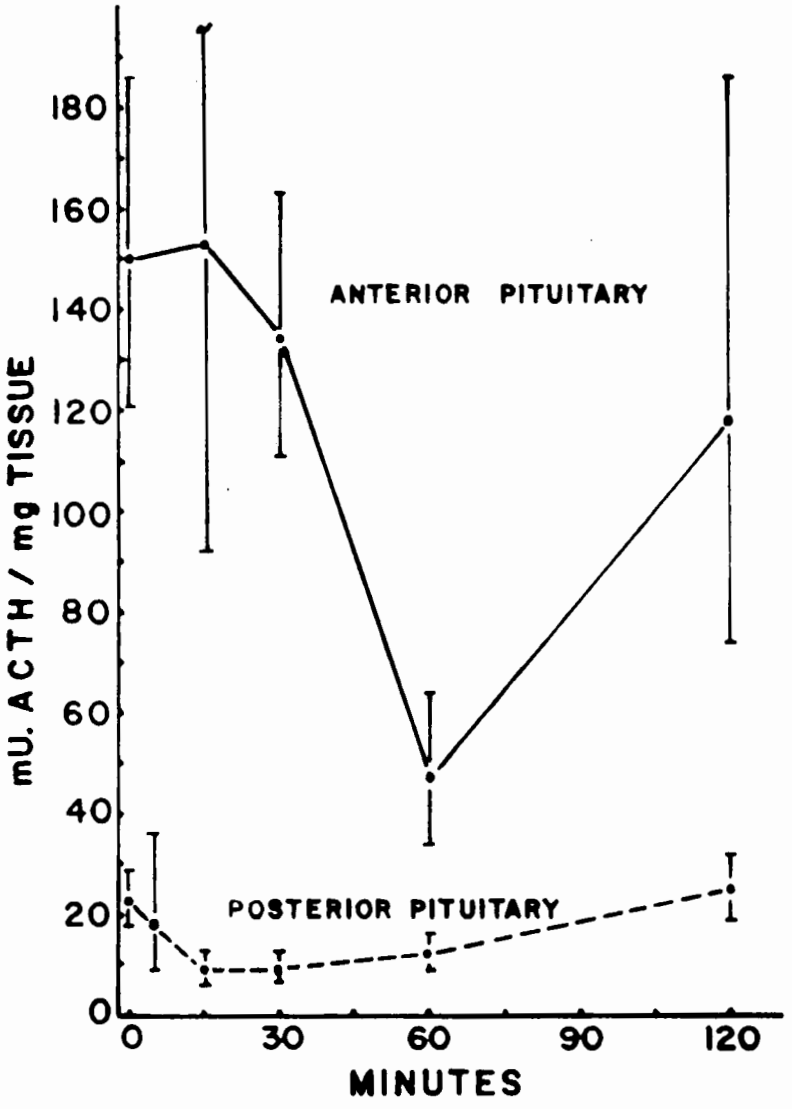

Figure 2: Changes in ACTH concentration of the rat pituitary lobes during sound stress.

2. Histamine stress.

Animals were trained to handling and to intraperitoneal injection by twice daily injections for four days prior to the experiment. On the fifth day histamine dihydrochloride, 1 mg. per 100 gm. body weight, was given intraperitoneally to each animal. The controls received only saline, the vehicle in which the histamine salt had been dissolved. At 30, 60 and 120 minutes after the injections, groups of 3 animals were sacrificed, their posterior and anterior pituitary lobes pooled for ACTH extraction and assay. The adrenals of the animals were extracted in pairs and the extracts assayed for ascorbic acid content.

The amounts of residual ACTH in the pituitary tissues at various time intervals after the injections are given in Table 7 and depicted in Figure 3. The histamine stress did not cause any statistically significant change in the ACTH content of the posterior pituitary gland, although there was an increase 30 minutes after the application of the stress. There was a rapid decline in the ACTH of the anterior lobe of the pituitary; at 30 minutes after the injection of the histamine the decrease was statistically significant. This is a 47% fall in the ACTH content and, for a 5 mg. anterior pituitary gland, represents a release of about 12.5 mU. of ACTH per minute, or a total of about 400 mU. in 30 minutes, if synthesis is neglected.

The concentration of ascorbic acid in the adrenals also fell sharply to reach its lowest level 30 minutes after the application of the

stimulus. The differences between the mean of the control concentrations and that of the concentrations 30, 60 and 120 minutes after the injections are all significant, $P < 0.01$. The differences between the means at 30, 60 and 120 minutes are not significant. These results are shown in Table 8 and Figure 4.

Table 7

Effects of intraperitoneal histamine on rat pituitary ACTH

Minutes after injection	Posterior Pituitary	Anterior Pituitary
	mU. ACTH/mg. (5% confidence limits)	
Controls	28.2 (19-57)	174.4 * (134-249)
30	46.5 (24-81)	100.8 * (81-126)
60	21.1 (13-34)	116.0 * (91-147)
120	16.2 (8-32)	139.7 (107-183)

* Geometric mean of two assays

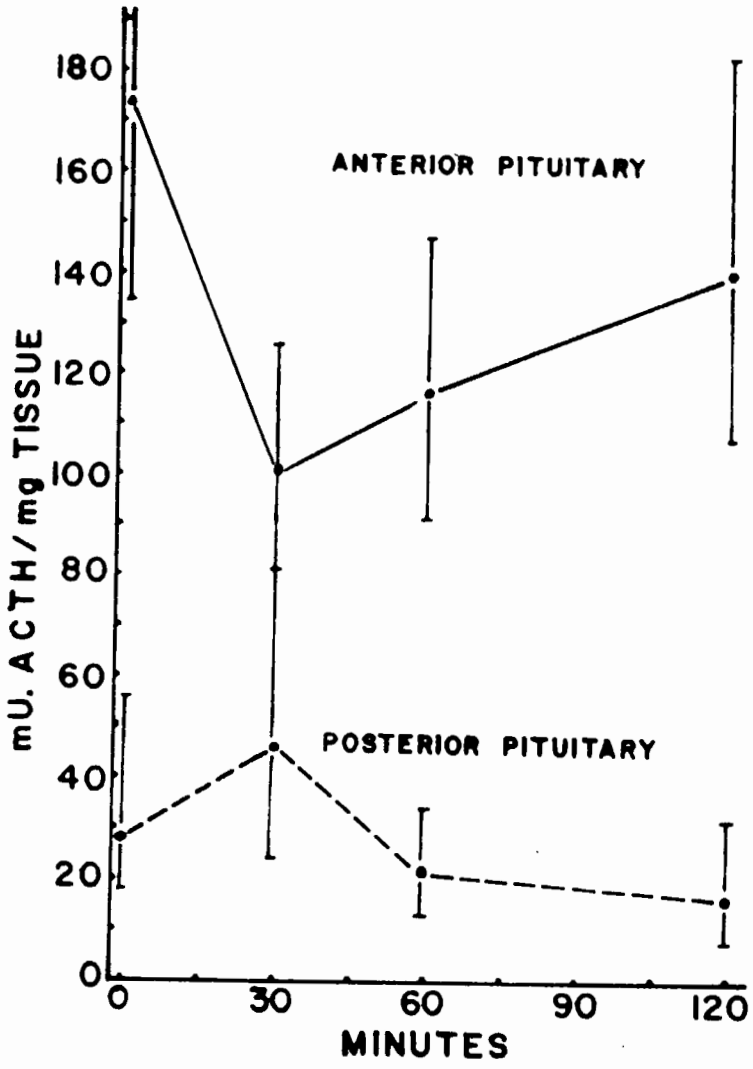

Figure 3: Changes in ACTH concentration in the rat pituitary lobes after intraperitoneal injection of histamine.

Table 8

Changes in adrenal ascorbic acid after intraperitoneal histamine

Minutes after injection	Adrenal ascorbic acid Individual responses	Mean ± S.E.	Fall
	mcg. per 100 mg.		%
Controls	500 502 458 518 495	494.6± 9.9	-
30	284 209 305 317 243	271.6±20.1	- 45
60	278 238 309 256 291	274.4±12.5	- 44.5
120	358 384 237 342 324	329.0±25.0	- 33.5

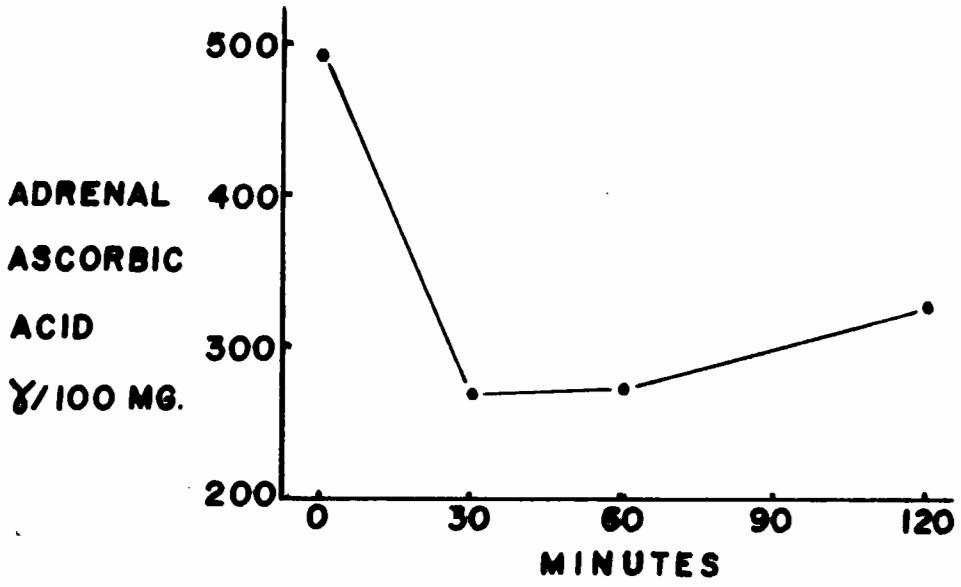

Figure 4: Changes in rat adrenal ascorbic acid content after intraperitoneal injection of histamine.

3. Cold stress.

Rats were subjected to an environmental temperature of 7°C. After 30, 60 and 120 minutes of exposure, groups of 3 rats were sacrificed and their posterior and anterior pituitary tissues pooled, extracted and assayed for their ACTH.

The ACTH concentrations of the pituitary tissues after these time intervals are shown in Table 9 and Figure 5. Exposure of the animals to the cold caused a gradual fall in the ACTH content of both the posterior and anterior pituitary lobes, but only the fall in the anterior lobe at 120 minutes was statistically significant.

Table 9

Effects of cold on rat pituitary ACTH

Minutes of exposure	Posterior Pituitary	Anterior Pituitary
	mU. ACTH/mg. (5% confidence limits)	
0	38.6 (25-61)	145.0 (117-180)
30	30.5 (24-39)	141.5 (94-213)
60	22.0 (14-34)	117.3 (97-140)
120	23.7 (16-35)	67.5 (39-118)

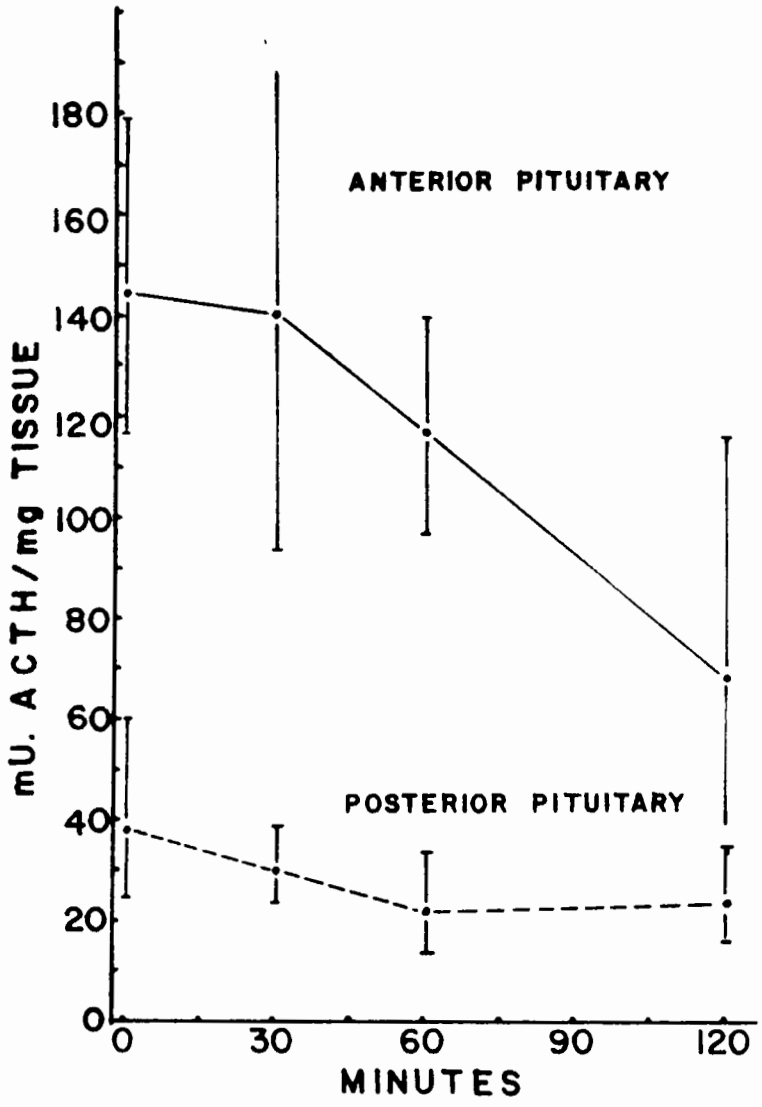

Figure 5: Changes in ACTH concentration in the rat pituitary lobes during exposure to cold.

C. Changes in rat pituitary ACTH concentration following injection of CRF and vasopressin.

1. Intraperitoneal injection of CRF and Pitressin.

The rats were acclimatized to the stress of intraperitoneal injection by dry injecting them for four days before the experiments. On the days of the experiments, one group of animals was injected with 0.5% acetic acid, a second group with Pitressin and a third group with either CRF standard or CRF purified. Sixty minutes after injection, the rats were sacrificed by decapitation, the anterior glands extracted and assayed for ACTH. The adrenal glands were extracted in pairs and the extracts analysed for their ascorbic acid concentration.

The intraperitoneal injection of 4-20 mcg. of CRF standard (total dose) caused a statistically significant fall of about 50% in the ACTH concentration of the anterior pituitary gland compared to the amounts found in the anterior pituitaries of the animals injected only with the diluent. CRF purified, at a total dose of 1.5 mcg., caused a similar fall in the ACTH concentration of the anterior lobe. Pitressin, at doses equipressor with the CRF injected(167,183), did not cause a statistically significant fall in the ACTH content of the anterior pituitary (Table 10).

The ascorbic acid content of the adrenal glands followed the same pattern as the anterior pituitary ACTH. The fall in adrenal ascorbic acid was greater after the CRF than after the equipressor doses of vasopressin, but the difference between the two treatments was not statistically significant.

No reactions to the injections were noted in the animals.

Table 10

Anterior Pituitary ACTH after intraperitoneal CRF and Pitressin

Material injected	Individual glands	Mean
	mU. ACTH/mg. (5% confidence limits)	
Vehicle	157 (122-202)	
	160 (127-202)	
	190 (120-300)	
	130 (82-205)	
	142 (113-176)	
	158 (112-223)	156 (142-172)
Pitressin, 80 mU.	150 (86-263)	
Pitressin, 200 mU.	123 (94-161)	
	115 (87-153)	119 (99-143)
CRF, standard, 4 mcg.	90 (57-142)	
	86 (47-156)	
20 mcg.	66 (52- 85)	
	84 (73- 95)	
	101 (49-208)	85 (76- 95)
CRF, purified, 1.5	90 (57-143)	
mcg.	82 (60-112)	
	89 (75-107)	
	86 (76- 98)	87 (80- 95)

Table 11

Adrenal ascorbic acid after intraperitoneal CRF and Pitressin

Vehicle	Material injected: CRF	Pitressin
	mcg. ascorbic acid per 100 mg. adrenal	
464	335	379
544	322	365
474	398	267
532	350	416
424	228	417
460	205	
437	390	
585		
562		
Mean±S.E.		
498±19.48	318±28.36	369±27.08

Comparison	Difference	"t"	P
Vehicle vs CRF	180	5.232	< 0.01
Vehicle vs Pitressin	129	3.846	< 0.01
Pitressin vs CRF	51	1.301	> 0.05

2. Intravenous injection of CRF and Pitressin.

Intravenous injections were made into the exposed femoral vein of nembutal-urethane anesthetized rats, with or without hydrocortisone pretreatment. In the corticoid treated animals, a saline-propylene glycol (5:1) suspension of crystalline hydrocortisone (Cpd.F) (Merck) was injected intraperitoneally at a dose level of 6 mg. per 100 gm. body weight. Four hours later, the diluent 0.5% acetic acid, Pitressin, 100 mU., and CRF standard, 1 and 2 mcg. total dose, were injected into the femoral vein. Other intravenous injections were made without surgical procedures by the method of Pearce (184) into the lateral marginal leg vein of nembutal anesthetized rats. Two dose levels of CRF standard, 0.05 and 0.01 mcg., were injected by this route in non-treated animals. Sixty minutes after the injections, the animals were sacrificed and the anterior pituitary tissues extracted and assayed for ACTH. The results are shown in Table 12.

One hour after the injection of the vehicle into the femoral vein of the non hydrocrotisone-treated animals, the ACTH level in the anterior pituitary had fallen to approximately 50% of normal, if 150 mU./mg. be accepted as the starting concentration. Undoubtedly the stress of the surgical procedure was the cause of this fall. The injection of a total dose of 2 mcg. of CRF in the femoral vein of non-treated animals brought about a much greater fall, significantly below that in the vehicle-injected animals. A dose of 100 mU. of pressor activity (Pitressin) was ineffective in the hydrocortisone-treated rats but CRF, at doses of 1 and 2

mcg., did cause a fall in the ACTH concentration of the anterior pituitary gland. However, the additional decrease with CRF was not statistically significant, when the absolute values are compared. As percentages, the CRF values represent 77% (57-109) and 60% (51-83) of the control.

Intravenous injections into the lateral marginal leg vein do not require any surgical procedures and produce minimal trauma in the animals. When CRF standard was injected by this method at very small doses, 0.05 and 0.1 mcg., falls of 21% and 46% in anterior pituitary ACTH were obtained. Statistically that obtained with the 0.1 mcg. dose level was significant.

No reactions were observed in the animals during or after the intravenous injections at the dose levels used.

Table 12

Anterior Pituitary ACTH after intravenous CRF and Pitressin

Treatment	Anterior pituitary ACTH concentration	
	mU. ACTH/mg. (5% confidence limits)	% control
Femoral vein injections:		
Vehicle	86 (50-151)	100 (58-176)
CRF standard, 2 mcg.	23 (15- 35)	27 (18- 41)
Hydrocortisone treated:		
Vehicle	178 (115-276)	
	166 (100-275)	
Mean	172 (132-224)	100 (77-130)
Pitressin, 100 mU.	174 (137-224)	101 (80-130)
CRF standard, 1 mcg.	132 (98-188)	77 (57-109)
CRF standard, 2 mcg.	104 (87-143)	60 (51- 83)
Lateral marginal vein injections:		
Vehicle	184 (141-239)	100 (77-130)
CRF standard, 0.05 mcg.	145 (116-181)	79 (63- 98)
CRF standard, 0.1 mcg.	100 (83-120)	54 (45- 65)

3. CRF time curve.

Nembutal anesthetized animals were injected via the lateral marginal leg vein with 1 mcg. of CRF standard; the controls were injected with saline. At 30, 60 and 120 minutes after the injection of the CRF, three animals were sacrificed; the anterior pituitary glands were individually extracted for ACTH, while the posterior lobes were pooled for extraction. The extracts were assayed against the extract of the pooled control anterior pituitary lobes, the dilution of each extract being adjusted such that each aliquot of assay solution would represent an equivalent amount of tissue. Later the concentration of protein peptide in each extract was measured by the method of Lowry. The adrenals were individually extracted and the extracts analysed for their ascorbic acid content.

Blanching and slight difficulty of breathing were noted in some of the animals in the first few minutes following the injection of the CRF.

Thirty minutes after the injection of the CRF, the anterior pituitary ACTH concentration had fallen to a level significantly below that of the control (Table 13). That of the posterior lobe had not. The anterior pituitary ACTH concentration continued to decrease and reached a level nearly 50% of control two hours after the injection. Sixty minutes after administration of the CRF, the ACTH concentration of the posterior lobe suddenly fell to 50% of control, but rose again rapidly to normal

level at 120 minutes.

As shown in Table 14 there were no large differences between the amount of protein peptide in the extracts of the anterior and posterior glands of the control animals and those of the CRF injected animals, although there were significant differences between their ACTH activity (Table 13).

The fall in anterior pituitary ACTH content was duplicated by the fall in adrenal ascorbic acid: 30 minutes after the injection of the CRF, a statistically significant depletion in the vitamin had occurred. The fall continued slowly to a magnitude of 42% of control at 120 minutes after the injection (Table 15). This change is depicted in Figure 6.

Table 13

CRF time curve

Time after injection	Tube		Anterior pituitary ACTH	Posterior pituitary ACTH
			% control (5% confidence limits)	
Control	CA 1		100	
	CP 1			100
30	EA 1		86.3 (67-110)	
	EA 2		61.4 (36-104)	
	EA 3		65.0 (52- 81)	
		Mean	65.0 (55- 77)	
	EP 1			91 (64-129)
60	EA 4		62.4 (40- 98)	
	EA 5		43.3 (26- 74)	
	EA 6		56.3 (40- 81)	
		Mean	54.0 (44- 65)	
	EP 2			44 (33- 59)
120	EA 7		55.3 (34- 90)	
	EA 8		67.0 (58- 77)	
	EA 9		38.2 (27- 54)	
		Mean	52.0 (45- 60)	
	EP 3			97 (70-133)

Table 14

Protein concentration in extracts and assay solutions

Sample		Tissue	Volume extract	Lowry protein concentration	Dilution for assay	Protein in assay solution
		mg.	µl.	mcg./100 µl.	100 µl. to	mcg./100 µl.
Control A.L.	CA 1	24.0	2400	94.7	2000	4.74
Exper. A.L.	EA 1	5.88	1000	57.56	1176	4.89
"	EA 2	5.43	"	42.72	1086	3.93
"	EA 3	6.49	"	58.88	1298	4.54
"	EA 4	6.40	"	60.40	1280	4.72
"	EA 5	6.10	"	60.68	1220	4.97
"	EA 6	5.20	"	52.56	1040	5.05
"	EA 7	4.60	"	52.12	920	5.67
"	EA 8	5.60	"	56.84	1120	5.08
"	EA 9	5.80	"	54.64	1160	4.71
Control P.L.	CP 1	3.90	500	61.60	633	9.73
Exper. P.L.	EP 1	3.12	500	51.16	520	9.84
"	EP 2	2.40	400	49.24	500	9.85
"	EP 3	2.60	400	52.28	542	9.65

Table 15

CRF time curve

Time after injection	Adrenal ascorbic acid	Mean ± S.E.
minutes	mcg./ 100 mg.	mcg./ 100 mg.
Controls	488	
	488	
	496	
	477	
	466	
	456	
	483	
	476	479± 4.59
30	390	
	411	
	233	
	254	
	405	
	397	348±33.39
60	400	
	372	
	319	
	322	
	236	
	218	311±29.49
120	186	
	246	
	262	
	289	
	348	
	339	278±25.06

Comparison	Difference	"t"	P
Control vs 30 minutes	130.5	3.87	< 0.01
30 minutes vs 120 minutes	70.0	1.67	> 0.05

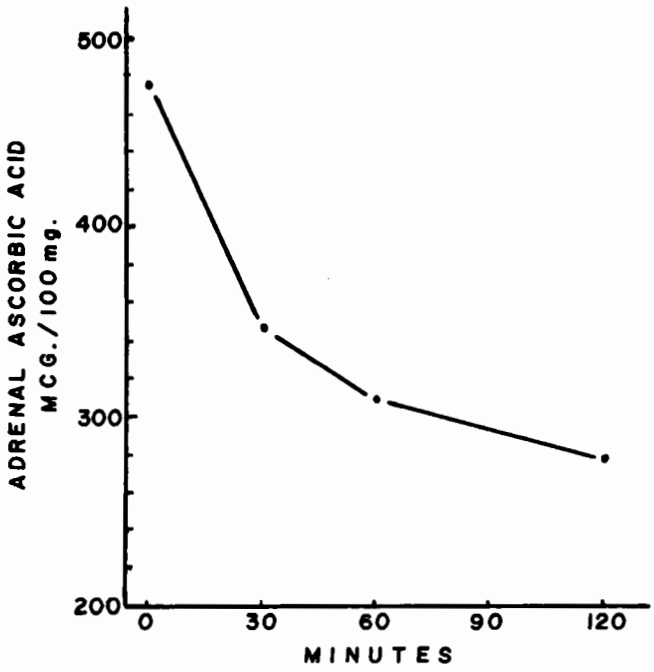

Figure 6: Changes in adrenal ascorbic acid after intravenous injection of CRF.

4. Intravenous synthetic arginine vasopressin.

Two dose levels, 0.01 and 0.1 mcg. (3.9 and 39 mU. pressor activity), of synthetic arginine vasopressin were injected via the lateral marginal leg vein into nembutal anesthetized rats. The controls received saline only. One hour after the injections, the animals were sacrificed; the individual anterior pituitary glands and the pooled posterior pituitary glands were extracted for ACTH with glacial acetic acid-dilute HCl. The extracts were assayed against the extract of the pooled anterior pituitary tissue. The adrenals were individually extracted and the extracts analysed for their ascorbic acid content.

As may be seen in Table 16, the injection of the synthetic hormone did not cause any release of ACTH from either the anterior or posterior lobe of the pituitary. The adrenals duplicated this lack of response, since there was no depletion in their ascorbic acid content (Table 17).

The animals showed no malaise after the injections of 0.01 and 0.1 mcg. of the synthetic arginine vasopressin. However, a dose of 1.0 mcg. (390 mU. pressor activity), injected by the same route and with the same rapidity (approximately 20 seconds), brought about immediate pronounced blanching, difficulty in breathing, fibrillation and death within three or four minutes.

Table 16

Pituitary ACTH after intravenous synthetic vasopressin

Total dose injected	Anterior Pituitary	Posterior Pituitary
	% control ACTH (5% confidence limits)	
0.01 mcg.	89 (68-117)	
	110 (93-130)	97 (84-112)
	95 (71-126)	
Mean	98 (86-113)	
0.1 mcg.	110 (88-138)	
	100 (77-130)	96 (79-116)
	104 (90-120)	
Mean	105 (95-112)	

Table 17

Adrenal ascorbic acid after intravenous synthetic vasopressin

Injection	Adrenal ascorbic acid Individual	Mean ± S.E.
Control: vehicle	455	
	467	
	462	
	457	460± 2.7
0.01 mcg. vasopressin	502	
	524	
	532	
	523	
	444	
	457	497±15.4
0.1 mcg. vasopressin	560	
	548	
	407	
	420	
	480	
	494	485±25.8

DISCUSSION

A. Extraction and assay procedures.

The work reported in this thesis is valid inasmuch as the following premiss is true: the material extracted from the pituitary tissue by the glacial acetic acid-dilute HCl technique and assayed by the *in vitro* method is ACTH.

Glacial acetic acid extracts of anterior pituitary tissue have been said to contain ACTH on the basis of the adrenal weight maintenance and adrenal ascorbic acid depletion assay methods (4,24,75). Similar extracts were also shown to cause steroidogenesis *in vitro* (33,185,186). Astwood and his co-workers (75) obtained a preparation with a 12% weight yield and with an average potency of 2.0 international units (I.U.) per mg. by extraction of dessicated hog anterior pituitary tissue with glacial acetic acid. Treatment of this material with oxycellulose and subsequent elution with 0.1 N HCl yielded an 80% recovery of the biological activity. The U.S.P. Corticotropin Reference Standard is also a crude glacial acetic acid extract of hog anterior pituitary tissue (187) with a stated potency of 1.14 I.U. per mg. In our hands, the material extracted from rat pituitary tissue by the glacial acetic acid-dilute HCl possessed the same assay characteristics as the U.S.P. Standard. The material was also active in depleting adrenal ascorbic acid (Table 2). Both the U.S.P. Reference Standard and the rat ACTH were adsorbed on, and eluted from, oxycellulose. When a potency of 150 mU. per mg. was assumed for the control rat anterior pituitary tissue in the CRF time curve experiment, the glacial acetic acid

extract had an activity of 1.5 units of ACTH per mg. (Table 18). This indicated a 12 - fold purification over the starting material, with a 10% weight yield. It should also be noted that stress stimuli were shown to cause significant depletions in the extractable activity (Tables 14 and 18), the hormonal activity was however independent of the weight of material extracted. This is quite understandable: if a minimum activity of 100 mU. per mcg. of pure corticotropin is assumed, a release of 300-400 mU. of ACTH from the anterior pituitary would affect its weight by only 3-4 mcg. and such a change would not be detectable.

Table 18

Characteristics of rat pituitary extracts

Sample	Tissue	Total material in extract	Weight yield	ACTH found	Total ACTH	Potency of extracts
	mg.	mcg.	%	% control	mU.	mU. ACTH/mcg.
Control A.L. I	24.0	2272.8	10.6	-	3600[1]	1.58
Exper. A.L. 1	5.88	575.6	10.2	86.3	761.5	1.32
" 2	5.43	427.2	12.7	61.4	500.1	1.17
" 3	6.49	588.8	11.0	65.0	632.8	1.08
" 4	6.40	604.0	10.6	62.4	599.0	0.99
" 5	6.10	606.8	10.1	43.3	396.5	0.65
" 6	5.20	525.6	9.9	56.3	439.4	0.84
" 7	4.60	521.2	8.8	55.3	381.8	0.73
" 8	5.60	568.4	9.9	67.0	562.8	0.99
" 9	5.80	546.4	10.6	38.2	332.3	0.61
Control P.L. I	3.90	308.0	12.7	-	97.5*	0.32
Exper. P.L. 1	3.12	257.8	12.1	91.0	70.9	0.28
" 2	2.40	197.0	12.2	44.0	26.4	0.14
" 3	2.60	209.1	12.4	97.0	63.1	0.30

[1] Based on an average potency of 150 mU. ACTH per mg. fresh anterior pituitary tissue.

* Based on an average potency of 25.0 mU. ACTH per mg. fresh posterior pituitary tissue.

These results suggest that the glacial acetic acid-dilute HCl treatment extracted the corticotropic activity from the pituitary tissues. Moreover the extraction appears to have been quantitative. Birmingham and co-workers (42) have shown that glacial acetic acid-dilute HCl extraction was more effective than dilute HCl extraction alone. Roberts (188) however, found the dilute HCl extraction method as effective as the glacial acetic acid-dilute HCl technique, provided the volumes of extraction solution were the same and thorough homogenisation of the tissue was carried out. As indicated in Table 2, acid-acetone extraction was as effective as that with glacial acetic acid-dilute HCl. The acid-acetone method used in these experiments was very similar to that developed by Lyons (189) and claimed to be quantitative by Reiss and Halkerston (190).

The ACTH concentrations in normal and stressed rat anterior pituitary tissue in our investigations were significantly greater than those previously obtained by extraction with various methods and assayed by the Sayers' adrenal ascorbic acid depletion technique (43,67-73,76).

This difference may have been due in part to the strain of rats used. Significantly, it has been recently reported by Kitay, Holub and Jailer (191) that the glacial acetic acid-dilute HCl extracts of the pituitary glands of male rats of the Sherman strain, assayed by a modification of the Saffran and Schally in vitro bio-assay technique, possessed a concentration equivalent to about 70 mU. of ACTH per mg. of fresh tissue. However, as may be seen in Table 2, a significant difference was also

obtained when the same extracts of one strain of rats were simultaneously assayed by the two methods.

The reasons for such a discrepancy are not clear. Birmingham et al. (42) have suggested that the steroidogenic/ascorbic acid depleting (S/AAD) activity ratio could be higher in native than in purified rat ACTH. This suggestion was supported by the results of Fortier (76) who found a mean S/AAD activity ratio of 2.64/1 for dilute HCl extracts of rat pituitary. Roberts (188) obtained a S/AAD activity ratio of 10/1 for both glacial acetic acid and dilute HCl extracts of rat pituitary. In our experiments, the mean S/AAD activity ratio was 9.6/1. Such results would suggest that the ascorbic acid depleting activity of ACTH is separated from its steroidogenic activity; it has already been suggested that the adrenal ascorbic depleting activity of ACTH is completely separate from the adrenal weight maintenance activity (110,192).

Roberts (188) has advanced two experimental conclusions to explain the discrepancy. He claimed (a) that the in vitro rat adrenal may be "markedly more sensitive to rat ACTH than to ACTH from other sources", whereas "in vivo, the rat adrenal gland does not appear to make this differentiation with respect to the ascorbic acid release" and (b) that "the in vitro assay procedure has a considerable lack of specificity", since the presence of large amounts of extraneous proteins (such as plasma or serum proteins) in the incubation flasks produced a non-specific release of steroids from the adrenal tissue.

Roberts' first conclusion was based on a series of comparative assays on rat pituitary extracts, a-Corticotropin and ACTH-protein. In the case of the rat pituitary extract, he found by the in vitro method a potency about 10 times that obtained by the Sayers method; for ACTH-protein, identical potencies by the two methods, and for a-Corticotropin, nearly twice the potency by the Sayers' method than by the in vitro technique. These results were not different from the ones previously obtained by our laboratory. We have already pointed the difference between our results on rat pituitary extracts and those obtained by the Sayers' assay. The agreement between potencies obtained by both methods on various crude and purified ACTH preparations has already been pointed out by Saffran and Bayliss (193), and more recently, by van der Vies (194). However, in a recent series of collaborative assays between several laboratories, serious differences were shown to exist between the potencies of a preparation of Corticotropin A_1 obtained by the Sayers' adrenal ascorbic acid depletion assay and those established by the in vitro technique (195). The report pointed to the low accuracy of the ascorbic acid assay and to the importance of the route of administration of the sample: the potencies obtained by the Sayers intravenous assays varied from 19 to 92 U./mg., by the Sayers subcutaneous assay, from 11-213 U./mg and by the in vitro technique, from 18-59 U./mg. The ascorbic acid assays also had poor indexes of precision: a range from 0.13 to 0.94 in the intravenous assays, from 0.13 to 0.65 in the subcutaneous assays and from 0.06 to 0.18 in the in vitro

assays. The possibility of inactivation of ACTH by blood enzymes when injected and the short half-life of 5.5 minutes of the hormone in the circulation have been mentioned (75,196). Finally, it should be stressed that the basis of the Sayers' method, that is the relationship between adrenal ascorbic acid and the stimulation of corticosteroidogenesis, has not been demonstrated. Recently Lundin and Holmdahl (197) questioned the specificity of the method: in their investigations, the intravenous injection of large doses of acid acetone or glacial acetic acid extracts of human placentae, even after oxycell treatment or boiling with alkali, produced significant adrenal ascorbic acid depletion in hypophysectomized rats. As the authors pointed out, "in the literature there is no report of any systematic investigation of possible unspecific effects from large doses of protein on ascorbic acid depletion in the adrenals of hypophysectomized rats".

Roberts' second conclusion was indeed a very serious objection to the validity of our results. The conclusion, "that the <u>in vitro</u> corticosteroid-release assay for ACTH may be both non-specific and non-responsive in the presence of interfering protein or protein-bound substances", was reached from results such as the dollowing: (a) the addition of 0.2 ml. of fresh normal, hypophysectomized or adrenalectomized rat serum to the incubation flasks stimulated a steroid release equivalent to that caused by the addition of 5 mU. of U.S.P. Corticotropin Reference Standard; (b) the addition of 0.2 ml. neutralized oxycellulose extracts equivalent

to 0.8 ml. of plasma obtained from normal or adrenalectomized rats also produced a total steroid release equivalent to that obtained with 2.5 mU. of U.S.P. ACTH; these fractions were not active in the Sayers' adrenal ascorbic acid technique; (c) the addition of 6 mg. dialysed and lyophilized preparations of whole plasma or of certain plasma fractions also produced non-specific steroid release, indicating ACTH-like activity in fractions inactive in the in vivo test; the non-specific steroid release was mainly due to an increase of 11-desoxycorticosterone (DOC).

Loraine (198) has emphasized that the assumption that "extracts prepared from blood can be conveniently assayed in terms of standard material prepared from sources other than blood" is not necessarily correct. And the same author stresses that "it is essential for investigators to determine by statistical methods whether a given assay procedure which they propose to use for estimations in blood satisfies the recognized criteria of validity". Unfortunately however, the data reported by Roberts (188) did not contain any of the criteria by which his experiments could be critically examined. Moreover, the actual weight of material which was placed on the adrenals in vitro in the blood fraction assays was very large, 6 to 150 mg. per 1.5 ml. of incubation medium. On the other hand, the standard preparations and the pituitary extracts were added in amounts of about 10 micrograms per 1.5 ml. of incubation medium (Table 14). Birmingham (199), in our laboratories, has shown that when rat adrenal tissue is incubated with blood plasma or serum, in quantities of 9 mg./100 µls or

over, a non-specific "release" of corticoids into the incubation medium results. However, the slope of the dose-response curve is very small and significantly different from that with ACTH or pituitary extracts. The same investigator has not been able to demonstrate the presence of DOC in the incubation medium, pointing to a major difference in the conditions of preparation and incubation of the adrenal tissue. In the in vitro assays carried out in our laboratory, the incubated rat adrenals responded to both porcine ACTH (U.S.P. Corticotropin Reference Standard) and to rat ACTH with the same log-dose response curve, identical slope, limits of error and index of precision.

In summary, therefore, no satisfactory explanation may be put forth to explain the large difference between the ACTH potencies obtained by the two assay procedures on similar or identical extracts of rat pituitary glands. Further investigations such as the following could be carried out: (1) the effects of increasing doses of non-ACTH proteins (such as extracted serum or plasma proteins) should be systematically investigated in both the in vitro and adrenal ascorbic acid depletion assay methods; (2) oxycell purified rat, beef, and hog ACTH extracts should be simultaneously assayed by the two procedures; (3) similar comparisons should be made on identical extracts inactivated by hot alkali and/or extensive enzymatic treatments; (4) the in vitro steroidogenic properties of rat pituitary ACTH should be tested on incubated adrenals of other species, such as guinea pigs, hamsters, hogs and cattle; (5) comparative assays should be run on ACTH from anterior pituitary glands

of other strains of rats; (b) the inactivation of ACTH, both *in vivo* and *in vitro*, should be carefully investigated using both assays.

B. Changes in ACTH content of the pituitary lobes with various stresses.

The results reported herein support the concept that stress causes a release of ACTH from the pituitary by a dual mechanism.

The release of ACTH from the anterior pituitary gland by stress stimuli had been demonstrated both by direct indices, such as depletion of sudanophilic material in the adrenal (8,9), changes in circulating eosinophils or lymphocytes (65,66), changes in adrenal ascorbic acid and cholesterol (24), rises in blood and urine corticoids (50,51), and by direct measures, such as the measurement of the residual ACTH in the pituitary of the stressed animal (67-72) or of the circulating ACTH in the blood of the stimulated animal (72,85,86). Our method of measuring the residual ACTH in the pituitary lobes at various time intervals during or after the application of the stress stimulus may be considered a direct index. Gemzell and his colleagues (40) have claimed that the pituitary ACTH reflected the changes in secretion and formation rate of the hormone. Again, it has been argued that "the best available criterion for the rate of release of ACTH was the content of corticotropin in the pituitary gland itself", when the changes in the concentration of the hormone in the blood could not be measured (200). It was thought preferable to make the measurements after small intervals and for a limited time only, since it had already been shown that ACTH

is released very rapidly from the pituitary after the application of the stress stimulus (72), and that a maximal effect could be detected one hour after the onset of the stress (4,72).

We have already discussed at length the difference between the potencies, in terms of mU./mg., found for normal rat pituitary extracts as assayed by the in vitro method and the Sayers' ascorbic acid method. The same comments, of course, may be applied to the quantitative values obtained for the pituitaries of stressed animals. The paucity of previous reports on ACTH content of the pituitaries after stress, the stimuli and the time relationships involved, the different methods of extraction and assay, made difficult direct comparisons. However when considered as percentages falls in ACTH, it was found that our results were not so widely divergent from those in the literature. Sayers' and his colleagues (67-69) observed a 30% fall in anterior pituitary ACTH one hour after the intraperitoneal injection of histamine and a 35% depletion after scalding. Gemzell (71) reported a 40% fall after subcutaneous epinephrine, Fortier (76) a 30% fall after splenectomy and a 22% decline after bilateral adrenalectomy, much the same figure as Gemzell et al (70). In our experiments, the falls in anterior pituitary ACTH one hour after the application of the stress stimuli were usually about 30%, although the maximal fall did not always occur at that time. These data and the time relationships involved in some previously reported and in our own experiments are compared in Table 19.

Table 19

Pituitary ACTH after various stresses

Stress	Time	Anterior Pituitary	Investigators
	hrs.	% initial ACTH	
Sound	½	102	Rochefort
	1	68	
	2	100	
Sound	½	90	"
	1	31	
	2	78	
Histamine i.p.	½	58	"
	1	66	
	2	80	
Cold	½	98	"
	1	80	
	2	47	
Splenectomy	½	100	
	1	72	
	2	65	
	4	63	Fortier (76)
	12	63	
	24	125	
	1536	115	
Epinephrine	½	-	
	1	71	
	6	53	Gemzell (71)
	24	62	
	48	60	
Histamine	1	70	Sayers et al. (69)
Scald	1	65	Sayers and Cheng (68)

Sound stress has been quoted and used as a neurotropic stress by previous investigators (3,4,6,54). The results obtained in our experiments confirm the work of Mialhe-Voloss (112) who had shown a decrease in the adrenal ascorbic acid depleting activity of the posterior pituitary lobes of rats also subjected to the stress of sound. It has been demonstrated, on a quantitative basis, that in animals subjected to the neurotropic stress the primary response was the release of the ACTH present in the posterior lobes and, after an interval of time had elapsed and apparently only when the posterior pituitary lobe ACTH stores had been nearly completely depleted, by a large and sustained secondary release of the anterior pituitary ACTH. On the other hand intraperitoneal histamine, classified as a systemic stress (3,4,6), affected the posterior pituitary ACTH stores only in that there was a transitory increase; the release of ACTH occurred only from the anterior pituitary lobe. The transitory increase in posterior pituitary ACTH may support the contention of Nowell and Jones (110) that there is a movement of ACTH from the anterior lobe to the posterior lobe during stress-induced release of the hormone. Cold, which was chosen as a "mixed" neurotropic-systemic stress, caused only a slow and gradual release of ACTH from both pituitary lobes; however, the fall was significant in the anterior lobe only, after two hours of exposure. The hormone release would perhaps have been more rapid with a greater severity of cold.

These results, based on a direct index of stress response,

support the theory of Fortier (6) that stresses may be divided into two main types, neurotropic and systemic. The neurotropic stresses were postulated to require "the participation of the hypothalamo-hypophyseal pathways", whereas the systemic stimuli would "imply the direct activation of the pituitary by one or several humoral agents". In experiments where the posterior lobe was shown or presumed to be atrophic or non-existent, as with pituitary transplants, stalk-sectioned glands separated from the hypothalamus by paper barriers, or after hypothalamic lesions which destroyed the median eminence and/or the pituitary stalk (6,7,54), there was a notable lack of response to neurotropic stimuli, or at the least, a very diminished one. Undoubtedly in these experiments the neural connections from the hypothalamus to the posterior lobe were also severed. Thus the neurotropic stresses could not effect a release of ACTH from the posterior pituitary lobe since the stimulus either could not reach the gland or the gland was atrophic or non-existent.

The demonstration of the participation of the posterior lobe in homeostatic responses was novel only in that it was shown to contain and release ACTH. It had already been shown that the posterior lobe responded to stress by a release of anti-diuretic hormone and oxytocin (94,100). It has been postulated that CRF is also stored in the posterior lobe and may be released from it to cause an increased secretion of ACTH by the anterior pituitary (5,88). The problem then of the release of the CRF from the posterior would need to be resolved, since the sudden release of ACTH thirty minutes after the initiation of the neurotropic stress suggested some

type of timing or control mechanisms. Gemzell and Heijkenskjöld (200) have proposed a reciprocal relationship between the blood level of a neurohumoral ACTH-releasing factor and of ACTH: an increase in the blood concentration of ACTH would inhibit the release of the neurohormone while a fall in the blood level of ACTH would stimulate the release of the factor from its source. Just as important, of course, is the problem of what initiated the release of ACTH (and probably concurrently of anti-diuretic hormone and oxytocin) from the posterior pituitary lobe. If very discrete lesions, such as to destroy only the neural connections between the posterior lobe and the remainder of the neurohypophysis, or if selective pharmacodynamic blocking of these neural pathways, were effective in preventing the ACTH response of the posterior pituitary lobe to neurotropic stress, then it might well be argued that the stimulus to the release was purely neural.

The problem of the source of the posterior pituitary lobe's ACTH has also puzzled us often. We had shown previously that post-mortem diffusion of ACTH from the anterior lobe to the posterior lobe could be ruled out (109); and in the present investigations, we were unable to detect, by our methods, any corticotropic activity in freshly extracted rat hypothalamic tissue. Mialhe-Voloss (107) was also unable to detect any adrenal ascorbic acid depletion activity in the hypothalamic tissue of cattle. There remain the possibilities that the posterior lobe is capable of synthesizing its own ACTH, or that the anterior lobe ACTH may be transported to the posterior lobe by undefined vascular routes. However no anterior pituitary-like cells can be detected in the posterior lobe so

that the possibility of ACTH originating in the posterior lobe seems improbable. The transport of anterior lobe ACTH through vascular channels to the posterior lobe where it is stored should be considered seriously. Landsmeer (111) has demonstrated, in the rat, fairly large and numerous anastomoses crossing the hypophyseal cleft between the anterior lobe and the posterior lobe, although he concluded that the blood flow in these anastomoses was directed towards the anterior lobe. Nonetheless it should be remembered that the posterior lobe of the pituitary has an extensive blood supply, and may well serve as a depot for transported material (54, 111,126,177). For example, it is known that thyroxine and triiodothyronine are concentrated in this gland (201).

The degree and the duration of the changes in the anterior pituitary ACTH brought about by the stresses employed here would seem to indicate that the gland, when stimulated to release ACTH, would respond in an all-or-none fashion. However, in terms of rates of release and of synthesis of the ACTH, little may be deduced from our results. Assuming a steady state in the pituitary gland of the non-stressed animal, with equal rates of synthesis and release, stress causes possibly a shift in the direction of acceleration of release with possible retardation or stoppage of synthesis. A return towards normal or above-normal levels could indicate an acceleration of synthesis and storage, with a decrease in the rate of release. Fortier (76) has discussed the possible relationships between these two rates, but as yet, no satisfactory data has been presented on this important problem. It is evident, moreover, that

elucidation of these questions must await sufficient knowledge on the cellular (or follicular) site of synthesis and secretion of the corticotropic hormone.

Kitay and colleagues (191) recently presented evidence that the blood level of corticoids may play an effective role in the regulation of pituitary ACTH and release, as Sayers had suggested. These investigators confirmed previous reports (70,76) that adrenalectomy was followed by a significant rise in pituitary ACTH content associated with pituitary hypertrophy, and noted that an increased blood level of corticoids, produced by cortisone administration, was followed by depletion of pituitary ACTH and adrenal atrophy, effects previously demonstrated in the dog by Farrell and Laqueur (202). Kitay, Holub and Jailer (191) suggested that the increased steroid level therefore produced a decrease both in ACTH synthesis and secretion. Fortier (76) reached much the same conclusions. On the other hand, our results would indicate that significant release of ACTH may occur even when presumably there is a high blood level of endogenous or exogenous corticoids. Guillemin (203) has recently shown a significant increase in the blood corticoids of the rat 15 minutes after the injection of ACTH or after the application of stress. Presumably therefore, one half hour after the beginning of sound stress, there was a high level of blood corticoids, due to the ACTH released from the posterior pituitary lobe (Tables 5 and 6). There was however, at this time, a significant release of the anterior pituitary ACTH (Figures 1 and 2). The same comments may be made of the continued decline in pituitary ACTH when a fairly large dose of CRF was

injected in the animals (Table 13). Moreover, as seen in Table 12, there was a decline in the pituitary ACTH content when the CRF was injected into animals previously treated with exogenous corticoid (hydrocortisone) at doses designed to block the pituitary-hypothalamus axis (4,24). Significantly also, it should be remembered that Kitay and his colleagues (191) and other investigators (76,202) made their measurements on animals injected with extremely large doses of corticoid, and for long periods of time. In summation, these results would suggest that a physiologically elevated level of blood corticoids does not block the release of ACTH from the anterior pituitary, possibly triggered by a neurohormone. However, abnormally high levels of corticoids, maintained over extended periods, seem to inhibit the pituitary synthesis and/or secretion of ACTH. To test this hypothesis, experiments should be conducted in which the effects of stress, both neurotropic and systemic, and of CRF injections would be studied in animals maintained for varying lengths of time at both physiologically and abnormally high blood levels of corticoids.

C. In vivo experiments with CRF and vasopressin.

The in vivo trials of CRF and vasopressin were carried out in a relatively non-specific system, viz. intact and untreated animals in all cases but one where hydrocrotisone injected animals were used. Intact and untreated animals respond to many stress stimuli and because of this sensitivity to stress, care was taken to exclude as many stressful factors as possible. The animals were well trained to accept intraperitoneal

injection and handling before the experiments, they were deeply anesthetized before intravenous injections, the control animals were treated identically to experimental animals and injected with the diluents and the time factors were scrupulously adhered to in all cases.

The injection of CRF, at very low doses, brought about a significant depletion of anterior pituitary ACTH, whereas vasopressin, at equipressor or equal weight doses to the CRF, did not cause any or very little, statistically never significant, changes in the ACTH content of the pituitary lobes. These results may be added to the growing body of evidence which would reject vasopressin as the neurohormone responsible for the release of ACTH. Synthetic arginine vasopressin did not provoke any changes either in the pituitary lobes ACTH content, or in the adrenal ascorbic acid. The doses were small, but were comparable, considering the degree of purity, to the doses of CRF. If vasopressin were an ACTH-releasing neurohormone, it should have shown an activity equal to or greater than the CRF. However, the lack of *in vivo* activity may have been due to the use of bovine, i.e. arginine vasopressin: the nature of rat vasopressin has not yet been demonstrated. However, the arginine vasopressin assayed *in vitro* caused a significant release of ACTH from the incubated pituitary tissue (204). Possibly the *in vitro* system is much more sensitive than the *in vivo* test. In this respect, a significant release of ACTH from the *in vitro* pituitary has been reported with a dose of CRF as low as 0.001 mcg., whereas *in vivo* 0.05 mcg. total dose did cause an ACTH release but not a statistically significant one. Intravenously 1 mcg.

of synthetic arginine vasopressin caused the rapid death of the animals; this was most probably due to the rapidity of injection of the pressor activity. Pitressin, on the other hand, showed consistent but slight ACTH releasing activity and considerable adrenal ascorbic acid depleting activity in the intact animals; it did not produce any changes in the hydrocortisone-treated animals. It has been claimed that the in vitro ACTH releasing activity of Pitressin is due to the presence of a contaminant (160); and recently, Pitressin was shown to possess adrenal ascorbic acid depleting activity in hypophysectomized rats (205).

On the other hand, every injection of CRF, intraperitoneal or intravenous, produced a fall in the pituitary ACTH content, though in a few cases the depletions were not statistically significant. In the experiment where the CRF was injected into hydrocortisone-treated rats, a relatively large dose of CRF did produce a fall in anterior pituitary ACTH. The neurohormone overcame the "corticoid block" whose effectiveness was shown by the failure of the surgical stress inherent to the experiment to cause any release of ACTH from the pituitary; the same surgical procedures, necessary to reach the femoral vein, produced in the intact animal an extensive depletion of the anterior pituitary ACTH. The degree of the falls of the ACTH produced by the injections of the CRF were similar to those produced by the application of stress stimuli (Figure 7). Similarly, the depletion of the adrenal ascorbic acid resembled the one produced by stress (Figure 8).

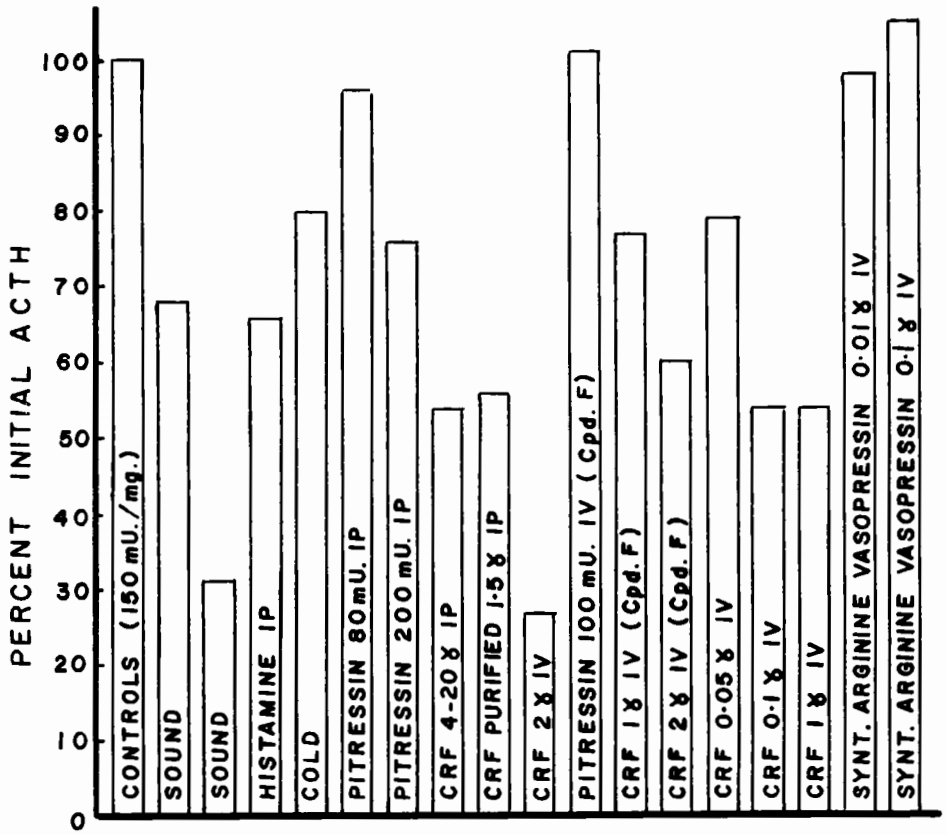

Figure 7: Percentage falls in anterior pituitary ACTH one hour after the application of various stimuli.

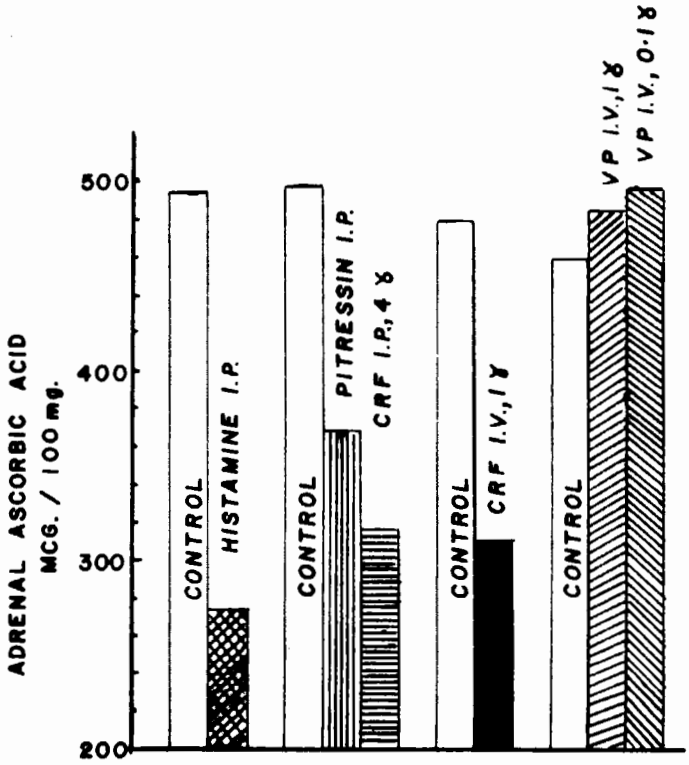

Figure 8: Depletion of adrenal ascorbic acid one hour after the application of various stimuli.

Because of the poor specificity of the test system, the in vivo effects of CRF may, of course, be construed as being due to the injection of a highly concentrated stress agent. However, the very small weight doses used, the degree and the duration of the effects (comparing for example the CRF time curve against the histamine curve in Tables 7 and 13), the activity in hydrocortisone-treated animals irresponsive to trauma would argue for a specific neurohumoral ACTH releasing factor. Moreover, if the activity of pure ACTH be assumed at 100 mU. per mcg., then the intravenous injection of 0.1 mcg. of this preparation of CRF caused a release of 4 mcg. of ACTH per 5 mg. anterior pituitary gland, or 40 times its own weight. Further in vivo experiments in specific systems are urgently needed to clarify the physiological activity of CRF in homeostatic responses.

SUMMARY

1. Acid-acetone and acetic acid extracts of rat pituitary tissues were of the same ACTH potency in the Saffran and Schally in vitro bio-assay.

2. In vitro bio-assays of acetic acid extracts of rat anterior pituitary tissue yielded values for ACTH that were about 10 times greater than values obtained by the Sayers adrenal ascorbic acid depletion assay. Smaller differences were obtained for extracts of beef and hog anterior pituitary tissue. The reasons for the discrepancy are not known.

3. Sound (neurotropic stress) produced a primary fall in the rat posterior pituitary ACTH content and a secondary depletion in the anterior pituitary ACTH.

4. Histamine (systemic stress) did not cause any significant alteration in the posterior pituitary ACTH content but produced an immediate and extensive fall in the anterior pituitary ACTH stores. The depletion of adrenal ascorbic acid was similar in degree to the changes in the pituitary ACTH.

5. Cold (mixed stress) caused a slow and gradual fall in the ACTH content of both lobes of the pituitary gland.

6. Intraperitoneal injections of low doses of a Corticotropin-Releasing Factor (CRF), but not of equipressor doses of Pitressin, brought about significant falls in anterior pituitary ACTH content. Significant

depletions of adrenal ascorbic acid were obtained after the injections of both CRF and Pitressin.

7. Significant decreases in anterior pituitary ACTH were obtained after intravenous injections of low doses of CRF in intact rats and of higher doses in hydrocortisone treated rats. Decreased levels of anterior pituitary ACTH and adrenal ascorbic acid were obtained from 30 minutes to two hours after intravenous injection of CRF.

8. Small doses of synthetic arginine vasopressin, injected intravenously, did not cause any changes in pituitary ACTH content or in adrenal ascorbic acid concentration.

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CLAIMS FOR ORIGINAL RESEARCH OR CONTRIBUTIONS TO KNOWLEDGE

1. Acetic acid extracts of rat pituitary tissue were shown to have the same corticotropic potency as acid -acetone extracts in the *in vitro* bio-assay. The *in vitro* potencies of acetic acid extracts were found to be *ten times* those obtained by the adrenal ascorbic acid method on the same extracts.

2. Sound (neutropic stress) produced a primary fall in the ACTH content of rat posterior lobes and a secondary depletion in anterior pituitary ACTH. Histamine (systemic stress) caused a depletion only in the anterior pituitary ACTH stores. Cold (mixed stress) slowly depleted the ACTH content of both pituitary lobes.

3. The Corticotropin-Releasing Factor (CRF), administered intraperitoneally or intravenously in minute doses to trained intact rats, caused significant depletions in anterior pituitary ACTH. The effect was also observed in hydrocortisone-treated animals. Vasopressin, at equipressor or same weight doses to the CRF, did not produce any significant falls in pituitary ACTH.