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The posterior pituitary and ACTH release

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THE POSTERIOR PITUITARY AND ACTH RELEASE

BUFORD LEE NICHOLS, JR.

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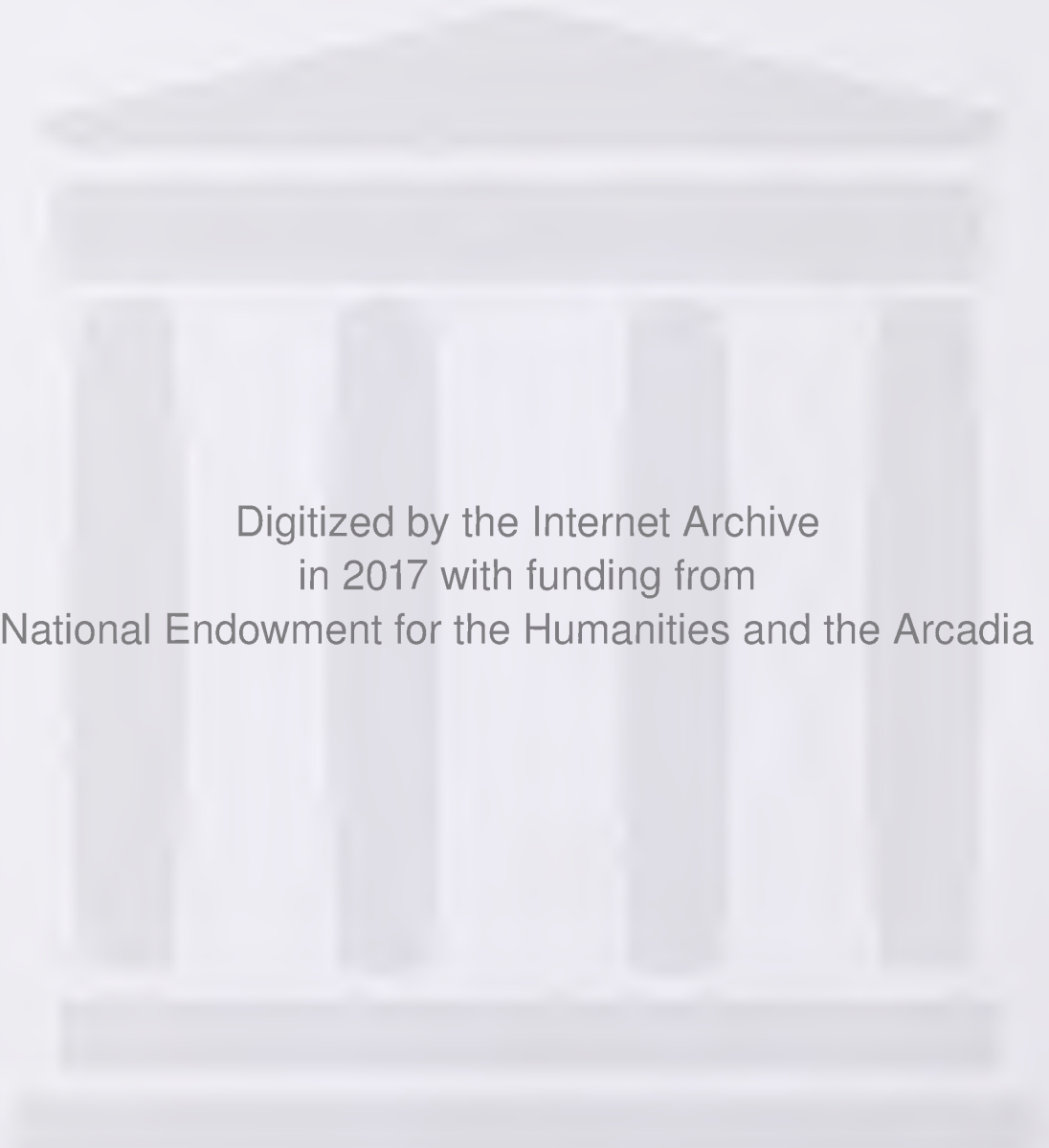

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THE POSTERIOR PITUITARY AND ACTH RELEASE

A DISSERTATION

PRESENTED TO

THE FACULTY OF THE SCHOOL OF MEDICINE

YALE UNIVERSITY

IN PARTIAL FULFILLMENT

OF THE REQUIREMENTS FOR THE DEGREE

DOCTOR OF MEDICINE

BY

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I am grateful to my wife for her abiding confidence in my work and her active participation in the preparation and typing of the thesis.

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PART I

Introduction

Historical

The historical origins of adrenal endocrinology date from the year 1564 when Eustachius while professor at the Collegia della Sapienza in Rome first described the presence of Glandulae renibus incumbentes.[1] The functional significance of these glands situated upon the kidneys lay dormant for nearly 300 years until Thomas Addison, one of the brilliant clinicians of Guy's Hospital, London, read a paper before the South London Medical Society in which he described melasma suprarenale, a disease of the adrenal glands. This was soon followed by a monograph: On the Constitutional and Local Effects of Disease of the Suprarenal Capsules.[2] Garrison, the medical historian, recognizes this book, regarded as a scientific curiosity at its time, as the inauguration of the study of diseases of the ductless glands, on the

level of the physiologic work of Claude Bernard.[3]

In the following year Brown-Sequard opened the doors to experimental endocrinology when he excised the suprarenal capsules of dogs. Since that time the failure of adrenalectomized animals to survive has been noted by many workers who have refined these observations. Ingle has tabulated these observations in a summary published in 1944 which follows on page 3.

Early efforts to study the controlling factors acting upon the adrenal cortex are those of Herring and of Hammett. These investigators found a fifteen to thirty-five per cent regression of the adrenal fifty days following thyroidectomy of rats.[5,6] In 1926 Smith demonstrated that experimental hypophysectomy caused profound atrophy of the adrenal cortex. This regression was marked and rapid with the adrenals losing half their weight in six days. This classic work was accompanied by the counterproof of the role of the pituitary when the adrenals of the hypophysectomized animals were maintained by daily transplantation of fresh pituitaries.[7,8] The contribution by Smith was followed after six years

EFFECTS OF ADRENAL REMOVAL[4]

Digestive

1. Loss of appetite.
2. Delayed or incomplete absorption from intestines.
3. Nausea; vomiting. Melena; ulceration of stomach, intestines.
4. Diarrhea.

Circulatory

Physical

1. Hemoconcentration - increased red blood cell count, volume; increased hemoglobin.
2. Decreased blood pressure.
3. Decreased blood flow.
4. Decreased blood volume.

Chemical

1. Decreased Na, Cl, bicarbonate, glucose in serum.
2. Increased K and nonprotein nitrogen.

Tissue

Physical

1. Muscular asthenia.
2. Reduction in muscle mass.

Chemical

1. Decreased Na in muscle cells.
2. Increased K and water in muscle cells.
3. Decreased glycogen in liver and muscles after fasting.

Renal

1. Increased excretion of Na, Cl, and bicarbonate.
2. Decreased excretion of K and total N.
3. Inability to excrete ingested water.

Growth

1. Hypertrophy of thymus and lymphoid structures.
2. Cessation of body growth.
3. Loss of weight.
4. Fall in body temperature.

Resistance

1. Decreased to all forms stress, toxins, infections, trauma, environmental changes.
2. Death in untreated animals.

by the purification of The Adrenotrophic Hormone of the Anterior Pituitary by Collip, Anderson and Thomson.[9] This established the role of the pituitary as the controlling gland regulating the adrenal cortex.

The control of corticotrophin release from the anterior pituitary was first studied by Selye who was actively engaged in research at the laboratory of Collip at the time of the early work on corticotrophin. This investigator tells of his joy at discovering that rat placenta when homogenized and injected into rats produced involution of the thymus and other signs of ACTH release. His feeling of discovery was soon crushed by the realization that such a reaction was completely nonspecific. Many substances including formalin produced the same effects. This early disappointment led to his interest in such nonspecific phenomena mediated via the anterior pituitary and resulted in the massive contributions to adrenal physiology from his laboratory in the past twenty-five years. This led to his historic hypothesis that the secretion of ACTH is the response evolved by the production of a peripheral catabolite in response to trauma or "stress".[10]

Many of the stresses used by Selye were "neurogenic" stresses, that is stresses acting through

the central nervous system. These nonspecific stimuli to ACTH release consisted of pain, intense sound, strong light, fear and rage. In 1944 Vogt observed that adrenal cortical hormone levels rise following the intravenous infusion of epinephrine in the dog or cat.[11] This discovery led to the further observation by Long and Fry that hypophysectomy prevents the appearance of the signs of cortical hormone release (depletion of ascorbic acid and cholesterol from the adrenals) which follows epinephrine injection.[12] Vogt later demonstrated that the isolated adrenal was not stimulated by epinephrine.[13] These facts indicated that epinephrine acts through the pituitary to produce increased secretion of cortical hormone.

In 1947 Long argued that since stresses of various types are known to result in the secretion of both epinephrine and ACTH, and since epinephrine is known to stimulate the release of ACTH it was reasonable to propose that epinephrine is part of the physiologic mechanism regulating ACTH release in response to stress.[14] This argument was strongly supported by the observation from his laboratory

that mild stresses were ineffective in the stimulation of ACTH liberation in the absence of epinephrine secretion.[15,16]

This concept of the control of the pituitary release of ACTH has come to be known as the Long and Vogt theory. Further research has limited the acceptance of this theory. Sayers demonstrated that sympathectomized animals resist stresses much more efficiently than do adrenalectomized animals.[17] Colfer, de Groot and Harris found that rabbits responded to stress after adrenal denervation by the normal release of ACTH as measured by changes of the white cell count in peripheral blood.[18] Adrenal demedulation was found ineffective in blocking ACTH release to emotional stress in work by Vogt.[19] This investigator also showed that plasma epinephrine was undetectable in adrenal-demedulated rats which responded to stress and that plasma levels of detectable quantities of exogenous epinephrine were ineffective in inducing ACTH release.[20] The demonstration by Guillemin that Dibenamine, a drug which blocks the action of epinephrine, does not block the release of ACTH to stress argues against the homeostatic

significance of this pharmacologic agent.[21] The added observation that epinephrine is inactive in stimulating the in vitro pituitary to release ACTH suggests that its activity in the intact animal must be at a site within the nervous system.[22] Epinephrine, therefore, may play a supporting role in the release of ACTH as a modulator of the response to stress by the nervous system which results in the ACTH stress response but it in itself is not the physiologic mediator of this response. Harris has commented that "It appears to be a system whereby an already increased secretion of ACTH, due to stress stimuli acting through the central nervous system, is reinforced."[23]

One of the classic relationships between endocrine glands has been the "push-pull" relationship which exists between the ovary and the anterior pituitary. The administration of estrogen inhibits the release of gonadotrophins while castration results in increased gonadotrophic excretion. As early as 1937 Ingle and Kendall had noted the atrophy of adrenal cortex following the administration of cortical extract. Subsequent studies showed that the administration of adrenal cortical extracts blocked

ACTH release.[24] These facts were used by Sayers to advance the view that ACTH secretion is regulated by the blood level of adrenal cortical hormone.[17]

Several experimental situations revealed that decreased blood levels of adrenal corticoids are not sufficient explanation for ACTH stimulation. Cheng et al. found that pituitaries transplanted to regions remote to the original site of the pituitary do not maintain adrenal weight.[25] Bush, Eik-Nes and Samuels found that dilution of the carotid blood by intra-arterial infusion of warm saline resulted in a fall in ACTH discharge rather than a rise.[26] More recently Sydnor and Sayers have observed that ACTH levels in the plasma of adrenalectomized rats may be raised by stress.[27]

Additional objections can be voiced to the physiologic significance of Sayer's theory on the basis of timing. Gray and Munson found that the injection of histamine produces ACTH discharge within three to ten seconds.[28] This interval is by far too brief for a change in plasma corticoids to induce ACTH release. Another observation is that intraperitoneal administration of hydrocortisone

although blocking ACTH release for approximately twenty-four hours is ineffective after that time although still present within the peritoneal cavity.[29] Moreover, Brodish noted that the pituitary would not respond to the removal of adrenal secretions for a period of two weeks following adrenalectomy.[30]

There is no question that cortical hormones influence the secretion of ACTH in a "push-pull" relationship; however, these relations form neither the main stimulus to the secretion of corticotrophic hormone nor the stimulus to changes in the rate of its release in conditions of stress. It is likely that this mechanism normally acts to set a more constant baseline of secretion against the background of which other factors modulate pituitary activity according to the needs of the organism.

Evidence for Neural Control of ACTH Release

What other possibilities exist for regulating the release of corticotrophin? The many observations of a nervous component to the stress response of the adrenal cortex suggest that the pituitary may be under direct nervous control. Indeed nerve fibers

have been described innervating the anterior lobe.[31] These fibers have been observed to atrophy after section of the cervical sympathetics, pituitary stalk and hypothalamic lesions.[32]

The school of Harris has repeatedly pointed out that the observation of neural fibers in the anterior lobe of the pituitary may be a staining artifact. It is reported by Harris that electron microscopy has failed to find such fibers.[23,33] Sayers has critically observed that the presence or absence of these fibers is yet far from proved.[34]

If for lack of evidence one disregards the direct neural supply of the anterior lobe as being a link in the response to environmental stress, we can conclude as has Long: "Therefore the mechanisms of secretion must be attributed to humoral factors that reach the gland through its blood supply."[35]

The discovery and description of a portal system connecting the adjacent nervous tissue with the anterior pituitary provides a link by which a humoral mediator could pass from nervous to glandular tissue. First studied by Popa at the suggestion of Professor Rainer of Bucharest these observations lay

unpublished until 1930.[11] He reported that the prominent vessels of the pituitary stalk were in reality portal vessels transporting from the pituitary to the brain.[36] Several years later Wislocki confirmed these observations and suggested that the direction of blood flow was from the infundibular stalk and median eminence of the brain to the anterior lobe of the pituitary.[37]

In 1950 de Groot and Harris suggested that some neural mechanism in the hypothalamus, the small area of brain to which the pituitary is attached, is largely responsible for maintaining and regulating the secretion of ACTH by the anterior pituitary. They postulated that hypothalamic nerve fibers liberate some chemical transmitter into the hypophyseal portal vessels which is carried to the anterior lobe to exert a specific influence over the activity of the gland.[38]

In general the evidence for the neural control of ACTH is drawn from the earlier observations of the rapidity of ACTH release following stress and the response to emotional and sensory stress both specific and conditioned. More definitive evidence

is offered by a number of experimental situations which seek to remove the neural influence by separating the gland by stalk section, transplantation or lesion of the adjacent neural tissue.

Section of the pituitary stalk has yielded equivocal evidence for the nervous control of ACTH release. The reason for these confused results has been attributed to a revascularization of the pituitary stalk after sectioning. de Groot reported that in mice he was able to correlate the revascularization with the return of ACTH release.[39] Attempts to prevent revascularization by the insertion of a barrier into the incision of the stalk allowed Fortier, Harris and McDonald to prevent the release of ACTH to cold and restraint but not to tissue trauma or adrenaline. Adrenal atrophy was observed under these experimental circumstances.[40]

Attempts at pituitary transplantation usually result in involution and fibrosis of the explant. Fortier found that these transplanted pituitaries failed to release ACTH in response to "neurotropic" stresses such as immobilization and intense sound or to maintain adrenal weight. He

did observe that these displaced organs removed from their hypothalamic connections would respond to "systemic" stresses such as unilateral adrenalectomy and histamine, histamine alone,and adrenaline and cold.[41]

Hypothalamic lesions have been found to block the ACTH release which follows a wide variety of stresses. This information has been summarized in Table 1 adapted from Fortier.[42]

The generalizations which can be drawn from these observations are more functional than anatomic for as can be seen in Table 1 there is much disagreement as to the anatomical site. The functional significance of the lesions lies in the proof of a relationship between neural and glandular elements of the mechanism of ACTH release. This, however, is tarnished by the failure of hypothalamic lesions to produce adrenal atrophy. Several groups have found that these adrenals will undergo atrophy when the animal received chronic treatment with hydrocortisone.[44,45] Other investigators have reported that hypothalamic lesions fail to block ACTH secretion in response to severe stress.[46,47,48,45]

Pharmacologic blockade of ACTH release has been

TABLE 1

HYPOTHALAMIC LESIONS BLOCKING ACTH RELEASE

Animal	Site of Lesion	Stress
Rat	Median eminence, posterior hypothalamus	Adrenalectomy, adrenalin, ether anesthesia
Rabbit	Zona tuberalis, mammillary body, posterior tuber cinereum	Immobilization
Monkey	Median eminence, posterior tuber cinereum, mammillary bodies	Adrenalin, formalin, trauma
Dog	Anterior median eminence, posterior tuber cinereum, mammillary bodies	Adrenalin, insulin, trauma, immobilization
Cat	Median and anterior eminence, posterior tuber cinereum, mammillary bodies	Adrenalin, trauma, formalin, histamine, hypertonic saline
Guinea pig	Nucleus ventromedialis	Diphtheria toxin[43]

reported by Briggs and Munson. This experimental procedure was found effective in preventing the response of the anterior pituitary to stimuli such as adrenaline, histamine and unilateral adrenalectomy.[49] Overdoses of adrenal cortical hormone produce blockade of ACTH stimulating procedures.[50] This treatment has been found effective in the blockade of the ACTH release to unilateral adrenalectomy. Plasma ACTH levels in ether anesthetized rats return to resting levels thirty minutes after induction. This preparation did not respond to stressful stimuli.[51] As the site at which the pharmacologic blockade occurs is unknown these approaches offer only inferential evidence to the neural control of the pituitary.

Electrical stimulation of the hypothalamus offers more direct evidence for a neural mechanism of ACTH release. This information is summarized in Table 2.

<u>In vitro</u> isolation of the anterior pituitary greatly simplifies the search for factors controlling ACTH release. Guillemin and Rosenberg found that the anterior pituitary of the rat was able to secrete

TABLE 2

STIMULATION PRODUCING ACTH RELEASE[42]

Animal	Site of Stimulation
Rabbit	Posterior tuber cinereum, mammillary body
Dog	Anterior median eminence, posterior tuber cinereum
Cat	Posterior tuber cinereum, mammillary body, anterior median eminence
Rat	Posterior hypothalamus

ACTH for prolonged periods only in the presence of hypothalamic tissue.[52] Saffran and Schally have used a simple pituitary incubation method to demonstrate that extracts of hypothalamic tissue are capable of stimulating ACTH release.[53] This method, however, is subject to criticism for tissues and extracts of other origins are capable of stimulating the production of ACTH. Barrett and Sayers have suggested that these extracts may act by inhibiting *in vitro* degradation of ACTH.[54] Although subsequent studies have disproved this particular argument, the significance of the *in vitro* results in the words of Guillemin, "will have to be assessed in some dynamic fashion *in vivo* lest they remain of purely esthetic value."[56]

Possible Neurohumoral Mediators

Zuckerman, the iconoclastic critic of the significance of the hypophyseal portal system, has correctly argued that the proof of a direct neuroglandular link must lie in the demonstration of the presumed chemical mediator.[57] Of early consideration were the known neurohumors, acetyl choline, adrenaline and histamine. The fact that the successful blockade of these stressors by pharmacologic antagonists

does not inhibit the release of ACTH speaks against their role as chemical mediators.[21] Recently Casentini *et al.* have suggested that acetyl choline might act *via* release of posterior pituitary anti-diuretic hormone.[58]

The presence of a humoral agent traversing the portal system is supported by the isolation of active plasma fractions from the portal vessels of dogs[50] and the venous return from the brain of hypophysectomized rats.[59] These fractions when injected into rats provoked ascorbic acid deletion and eosinopenia respectively.

Several attempts to isolate a more specific agent brought early encouragement to those investigating these neurohumoral mechanisms. Slusher and Roberts reported the isolation of a lipid concentrate from the posterior hypothalamus which depleted adrenal ascorbic acid in normal rats.[60] This lipid extract has received further study in the laboratory of DeWied who found the substance active in intact rats but not in those with hypothalamic lesions blocking the ACTH response to stress.[61]

Using the *in vitro* technique described

earlier Saffran *et al*. and Guillemin *et al*. were able to isolate an activity from hypothalamic and posterior pituitary tissue which appears specific for the release of ACTH. This active principle termed corticotrophin releasing factor (CRF) has been demonstrated to be a peptide distinct from vasopressin or oxytocin although probably related to them by amino acid composition.[62] Small doses of this material, inactive in hypophysectomized animals, stimulate the *in vivo* adrenal in animals where the stress response has been inhibited: the hypothalamic lesioned rat,[63] the nembutal-morphine blocked rat[63] and the steroid blocked rat.[64] Although Saffran considers the fact that CRF is found in the posterior pituitary of physiologic significance, Guillemin, who found more activity in hypothalamic tissue, considers the isolation of CRF from posterior pituitary extracts factitious and probably the result of contamination with median eminence tissue.[65,64]

PART II

Survey of the Literature

Historical

Anatomical knowledge of the pituitary gland dates from the Classical Period. Rufus of Ephesium (c.100 A.D.) was credited by Vesalius with the discovery of the gland.[66] Subsequent anatomists contributed little to the description or significance of this organ after Galen (130 - 200 A.D.) named it the pituitary. This organ, he taught, acted as the filter through which impurities (pituita or phlegm) were discharged by the brain. This early role of the gland is prophetic of the humoral significance it has today. It is also symbolic of the Galenic physiology that lasted fifteen centuries. In 1778 when Soemmerring issued his inaugural dissertation, De Basil Encephali et Originibus Nervosum Caranio Egredientium, he could list seventeen names for the pituitary gland but could only quote Galenic doctrines concerning its function.[67]

The discovery of the anatomic dichotomy of the pituitary gland was published in 1724 by Santorini (1681-1737).[68] This cleavage of the gland was subsequently explained by Rathke (1838) who demonstrated an epithelial origin for the anterior lobe and neural origin for the posterior lobe.[69]

The first experimentally demonstrated function of the pituitary gland was discovered by Oliver and Schäfer (1894) who found that extract of this gland possessed a hypertensive activity.[70] Four years later Howell was able to prove that this activity was a property of the posterior pituitary.[71] This established the physiologic independence of the lobes of the hypophysis.

From the teleologic view this divorce of neural and glandular elements so closely proximated anatomically was far from satisfying. In view of the poor innervation of the epithelial hypophysis Celestino da Costa, the Portuguese histologist, in 1942 wrote: "One has to admit that the neural control of this organ is effected by intermediaries which accumulate in the posterior lobe and pass through the circulation to the anterior lobe."

In his view the physiologic relation of the two hypophyseal lobes is one of the most exciting questions in the study of the pituitary.[72]

The possibility that the posterior lobe of the pituitary may provide the neuroglandular link for activation of the anterior lobe was entertained by Hensey and Markee as early as 1933 when they postulated that "the pathways from the hypothalamus must activate the posterior lobe of the hypophysis which in turn may exert an influence on the anterior lobe by hormonal transmission."[73] This relationship has become of more than theoretical interest since vascular links between the anterior and posterior glands have been demonstrated in the rat,[74] dog,[75] rabbit, cat, guinea pig[76] and man.[77] The functional significance of these intralobar vessels has been demonstrated by their ability to maintain the integrity of a significant volume of anterior pituitary following infarction of the stalk of the anterior lobe in the rat, sheep, goat, baboon, rhesus monkey and man.[78]

At the present time evidence has been advanced to demonstrate a role of the neurohypophysis

in the control of leuteotrophic,[79] thyrotrophic,[80] somatotrophic[81] and adrenocorticotrophic hormone release. The evidence that ACTH may be regulated by the neurohypophysis lies along three lines: first that the administration of exogenous vasopressin results in ACTH discharge; second that ADH and ACTH are released or inhibited simultaneously; and third that neurohypophysectomy blocks the release of ACTH.

Stimulation of ACTH Release by Exogenous ADH

The repeatedly observed effect of exogenous vasopressin upon adrenal activation is the best documented of the arguments for its role in ACTH release. The effect of posterior lobe extracts upon the adrenal cortex was first noted by opotherapists. Baduel as early as 1908 noted adrenal cortical proliferation resulting from administration of succo de lobo posteriore ipofisurie.[82] In the following year Delille published results of experiments often said to be the first to demonstrate the corticotrophic effect of the pituitary gland. It is not generally appreciated that he attributed

this activity to the pars nervalis. He demonstrated that the administration of either whole extract or posterior pituitary extract doubled adrenal weight, caused hyperplasia of adrenal cortex and pituitary basophilia[83] (confirmed by Martini et al., 1956).[84]

The investigations of the opotherapist lay fallow for more than two decades until their studies were confirmed by several investigators seeking to find in the pressor activity of the posterior lobe a possible etiology of vascular disease. Moehling and Osius in 1931 and Hantschumann in 1937 noted the hypertrophy of the zona fasciculata of the suprarenal cortex in response to vasopressor fractions of the pars neuralis.[85,86] In 1935 Moszkowska noted the presence of corticotrophic activity of the pars neuralis when injected into normal rabbits. She attributed this to diffusion of colloid from the anterior lobe.[87]

In his attempts at ACTH purification Sayers noted in 1943 that posterior lobe contamination of ACTH produced an additive adrenal response in the normal animal. This effect was localized in the vasopressor fraction and was abolished by

hypophysectomy of the recipient animal.[88] Parks some eight years later also remarked about this property of the posterior lobe extracts.[89]

The first intensive investigation of this activity of vasopressin was published by Eser and Sipahiogla who demonstrated an eosinopenia when this material was injected into normal rats. They demonstrated that this phenomena was inhibited by hypophysectomy but not by unilateral adrenalectomy or adrenal demedulation. On the basis of the anatomical proximity of the pituitary lobes they postulated that vasopressin may be one mediator of ACTH stimulation.[90] In the same year Nagareda and Gaunt noted similar adrenal activation while studying the apparent reciprocal nature of ADH and ACTH at the renal level.[91]

In the following year Stutinsky and his collaborators demonstrated the posterior lobe activity in a number of commercial preparations and were able to show that the adrenal activating activity is parallel to the vasopressor activity thus concluding that the property must be due to vasopressin.[92]

At the present time there are some twenty four published experiments describing the adrenal

activation following injection of vasopressin in normal animals (Table 3). The indices of this activity have been varied: eosinopenia, anti-insulin effect, adrenal weight, adrenal histologic changes, adrenal content of cholesterol and ascorbic acid, P^{32} uptake by the adrenal, plasma and urinary steroids and plasma or pituitary ACTH. The preparations of vasopressin have ranged from posterior lobe extracts to synthetic (and therefore presumably pure) arginine and lysine vasopressin. In addition it has been shown that inactivation of the pressor activity parallels inactivation of ACTH releasing activity.[109] Although we can conclude from these studies that vasopressin stimulates ACTH release we must also discuss the specificity of this effect and its physiologic significance.

Because of the possibility that vasopressin may act as a nonspecific stressor preparations have been employed in which the ACTH response to various stresses has been inhibited or abolished (Table 4). The twenty-three published attempts to demonstrate a specificity of response have employed various preparations which include: the immature rat, the steroid block, the morphine-nembutal block,

Table 3

EFFECT OF VASOPRESSIN IN THE NORMAL ANIMAL[a,b]

Vasopressin	Dose U.	Animal	Index
Pitressin	7.5/15 days(SC)	Rat	Adrenal weight, lipid depletion[84]
Pituitrin	0.28-0.5(SC)*	Rat	Cytochemical changes in adrenal cortex[93, 92]
Pitressin	1.0-5.0(IP)	Rat	Adrenal cholesterol depletion[94]
Pitressin PNH	0.1-0.2(IP) 0.83-1.66(IP)	Mouse	Anti-insulin effect[88]
Pitressin Crude VP Purified VP Vasopressin A,B,C,(?) Pituitrin(DNZ) LVP	0.7-1.25(IV,SC,IP) 0.4 (SC) 0.4 (SC) 0.5 (SC) 0.4 (SC) 0.3 (IP)	Rat	Adrenal ascorbic acid depletion[49,61, 95, 96,97,92,98,99]
Saline extract post.pituitary Pitressin Highly purified	--- (IP) 3.0 (IP) 8.0 (IP)	Guinea pig	Urinary neutral reducing lipid[100]
Highly purified LVP	0.13-2.12(IC)	Dog	Plasma 17-OH corticoids[101]
Pitressin LVN-2 AVN-2 Synthetic LVP	2.0-5.5 (IV) 2.0 (IV) 2.0 (IV) 2.0 (IV)	Human	Plasma 17-OH corticoids[102,103, 104]
Pitressin	0.2 (IP)	Rat	P^{32} uptake by adrenals[105]

Pitressin	2140/100days (SC,IP)	Rabbit	Adrenal weight[85]
Tonephin		Rabbit	Adrenal weight[86]
Vasopressin (DNZ)	0.2-1.0%(SC)	Mouse	Cytochemical changes in adrenal cortex[106]
Post.Pit. extract		Rabbit	Adrenal weight[87]
Infundin	0.005*(SC)	Rat	Eosinopenia[90]
Postuitrin	0.1-2x0.25(SC) 0.01*(SC)		
Pitressin	0.08-0.1*(IP,IV)	Rat	Adrenal ascorbic acid depletion, pituitary ACTH content(invitro assay)[107]
Synthetic AVP	0.2 (IP) 0.04-0.4*(IV)		
Pitressin	0.04* (IC)	Rat	Plasma ACTH activity (adrenal ascorbic acid assay)[108]
Synthetic LVP	0.08-0.2(IC)% 0.2 (IC)%		

a. Symbols:

% - dose stated per 100 gm. body weight.
SC- subcutaneous injection.
IP- intraperitoneal injection.
IV- intravenous infusion or injection.
IC- intracarotid infusion or injection.
* - ineffective.
local - direct application to intraocular transplant.
perf. - perfusion per minute.
VP - vasopressin.
LVP - lysine vasopressin.
AVP - arginine vasopressin.
(?) - preparation of vasopressin not stated.
LS - lymph sac of frog.

b. Preparations of Vasopressin:

Pituitrin, Pitressin - trademarks of Parke-Davis.
Postipofisan - trademark of Richter.
AVN - 2,3,5 arginine vasopressin purified by du Vigneaud.
LVN - 2 lysine vasopressin purified by du Vigneaud.
PNH - Pars neuralis hormone prepared by Van Dyke.
Pituitrin(DNZ) - product of Dai-Nippon Zoki Institute.
Vasopressin(DNZ) - product of Dai-Nippon Zoki Institute.
Tonephin
Infundin
Postuitrin

Table 4

THE EFFECT OF VASOPRESSIN IN ANIMALS BLOCKED TO STRESS[a]

Vasopressin	Dose U.	Animal	Index
	Immature		
Pitressin PNH	35/3days(IP) 25/3days(IP)	Rat	Adrenal hypertrophy lipid depletion[88]
Pitressin	1-5 (IP)	Rat	Adrenal cholesterol depletion[88]
Pitressin PNH	35 (IP) 25 (IP)	Rat	Thymus involution body weight[88]
	Steroid Blockade		
Pitressin LVP Highly purified AVP Vasopressin(?)	0.2,0.3-2.4(IV) 1.25(IP) 0.012(IC) 0.3(IV) 0.25*(IP) 0.5-1.0(IP) 0.1	Rat	Adrenal ascorbic acid depletion[50,61, 51,96,109,99,111,112]
Pitressin	0.1*(IV)	Rat	Pituitary ACTH content(in vitro assay)[107]
Pitressin	0.2*(IP)		P^{32} uptake by adrenals[105]
	Prolonged Ether Anesthesia		
Pitressin Lyophilized Pitressin AVN-5	5.0(IV,IC) 5.0 (IV) 5.0 (IV)	Rat	Plasma ACTH activity (adrenal ascorbic acid assay)[51]

Morphine-Nembutal Block			
Pitressin Highly purified	0.07-0.2*,0.25(IV) 0.006-.04* 0.08 -.40	Rat	Adrenal ascorbic acid depletion, plasma corticosterone[49,61,113,111,63]
Chloropromazine Block			
Vasopressin(?)	0.1(IV)	Rat	Adrenal ascorbic acid depletion[114]
Reserpine Block			
Pitressin	0.3(IV)	Rat	Adrenal ascorbic acid depletion[115]
Dibenzyline Treated			
Vasopressin(?)	0.1	Rat	Adrenal ascorbic acid depletion[111]
Hypothalamic Lesion			
Pitressin	0.1,0.2,0.61(IV)	Rat	Adrenal ascorbic acid depletion[61,116,117,118]
Synthetic LVP	0.1(LS)	Frog	Molting[119]
Highly purified LVP	0.004-.032*(IV) 0.064-.128 (IV)	Rat	Plasma corticosterone[63]
Pituitary Transplant			
Pitressin Postipofisan	0.01-0.3(IP,local) 0.3(IP)	Rat	Adrenal ascorbic acid depletion, eosinopenia[120,99]
Decerebrate and Ether Anesthesia			
Pitressin	5.0*(IV)	Rat	Plasma ACTH (adrenal ascorbic acid assay)[121]

In Vitro			
Pitressin Synthetic LVP Synthetic AVP Highly purified LVP	0.0001 0.00067 0.001 0.0005	Rat	Saffran-Schally assay[110,122]
Pitressin AVN-2	1.0 1.0*	Rat	Tissue culture (adrenal ascorbic acid depletion)[52]
Pitressin	1.0-2.0	Rat	Pituitary incubation(adrenal ascorbic acid depletion)[112]
Neurohypophysectomized			
Pitressin	0.3(SC)	Rat	Adrenal ascorbic acid depletion[115]
Decapitate			
Pitressin	0.5-5(IV)	Rat	Plasma ACTH(adrenal ascorbic acid assay)[118]
Stalk Section			
Pitressin	1 2x3days	Dog	Blood ACTH, plasma 17-OH corticosteroids, Cytochemical changes in adrenal cortex[123]

a - see Table 3 for footnotes.

chloropromazine block, prolonged ether anesthesia, hypothalamic lesion, ocular pituitary transplants, decerebrate and ether anesthetized rats, neurohypophysectomy and pituitary stalk section. In each of these experimental situations the preparation has shown an inability to respond to one or more stressing procedures indicating some degree of isolation of the pituitary gland from its afferent stimuli. The demonstration that vasopressin is specifically active in these circumstances makes the conclusion that the material acts directly upon the pituitary seem justified. This conclusion is strengthened by the observation that synthetic lysine vasopressin has a corticotrophin releasing effect upon the pituitary <u>in vitro</u>.[110] It is noteworthy that the systemic doses necessary for adrenal activation are in general the same as those used in the normal animal.

The necessary confirmation of the site of vasopressin activation lies in the demonstration that the posterior lobe hormone has no direct effects upon the adrenal. This has been shown repeatedly to be the case when the indices of thymic involution, eosinopenia, adrenal weight and content of lipid, cholesterol or ascorbic acid,

Table 5

THE EFFECT OF VASOPRESSIN IN THE HYPOPHYSECTOMIZED ANIMAL[a]

Vasopressin	Dose U.	Animal	Index
Pitressin	1.6(IV)*	Rat	Adrenal ascorbic acid depletion (64% block)[50]
Pitressin	5 (IP)*	Rat	Adrenal ascorbic acid depletion (92-98% block)[116,117]
Pitressin	0.3(SC)* 0.2(IV)* 0.5(SC)*	Rat	Adrenal ascorbic acid depletion (100% block)[61,115,92]
Pitressin	0.2(SC)*%	Rat	Adrenal ascorbic acid depletion (100% block)[126]
Pitressin	0.3(IP)*	Rat	Eosinopenia (100% block)
Postipofisan	0.3(IP)*		Adrenal ascorbic acid depletion (99% block)[120]
Pitressin	7.5/15days (SC)*	Rat	Adrenal weight lipid depletion (100% block)[84]
Pitressin	5 (IP)*	Guinea pig	Urinary neutral reducing lipid (100% block)[100]
Pitressin AVN-5 Synthetic AVP	1-5(IV) 2.5-5(IV) 1-5(IV)	Rat	Adrenal ascorbic acid depletion[118]
Pitressin PNH	1x14days(IP)* 75x 3days(IP)* 833x14days(IP)*	Rat	Adrenal weight, lipid maintenance[88]

Pitressin PNH	1x14days(IP)* 75x 3days(IP)* 833x14days(IP)*	Rat	Adrenal cholesterol depletion[88]
Pitressin PNH	1 (IP)* 833 (IP)*	Rat	Thymic involution[88]
Pitressin Purified VP Synthetic VP	0.2-20(IV)	Dog	Adrenal vein 17-OH corticoids[124]
Synthetic VP	0.1-0.4(perf)	Dog	In situ perfusion of adrenal, 17-OH corticoids[125]
Pitressin	0.2*(IP)	Rat	P^{32} uptake of adrenals (64%block)[105]
AVP	0.3*(IV)	Rat	Plasma free corticosterone[152]
Infundin	2x0.02*	Rat	Eosinopenia[90]
Post.Pit. extract	300-600/13days	Rat	Adrenal hypertrophy[127]
Pitressin	1.0-4.0	Rat	Adrenal ascorbic acid depletion[112]

a - see Table 3 for footnotes.

and peripheral plasma or urinary steroids have been used.(Table 5). Using the most direct approach to the problem Nelson and Hume in 1957 and Hilton in 1959 studied the effect of vasopressin on adrenal venous effluent steroid content. Working with hypophysectomized dogs Nelson and Hume found that 0.2 to 20 units, when given intravenously stimulated corticogenesis.[124] Hilton using the isolated adrenal perfused by hypophysectomized donor blood found that the addition of as little as .00001 u/ml. to the perfusing blood stimulated corticogenesis. Although larger doses produced more marked stimulus this dose appeared to be near threshold in its effect.[125]

In order to compare this very interesting observation with other experimental results we can convert this dose to the equivalent dose which is injected systemically. Disregarding the half life of vasopressin in the blood we can calculate that in a 20 kg. dog (with a blood volume of 78ml./kg.) the equivalent dose would be 0.015 units administered intravenously. Of necessity the dose would be larger proportionately if it were to perfuse the adrenal with a constant level of 0.00001u./ml.

In our experiments in the intact dog this dose produced antidiuresis and doses less than 0.130u. were incapable of adrenal activation.[101] The observed absence of antidiuresis in the presence of increased corticogenesis[101] makes the physiologic significance of circulating vasopressin in the direct activation of the adrenal gland very remote.

In another experimental approach Sayers found that the administration of 0.5 to 5 units of pitressin to decapitate animals maintained by artificial respiration resulted in adrenal ascorbic acid depletion. This was confirmed in 24 hour hypophysectomized rats using 1 to 5 units of pitressin and purified or synthetic arginine vasopressin. His studies led him to conclude that the vasopressin did not act directly upon the adrenal but acted to release "bound" ACTH from its captive sites, principally in the kidney.[121] In view of Nelson and Hume and Hilton's observations of an unequivocal local effect on the adrenal this postulate seems unnecessary.

Nagureda and Gaunt were the first to question the physiologic significance of ACTH releasing effect of vasopressin when they studied the phenomena in 1951.

In their classic work they noted that the dose of pitressin necessary for adrenal activation was 20 to 80 times that reproducing physiologic antidiuresis (0.005 units).[95] Shannon and later Lausen studied the rate of ADH liberation in dog and man and found that the physiologic rate of secretion fell between 0.001 to 0.005 and 0.007 to 0.050 units respectively.[128,129] It is readily appreciated from Tables 3 and 4 that all of the doses administered systemically were in excess of the physiologic range. This forms one of the serious objections to the ADH theory of pituitary stimulation.

Although this argument against the significance of ADH in activating the adrenal may be partially on the basis of aesthetics, some attempts have been made to quantitate the threshold necessary to produce ACTH release. Arimura found that the threshold for pituitary activation as measured by plasma ACTH activity fell between 0.04 and 0.08 units in the rat.[108] In our work we have noted the threshold to be between 0.16 and 0.26 units in the dog. In both experiments the vasopressin was administered <u>via</u> the carotid artery. Using highly purified lysine vasopressin the experiments

with unanesthetized dogs we were able to calculate that a difference of 3.5×10^3 to 7.1×10^3 molecules/second existed between the dose reproducing physiologic antidiuresis and that producing ACTH release.[101] Knowing that the threshold for *in vitro* pituitary stimulation is approximately one thousandth of the *in vivo* threshold dose in the rat it can be argued that dilution may account for the necessarily high dose required to produce a sufficient concentration of vasopressin at the pituitary.[110,63,108,130] However by the same reasoning the endogenous ADH would have to reach this level to stimulate ACTH release. This would dictate a maximal antidiuresis each time ACTH is discharged. Such a parallel discharge of vasopressin was not observed experimentally in our unanesthetized hydrated dogs,[101] sensitive to as little as one or two milliunits of purified lysine vasopressin. In view of these observations and the enormous doses of exogenous vasopressin it can only be concluded that the physiologic significance of the ACTH releasing and adrenal stimulating potential of the posterior lobe hormone is still only hypothetical.

The Coincidence of ADH and ACTH Release

The second major tenet of those who have postulated ADH as the mediator of ACTH discharge by the pituitary is that the two hormones are released synchronously and inhibited synchronously. Rothballer appears to have been the first to advance this argument. This investigator based his hypothesis on morphologic evidence of depletion of granules (presumably ADH) from the posterior pituitary to stimuli evoking ACTH discharge. Attention was also directed to the similarity of stressors releasing the two hormones.[131]

Mirsky, Stein and Paulish published a series of experiments in which they observed rapid variations of the antidiuretic activity of peripheral blood following traumatic or emotional stresses. Working with the rat they noted a 120 per cent rise in antidiuretic activity 30 seconds after exposure to intense noise. A similar rise of activity was observed following faradization or intraperitoneal injection of histamine. The administration of cortisone or bilateral adrenalectomy produced changes in the level of antidiuretic substance

which paralleled the alterations in ACTH release. In addition they observed that changes in antidiuretic activity occur in hypophysectomized and adrenalectomized animals.[132] These experiments were interpreted as evidence that ADH may mediate ACTH release. The estimates of antidiuretic activity by Mirsky et al.have been subject to criticism by Van Dyke who found their assay method to be non-specific.[133] The observations of this group on the relationship between antidiuretic activity of plasma and ACTH release are therefore of uncertain significance.

In an attempt to investigate the possible relationship of the two hormones we simultaneously studied the plasma corticoid and antidiuretic responses of unanesthetized dogs to hypertonic saline injected into the carotid artery. With these more direct criteria for hormone release no correlation was demonstrable between antidiuresis and corticogenesis after endogenous ADH release. Although the antidiuretic response varied from moderate to maximal, elevation of plasma corticoids did not occur. Additional observations made of plasma corticoids during ADH inhibition by hydremia failed to show any parallel between the

parameters.[101] McDonald has noted similar disassociations in the human.[134]

In recent work Dingman and his coworkers were able to selectively stimulate the diencephalan of dogs producing either antidiuresis or ACTH release as indicated by increased corticoid levels in the adrenal effluent blood.[135]

George and Way observed that whereas morphine induced both ACTH and ADH release only less than one hundredth of the dose was required for stimulation of ADH discharge.[136] They found that the equivalent effect of the larger ACTH releasing morphine dose could be reproduced with aspirin without any discharge of ADH.[137]

These data appear to justify the conclusion that ADH and ACTH are independently released. The other side of the argument remains to be discussed. Are the two hormones blocked simultaneously? McCann and Brobeck created electrocoagulative lesions of the hypothalamus in rats.[116] These lesions which inhibited normal adrenal ascorbic acid depletion to stress interrupted in each case the supraoptic-hypophyseal tract which Fisher et al. had shown to be essential for the release of ADH.[138] The consequent diabetes insipidus in these rats failing to respond to stress

by the release of ACTH was taken as strong evidence that ACTH release is mediated by ADH. However, in the hypothalamic lesioned rat adrenal atrophy was not observed but rather adrenal hypertrophy which paralleled the severity of diabetes insipidus.[138]

Using plasma corticosterone levels instead of adrenal ascorbic acid depletion as an index of ACTH release we reinvestigated this apparent relationship. In a series of more than 600 rats minute hypothalamic lesions were produced in the area of the median eminence. This lesion inhibited the stress response of plasma corticoids in about one fourth of the animals. These animals also demonstrated diabetes insipidus in all but very few cases. However, when the 303 animals with diabetes insipidus are studied as a whole only half of these demonstrated a block of ACTH release. It is also noteworthy that in this series of animals demonstrating inability to release ACTH adrenal atrophy was observed.[91] The discrepancy between these observations and those of McCann may be due in part to the different indices of ACTH release; however, others using adrenal ascorbic acid depletion as the index of adrenal activation have also noted the lack of correlation between ADH

and ACTH blockade.[136,139,140,91] This discrepancy was also noted in humans with diabetes insipidus who demonstrated no impairment of the ACTH response to exercise.[141]

Observing the antidiuretic response in rats DeWied and Mirsky found that though prednesolone administration effectively inhibited the ACTH release following pain or the injection of histamine or nicotine the antidiuretic response to these stresses was intact.[96] In contradistinction, McCann et al. noted that hydrocortisone significantly although incompletely blocked the antidiuresis following stress.[44] However, the apparent discrepancy does not eclipse the major agreement that endogenous ADH can be released in the animal with effective steroid blockade of ACTH release.

The Effect of Neurohypophysectomy on the Stress Response

A direct approach to the relationship between vasopressin and ACTH release has followed the classic modes of endocrine study: ablation of the posterior lobe. The neurohypophysectomized animal has been found refractory to several stresses which produce ACTH

discharge in normal animals. The results of these studies are summarized in Table 6. Although the response to epinephrine and cold was uniformly inhibited by neurohypophysectomy the other stresses varied in their responses between laboratories. Nowell found bell ringing to be an effective stress in the neurohypophsectomized rat[115] whereas Smelik and DeWied found such "psychic stimuli" ineffective.[142,143] Fisher and De Salva have found that the removal of the posterior lobe blocked the response to epinephrine as indicated by ascorbic acid depletion but not as measured by plasma free corticosterone.[144] In each study the neurohypophysectomized animal developed diabetes insipidus which was used along with histologic criteria as index of successful ablation of the pars nervosa. In addition significant adrenal hypertrophy was noted by most investigators. Arimura attempted to define the factors contributing to the unresponsiveness of the neurohypophysectomized preparation. He discovered that the adrenal hypertrophy also occurred in rats with chronically implanted carotid cannulae and that these animals failed to respond to electrical shocking. The two preparations differed, however, in

Table 6

EFFECT OF NEUROHYPOPHYSECTOMY
ON RESPONSE TO STRESS

Effective Stress	Blocked Stress	Animal	Index of Response
Bell ringing Pitressin Nicotine	Cold Hypertonic saline	Rat	Adrenal ascorbic acid depletion[115]
	Epinephrine Electrical shocking	Rat	Adrenal ascorbic acid depletion[108]
Unilateral adrenalectomy	Cold(diminished) Bell ringing Epinephrine(IM,IV)	Rat	Adrenal ascorbic acid depletion[142]
	"Psychic stimuli" (diminished)	Rat	Plasma free corticosterone[143]
Epinephrine		Rat	Plasma free corticosterone
	Epinephrine		Adrenal ascorbic depletion[144]

the resting level of plasma ACTH which was high in the neurohypophysectomized animal but normal in the cannulated animal.[108] This observation raises the possibility that the inhibition of stress response may not be a specific response to neurohypophysectomy.

Inhibition of ACTH Release By Exogenous ADH

Although major emphasis has been given to the stimulatory effects of vasopressin on ACTH release the material also possesses an ability to inhibit ACTH discharge from the pituitary. The principal results of these experiments are summarized in Table 7. Doses of 0.02 units inhibit various stresses when given to rats and mice 30 minutes before the onset of the stress.[126,106] The mechanism of this inhibition has been extensively studied by Arimura. This investigator found that the timing of the premedication was critical with deviations from the 30 minutes before stress schedule failing to inhibit the stress response. Synthetic lysine vasopressin was found to be equally effective. With the dosage employed there was no change in the hematocrit or in the pituitary portal vessels when under direct observation. The phenomena

Table 7

THE INHIBITORY EFFECTS OF VASOPRESSIN UPON STRESS[a]

Vasopressin	Dose U.	Animal	Stress	Index
Vasopressin (DNZ)	0.02-1% (SC)	Mouse	Cold Epinephrine	Adrenal lipid depletion[106]
Pituitrin (DNZ)	0.02-.04% (SC)	Rat	Pain Cold	Adrenal ascorbic acid depletion[98]
Pitressin Synthetic LVP	0.02%(IV) 0.02%(IV)	Rat	Electrical shocking Epinephrine Laparotomy* Unilateral adrenal-ectomy* Immobiliza-tion*	Adrenal ascorbic acid depletion & blood ACTH(ascorbic acid depletion assay)
			Hypertonic saline(SC)	Eosinophil depletion[108]

a - see Table 3 for footnotes.

could not be the result of a refractory period following the administration of the premedication for small doses of epinephrine administered 30 minutes before stress did not inhibit the response. Inactivated lysine vasopressin was also without effect.[108]

The physiologic role of this phenomena is not apparent but greater significance should be attached to these observations for they occur with doses of vasopressin which as Kurokawa has noted approach the physiologic level.[106]

Itoh et al. found that hydrated rats were more responsive to stress and that dehydration reduced the adrenal response to the same stresses: epinephrine, histamine and cold. Under these conditions no change in adrenal sensitivity could be detected.[145] Arimura observed that dehydration of rats suppressed their response to electrical shocking and unilateral adrenalectomy. In his hands hydration seemed to also suppress ACTH release although producing hypertrophic adrenals.[108] These observations in general support the thesis of these investigators that ACTH may be inhibited by ADH.

The possibility that ADH release may be inhibited by the adrenal hormones has also received intensive

study. Gaunt, Lloyd and Chart found that hypothalamic antidiuretic activity was depleted by stress and increased by hydrocortisone administration. Parallel changes occurred in the posterior lobe antidiuretic activity except in the hydrocortisone treated animal where the material strikingly accumulated. The changes in activity were reflected in changes in secretory morphology.[146] Schapiro et al. studied the urinary 17-OH steroid excretion in guinea pigs and found that steroid production did not parallel the changes in antidiuretic activity of the hypothalamus or posterior pituitary.[147] This evidence speaks against a physiologic role of ADH in the regulation of ACTH release and also suggests that the level of endogenous adrenal steroids may never be sufficiently elevated to produce the ADH inhibiting effect seen with exogenous steroids.

PART III

Apologia

In the previous section the evidence for a physiologic role of vasopressin in ACTH release has been reviewed extensively. It is apparent from this study that two of the three arguments for such a role of the posterior pituitary hormone can now be discounted. The failure of parallelism between ACTH and ADH release or inhibition is well established and evidence has been produced that indicates that although exogenous vasopressin activates ACTH release this is of pharmacologic rather than homeostatic importance.

Having been active in the investigation of both of these phenomena it is but logical that we should also study the argument that neurohypophysectomy is effective in inhibiting ACTH release. As is seen in Table 6 the results of previous experimental work on this problem are far from conclusive. The cause of these inconsistent results was inapparent at the

outset of our investigations but it was our hope that the use of plasma corticosterone rather than adrenal ascorbic acid depletion in the rat would give insight into these problems.

As ancillary problems the corticosterone resting levels and stress responses of normal rats have been studied. The hypophysectomized animal has been studied and the possible direct effect of vasopressin upon the adrenal evaluated. These are fundamental observations necessary for the evaluation of the neurohypophysectomy experiments and for understanding the normal physiology of ACTH release.

The various indices of ACTH release are tabulated on the following page. The direct measurement of this hormone is dependent upon bio-assay techniques at the present time. In as much as these bio-assay techniques are dependent upon stimulation of <u>in vivo</u> or <u>in vitro</u> adrenal tissue they have no technical advantages over the determination of ACTH release as indicated by the endogenously stimulated adrenal gland. The use of direct chemical analysis of blood steroid is not only of greater esthetic value than other techniques but of greater physiologic

significance; for it allows the definition of ACTH as that hormone which stimulates the production of adrenal steroids.

Indices of ACTH Release*

I. Primary measurement:
   A. ACTH content of pituitary (bio-assay).[148]
   B. ACTH content of blood (bio-assay).[27]
   C. ACTH content of incubation media (bio-assay).[22]

II. Secondary measurement:
   A. Morphologic changes of adrenals.
      1. Hypertrophy.[149]
      2. Histologic changes in stainable lipids.[150]
   B. Chemical changes of adrenals.
      1. Cholesterol content.[151]
      2. Ascorbic acid content.[151]
      3. Corticosterone content.[152]
   C. Chemical changes of body fluids.
      1. Blood cholesterol.[94]
      2. Blood ascorbic acid.[153]
      3. Blood corticoids.[152]
      4. Incubation media corticoids.[22]
      5. Urinary corticoids.[147]
   D. Biologic Activity.[154]
      1. Bio-assay of blood corticoids.
      2. Bio-assay of urinary corticoids.

III. Tertiary measurement
   A. Lymphoid tissue depletion.[155]
   B. Circulating lymphocytopenia.[156]
   C. Circulating eosinopenia.[157]

* Only key references listed.

PART IV

MATERIALS AND METHODS

Experimental Animals

1. Normal

Male rats of the Sprague Dawley strain weighing 180-240 gm. were purchased from Charles River Breeding Laboratories, Brookline, Mass. They were received at least 48 hours before use.

2. Hypophysectomized

In each of the following groups hypophysectomy was confirmed by gross examination of the sella at autopsy.

a. 48 hour and 60 hour rats. - This group was composed of Sprague Dawley male rats weighing 180-200 gm. hypophysectomized by the parapharyngeal approach by Charles River Breeding Laboratories and shipped the day before the 48 hour post-operative experiment. The same animals were used again at 60 hours post-operative.

b. 10 week rats. - These animals were females weighing between 230 and 300 gm. In the first post-operative week several had received one dose of thyroxin in a classroom demonstration. The studies on these animals were conducted at 10 weeks post-operative.

c. 4 hour rats. - This is a small group of male Sprague Dawley rats from Charles River Laboratories. The body weights ranged from 310 to 350 gm. The pituitary was removed by aspirating the hypophysis with an 18 gauge needle after entering the sella by way of the auditory canal.

3. Neurohypophysectomized

Male rats weighing 180 gm. were operated upon by Charles River Breeding Laboratories. A parapharyngeal approach similar to that employed in hypophysectomy was used. The animals were shipped on the first post-operative day. Clinical records were kept on each rat of weight gain and daily water consumption. At autopsy the adrenals were weighed and the sella contents removed for histologic study.

Care of Animals

1. Domiciliary Care

When received by railway express from the breeding laboratory the animals were housed in communial cages where they received water and Purina (R) dry rat feed ad libitum. The animals were fed and cages cleaned each morning. The light cycle consisted of an eleven hour day lasting from 7 AM to 6 PM and the room temperature was maintained at 80 ± 2°F.

2. Isolation before Experiments

a. Rat room. - When resting B levels were determined on rats in the rat room the animals had been isolated in individual cages overnight. Although the experimental animals had food and water the rest of the animals were not fed until after the experiment was completed and an attempt was made to reduce the traffic through the room.

b. Laboratory. - Animals were isolated overnight in individual cupboards in the experimental laboratory. The cupboards were approximately 18x20x20 inches in

dimension and had tightly fitting doors. The animals were placed in small cages in each cabinet and provided with food and water ad libitum. There was no control of temperature, light or noise.

c. Special isolation room. - This sound proof room was used for all but the preliminary experiments reported in this thesis. It consisted of a 10x12 foot room lined with acoustic tile with double doors separating the room from a sound proofed passageway and the hall. The room was air conditioned with the temperature maintained at 80 ± 2°F. The lights were controlled by the same time clock as the main rat room. Animals were isolated overnight in individual cages placed randomly about the room. The animals were decapitated over a sink in the corner of the room. During the isolation and experimental period the room was locked and no intrusion permitted.

Stressing Procedures

1. Electrical Shocking

Animals were isolated in individual cages overnight in the special room. The individual cages were equipped with wire grids on the floors insulated

from the rest of the cage. On the morning of the experiment the room was unlocked and the experimenter silently entered the sound proofed room. With a Havard Inductorium set at 2 cm. 3 volts were passed into the coil and thence through the grids. Each unanesthetized animal was given 30 seconds of electrical shocking followed at a 2 to 2½ minute interval by a repeat 30 seconds. The shocking was intermittent at the experimenter's discretion. Fifteen minutes after the onset of the first shocking the animal was gently removed from its cage and quickly decapitated. Blood was collected in the usual manner and the trunk saved for dissection of adrenals.

2. Unilateral Adrenalectomy

The animals were isolated overnight in individual cages in the special room. The next morning the special room was entered cautiously by the experimenters who gently removed each animal from its cage and injected nembutal intraperitoneally. The use of sharp gauge 25 half inch needles allowed this to be done with a minimum of reaction from the animal. It was noted in preliminary studies that rats squealing when injected seemed to have higher resting values.

Twenty minutes after injection of the nembutal the anesthetized animal was stressed by opening the left flank and removing the left adrenal. The incision was closed with skin clips. Twenty minutes after stress the rat was decapitated and blood collected in the usual manner.

3. Sham Unilateral Adrenalectomy

This experimental procedure was performed in a manner parallel to the unilateral adrenalectomy. The animal was anesthetized and the skin opened in identical techniques; the adrenal was located and manipulated before the incision was closed with skin clips. Decapitation for the collection of blood samples took place 20 minutes after the stress.

4. Splenectomy

This procedure also parallels the unilateral adrenalectomy with the exception of splenectomy instead of adrenalectomy. The animals were sacrificed 20 minutes after stress.

Collection, Preparation and Storage of Plasma

1. Collection of Samples

In the experiments upon the normal and neuro-

hypophysectomized animals , the animals were gently and quickly decapitated with large scissors. Blood was collected from the trunk into test tubes containing a few crystals of powdered heparin. When the animals were thus exsanguinated the tube was twirled to dissolve the heparin and centrifuged within twenty minutes.

When the experiments upon hypophysectomized rats were performed the blood was taken also from the external jugular vein under anesthesia. A short incision was used to bare the vein and skin clips used to close the wound. The blood was collected in a dry syringe and immediately transferred to tubes containing crystalline heparin.

2. Preparation and Storage of Samples

Whole heparanized blood was centrifuged in an anglehead centrifuge at a moderate speed for five minutes. The plasma was removed by Pasteur pipettes and transferred to small test tubes. These were capped with a bit of paraffin membrane and immediately stored within a deep freeze. On occasion clots were encountered which were broken up, recentrifuged and the serum collected as above. The use of serum did not appear to effect the experimental results.

The samples were stored in a deep freeze until the time of analysis. This seldom was greater than 24 hours. They were removed and brought to room temperature about 30 minutes before the start of the analysis procedure. A preliminary experiment had demonstrated no effect of freezing and thawing upon the plasma samples.

Analytical Method for Corticosterone

1. Technique

The method used for corticosterone (compound B) determinations in this thesis is that of Silber, Busch and Oslapas[158] as modified by Guillemin et al.[152] Its principle is the development of fluorescence by the steroid when treated with sulfuric acid. By employing differential extractions before the acid treatment the test is quite specific for corticosterone. Corticosterone is the predominant adrenal steroid in the rat.

a. Standards. - Approximately ten mgm. of the free alcoholic form of Corticosterone (Upjohn Co., U 4460) were weighed to the nearest one hundredth mgm. This was dissolved and diluted in redistilled absolute ethanol to a working standard containing 0.25 µg. per ml.

the working standard was read against a redistilled ethanol blank at 241 mμ.in the ultraviolet range of a spectrophotometer and the concentration determined to be 91% of the volumetric dilution. Chromatographic studies with the isotope dilution technique demonstrated that the standard had approximately 90% of the concentration based on volumetric dilution. Because calculations are based on the volumetric concentration this introduces a 10% error in the absolute values reported subsequently although it does not influence interpretation of the dynamic studies. The standard was tightly sealed with a paraffin film and stored at refrigerated temperatures. It was brought to 20°C. for pipetting purposes.

b. Equipment. - A Farrand, Model A, Fluorometer was used for fluorometric readings. The primary filtered wave length was 436 mμ. and the secondary filtered wave lengths were from 530 to 545 mμ. A neutral density filter was used in the primary to reduce the intensity of the emitted light and subsequent phototube fatigue. A pinhole diaphragm was used prior to the installation of the neutral density filter with equal success.

c. Reagents. -

(1). Fresh glass distilled H_2O.

(2). Iso-octane (2,2,4-trimethylpentane) practical, Eastman Kodak.

(3). Chloroform, Fisher Scientific Co., redistilled from K_2SO_4.

(4). Sulfuric Acid, A. R., Analyzed Reagent.

(5). Absolute ethyl alcohol, redistilled from 2, 4-dinitrophenylhydrazine.

(6). Sodium hydroxide, 0.1 N solution.

(7). Dow-Corning silicone stopcock grease.

d. Outline of procedure. -

(1). Corticosterone Standard (0.25 µg./ml.): 1.0 and 0.5 ml. pipetted to extraction-centrifuge tubes and evaporated to dryness. Brought to 4.0 ml. with H_2O.

(2). Plasma sample: 0.2 ml. delivered to extraction-centrifuge tube containing 4.0 ml. H_2O.

(3). Iso-octane: 4.0 ml. added to each tube, shaken gently 15 seconds and centrifuged 5 minutes, iso-octane discarded.

(4). Chloroform: 5.0 ml. added to each tube, shaken vigorously 30 seconds and centrifuged 3 minutes, aqueous layer (top) discarded.

(5). 0.1 N NaOH: 0.5 ml. added to each tube, shaken vigorously 15 seconds and centrifuged 3 minutes.

(6). 30 N Sulfuric Acid: 4.0 ml. of chloroform

layer transferred to clean extraction-centrifuge tubes containing 1.2 ml. H_2SO_4*, shaken 30 seconds and centrifuged 3 minutes.

(7). Reading: 1.0 ml. of acid (bottom) layer transferred into cuvettes. Read 30 minutes from *, standard set at 40 on galvanometer scale.

e. Calculations. - Using linear graph paper with fluorescence along the ordinate and concentration along abcessa, three points (two standards and blank) were connected in a straight line. The sample concentration was read from the graph set for the particular standards and blank. The curves are of the type y=a+bx. The blank readings ranged from 4 to 8 in the experiments here reported. As experience was gained with the technique consistent blank values of 4 ± 0.5 were obtained. With the 0.25 µg. B standard set at 40 the 0.125 standard gave a reading of 22.6 ± 0.36 (fluorometric scale reading ± SE) in 34 experiments. The variations were related to variation in blanks. The absolute corticosterone concentrations were multiplied by 500 and expressed as µg. corticosterone/ 100 ml. plasma (µg.%).

2. Validation of Method

This method has been amply studied and described

in the literature[152,158] however, several standard procedures were followed in the early experiments to determine the validity of the work in our laboratory.

a. Recovery curves. - Linear recovery curves were obtained in five experiments in which corticosterone was added to H_2O; three where B was added to normal plasma or serum and one with B recovered from adrenalectomized plasma.

b. Replicate determinations. - Excellent replicate determinations were obtained from plasma and from corticosterone added to H_2O or adrenalectomized plasma. As an example when pooled plasma was analyzed in nine aliquots the mean concentration was 38 ± 3 µg.%B.

c. Pooled plasma. - As a control of constancy between analyses the plasma remaining from preceeding determinations was pooled, mixed thoroughly for 60 minutes and pipetted in 0.5 ml. aliquots which were preserved in the deep freeze. One of these aliquots was thawed and run alongside of the experimental plasmas in each determination. The mean value for 27 analytic runs was 12.6 ± 0.37 µg.%B.

Analysis of Adrenal Ascorbic Acid Concentrations

1. Collection of Adrenals

The adrenal glands were removed and dissected clean of fat. They were weighed to the nearest tenth mgm. and immediately placed in 6% trichloracetic acid solution and ground with a frosted glass rod. The tubes were then covered with paraffin film and preserved in a refrigerator until the analyses were performed (usually each Friday).

2. Analysis of Ascorbic Acid

The method of Roe and Kuether was used for the determination of ascorbic acid content (AAA) of the glands.[159] This technique involves the oxidation of the reduced acid with norite and the formation of the osazone with 2,4-dinitrophenylhydrazine. After the color has developed the absorption was read at a wave length of 590 mμ.

3. Calculations

The absolute concentration was divided by the gland weight in mgm. and multiplied by 100 to

express the concentration in mgm. of ascorbic acid/ 100 gm. adrenal weight (mgm.%).

Statistical Techniques

1. Analysis of Standard Error and Significance of Data

The analysis of single groups of data was routinely undertaken to obtain an index of the significance of the observed phenomena. The standard error (SE) was calculated with the following formula:

$$SE = \sqrt{\frac{\xi d^2}{N(N-1)}}$$

where ξd^2 is the sum of the squares of deviations about the mean value and N is the number of observations. The mean was divided by the standard error to determine the "t" value of the data. The per cent level of significance (P) was derived from student's tables.

2. Analysis of the Significance of Difference between Groups

The derivation of "t" values used for this thesis was from the following formulae:

$$S = \sqrt{\frac{\xi d_1^2 + \xi d_2^2}{N_1 + N_2}}$$

Where S is the square root of variance, Σd^2 is the sum of the squares of deviations about the mean and N is the number of observations in group 1 and group 2.

$$SE = \sqrt{\frac{N_1+N_2+2}{(N_1+1)(N_2+1)}}$$

Where SE is the standard error and S, N_1 and N_2 are the same as above.

$$t = \frac{M_1-M_2}{SE}$$

Where M_1 and M_2 are the means of groups 1 and 2 and SE is the standard error as above. Using student's tables the per cent level of significance (P) is calculated from the "t" value.

PART V

Results

Studies of the Resting Corticosterone Level of Normal Rats

The neural factors involved in ACTH release were reviewed briefly in the Introduction. Among the various neural stimuli effective in stimulating ACTH release the environmental stress is well established. The susceptibility of the rat to environmental factors is especially evident when the plasma corticoids are used as the index of ACTH release. Knowledge of the sensitivity of the rat to environmental stresses led us to the most rigorous of environmental controls before uniform and physiologic resting B levels could be observed. In the following paragraphs the steps toward the attainment of resting plasma B levels are outlined as they developed in this laboratory. The results of the studies on resting levels are collected in Table 8.

1. Rats Decapitated in Rat Room

These observations were made on rats kept under various conditions in the regular rat room of the animal house. The animals were usually fed before 8:00 A.M., however, on the day of experiment the animal keeper was requested to stay out of the room until the completion of the experiments. The rats were killed by decapitation after being gently removed from their cages. In all of the experiments with normal animals reported in this thesis the blood samples were taken between 9:00 A.M. and 12:00 Noon.

a. Preliminary observations. - The effect of housing the rats in community cages was superficially determined in the following experiments. When four rats were sequentially removed from a single community cage their plasma B levels were 16, 14.5, 23, and 36.5 µg.% respectively (Exp.32). This seemed to indicate that the process of removing animals from a community cage was stressful to their neighbors. Other factors were also in effect, however, as demonstrated by two rats who were randomly selected from two separate community cages. The levels of corticosterone were at the

Table 8

Resting Corticosterone Levels of Rats
in Various Environments

Isolation Procedure	Mean B µg.% Level ± SE	Range of values µg.%	Number Animals	Protocol Number	Significance of data (P)	Significance of difference(P)
1.Rat Room	17.6 ± 3.7	7.4 - 37.0	8	33,38, 44,45	<0.01	vs.2. 0.02
2.Laboratory	42.2 ± 10.0	5.5 - 93.0	8	38,39 40,41	<0.01	vs.3. 0.01
3.Special Room	13.7 ± 0.86	5.5 - 19.0	32	50,52 53,64 66,67	<0.01	vs.1. 0.10

extremes of 63.0 and 12.5 µg.% (Exp. 38).

b. Isolation in individual cages. - In a group of eight rats isolated overnight in small individual cages a mean B level of 17.6 ± 3.7 µg.% was observed. These animals had been sacrificed between 11:20 A.M. and 12:20 P.M. This observed value approaches that reported in the literature, however, a wide range of values led to the search for a more reproducable method of obtaining a resting corticosterone blood level.

2. Rats Decapitated in Laboratory

Because of the practical difficulty of controlling the traffic through the regular rat room an attempt was made to reach more stable resting levels by isolating rats in dark individual cupboards in the experimental laboratory. In this way it was hoped that human stimulus to the animals would be at a minimum. The animals were kept in small cages with food and water. They were placed in the cages the previous evening and the cabinet doors were closed. Between 11:00 and 12:30 the next morning they were gently taken from the cupboards and decapitated.

The plasma B levels had a mean of 42.2 ±10.0 with extreme variations in range (5.5 to 93.0 µg.%). These experiments were complicated by several uncontrolled variables such as heat and noise.

3. Rats Decapitated under Special Precaustions

The difficulty in obtaining baseline plasma B levels enumerated above led to the use of a constant temperature, sound proof room with the same light cycle as in the regular rat room (see description under Methods). The rats were placed in individual cages with food and water the evening before the experiment. Between 10:30 and 11:30 the following morning the rats were gently removed from their cages and quickly decapitated. The mean plasma B level for these animals was 13.7 ±0.80 µg.%. This agrees with other reported resting values in rats. If one excludes a value of 29 µg.% in an animal which was obviously ill with respiratory distress the range of values for the 32 animals was 5.5 to 19.0 µg.%.

4. Analysis of Differences in Resting Levels

The mean resting levels of animals isolated in the rat room and the special room are not statistically

different, however, the range of values varied much less among the animals isolated in the special room. Both of these levels were significantly lower than the mean plasma B level of rats isolated overnight in the laboratory (Table 8).

Studies of the Rat Resting Corticosterone Level under Nembutal Anesthesia

The effect of nembutal anesthesia in the normal rat was studied in order that a baseline for operative stresses might be available. These results are summarized in Table 9. The nembutal was given intraperitoneally.

1. Transportation to Laboratory and Anesthesia

This group of four rats was carried from the rat room to the laboratory in a common cage. The rats were anesthetized and blood was drawn from the external jugular vein 30 minutes after the nembutal administration. A mean resting level of 42.3 µg.% B was observed under these conditions.

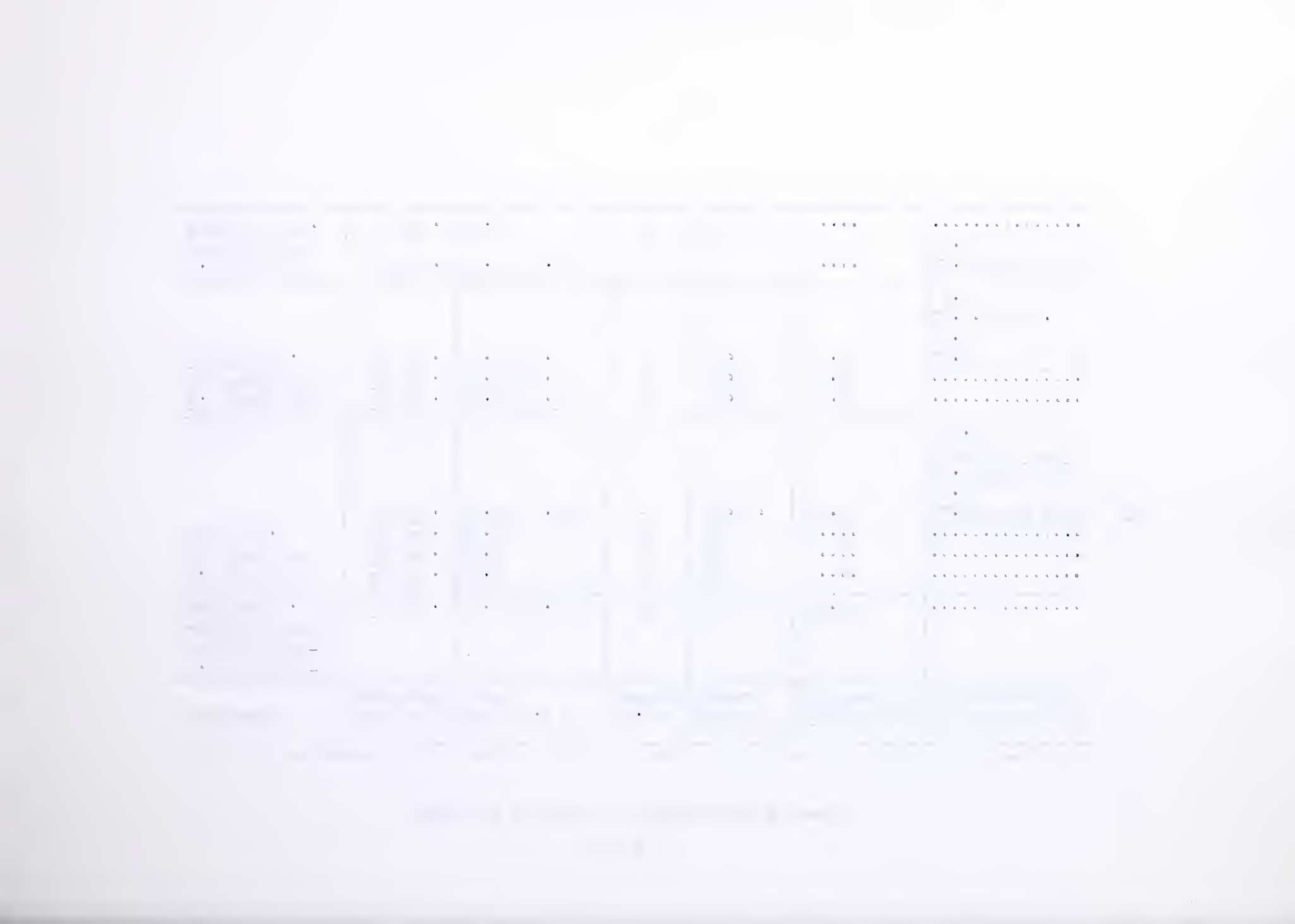
2. Isolation and Anesthesia in Rat Room

The administration of nembutal was followed at ten minute intervals by decapitation and blood

Table 9

Effect of Nembutal on Corticosterone Levels

Procedure	Time after Anesthesia	Mean µg.% B ± SE	No. of Animals	Protocol Number	Significance of Data (P)	Significance of Difference (P)
1. Transportation to laboratory before anesthesia.	30 min.	42.3 ± 1.6	4	29	<0.01	.....
2. Isolation overnight in rat room.	10 min.	26.5	2	33	....	.....
	20 min.	31.1	2	33	....	.....
	30 min.	17.0	1	33	....	.....
	40 min.	21.2 ± 3.23	10	33,43, 45	<0.01	vs.resting level 0.50 vs.special room resting level <0.01
3. Isolation overnight in laboratory.	15 min.	44.0 ± 13.3	4	39,40	0.05	.....
	30 min.	63.9 ± 3.7	4	39,40	<0.01	.....
	45 min.	35.4 ± 11.7	4	39,40	>0.05	vs.resting level 0.50 vs.2.(40min.) 0.50
4. Isolation overnight in special room.	40 min.	15.8 ± 2.2	3	68	....	vs.resting level 0.50
	80 min.	20.0	2	68	....	.....



collection in fifteen animals. The few scattered observations before forty minutes indicated an initial rise at twenty minutes dropping to a mean plasma B level of 21.2 µg.% at forty minutes. This level while statistically identical with that obtained under resting conditions in the rat room was significantly elevated when compared to the special room conditions.

3. Isolation and Anesthesia in Laboratory

When rats, maintained overnight in the laboratory, were submitted to nembutal anesthesia there was a peak of 63.9 µg.% plasma B at 30 minutes which was significantly higher than the resting level under these circumstances. By 45 minutes the level had returned to 35.4 µg.% B which was statistically indistinguishable from the laboratory resting levels and the 40 minute nembutal level of rats anesthetized in the rat room.

4. Isolation and Anesthesia in Special Room

In a small number of rats used as controls for other experiments the nembutal response was determined at 40 and 80 minutes. The 40 minute levels appear indistinguishable from resting levels in the special room. The 80 minute level seems somewhat

higher; however the mean value of two determinations is skewed upward by a value of 31.5 µg.% B in one of the animals. This animal had responded to the nembutal injection with a squeak.

Studies of the Plasma B Levels of Hypophysectomized Rats

1. Resting Hypophysectomized Animals

a. 4 hour hypophysectomized males. - A smaller group of 4 male rats were hypophysectomized by the ear approach. Four hours later under ether anesthesia blood samples were removed from the external jugular vein. The mean plasma B level in this group was 16.92 ± 2.13 µg.%.

b. 48 hour hypophysectomized males. - A group of 7 male rats hypophysectomized by the parapharyngeal approach were anesthetized with nembutal in the laboratory and blood samples removed from the external jugular vein. The mean level of fluorescence interpreted as corticosterone was 3.8 ± 1.36 µg.%. The range of values was from 0.0 to 9.0 µg.%.

c. 10 week hypophysectomized females. - This group of

large female hypophysectomized animals had been used eight weeks previously in classroom experiments where half had received thyroxin. Clinically all appeared in good health and showed signs of pituitary insufficiency. Blood was collected after decapitation in all but one animal from whom the blood was collected from the aorta under nembutal anesthesia. The mean corticosterone plasma levels in these eight animals was 19.0 ± 3.4. The range of values was from 10.0 to 40.0 µg.%. The difference between the plasma B level of these animals and the 48 hour hypophysectomized males is highly significant.

2. Effect of Phlebotomy in 5 Day Hypophysectomized Rats

These rats, hypophysectomized by the parapharyngeal approach, three days previously had been subjected to nembutal anesthesia and the withdrawal of blood for resting levels. On the day of experiment they were again anesthetized with nembutal and the tip of their tails removed. During the next hour 2.5 to 3.0 ml. of blood was milked from the tail following which a sample was removed from the aorta for corticosterone determination. The mean plasma B level in these five animals was 3.4 ± 0.33 µg.%. This was not

significantly different from the resting values obtained in 48 hour hypophysectomized males.

3. Effect of Pitressin on Hypophysectomized Rats

a. Effect in 4 hour hypophysectomized male rats. - A group of four rats were hypophysectomized by the ear approach. Four hours later a baseline blood sample was removed from the jugular vein under ether anesthesia (see Methods). Following this 0.5 unit of Pitressin was injected into the external jugular vein over a two minute interval. These animals blanched and developed bradycardia but did not develop respiratory difficulty. The rats weighed from 310 to 320 gm. Fifteen minutes after the injection blood was again taken for plasma B determination. The mean level of corticosterone following the drug was 12.04 ±3.04. This was not significantly different from the resting level.

b. Effect in 48 hour hypophysectomized male rats. - Eight animals (175-210gm.) were maintained under nembutal anesthesia during the experimental period. Following the removal of jugular venous samples for baseline determinations (see Methods) the animals

received 0.5 and 1.0 units of Pitressin in the external jugular vein over a two minute period. The immediate response to this drug was in each case a marked peripheral vasoconstriction, labored respiration and bradycardia. Despite being maintained by artificial respiration and tracheal suction three animals receiving 5.0 units of Pitressin died in what appeared to be right heart failure. The symptoms observed with the two smaller doses of Pitressin seemed identical. The mean plasma level of corticosterone 15 minutes following the half unit dose was 3.6 µg.% and that following the one unit dose 7.6 µg.%. When these data are combined they are statistically identical with the resting levels. There was no significant difference between these results and those following phlebotomy in the 5 day hypophysectomized male rats.

4. Analysis of Observations upon Hypophysectomized Rats

A low basal level of corticosterone was noted in 48 hour hypophysectomized male rats. This level was not increased by phlebotomy or by the injection of large doses of Pitressin. The basal level of 10 week post-operative hypophysectomized female rats was

significantly elevated. The control levels of 4 hour hypophysectomized males were also elevated. This observation is not in accord with subsequent unpublished observations by Brodish[139] and is most likely due to analytical error. The results of these studies are compiled in Table 10.

Studies of the Plasma Corticosterone Response to Stress of the Normal Rat

The stress response of the pituitary - adrenal axis has been the subject of many investigations; most with criteria indicating only a qualitative response. In order to quantitate the adrenal steroid increase following endogenous ACTH release the following studies of the normal response to stress were studied. The data is summarized in Table 11.

The animals were subjected to various stresses and at the stated intervals trunk blood was collected after decapitation. All of these experiments were performed in the special sound proof and air conditioned room. Adrenals were also removed in some experiments with the view of correlating ascorbic acid levels of this gland with the corticogenic response to stress.

Table 10

Corticosterone Levels of Hypophysectomized Rats

Description	Sex	Post-op. Period	Mean µg.% B ± SE	No. of Animals	Protocol Number	Significance of Data (P)	Significance of Difference (P)
1. Resting	M	4 hours	16.9± 2.1	4	72	....	.....
	M	48 hours	3.8± 1.4	7	47,48	<0.05	vs.10wk.<0.01
	F	10 weeks	19.0± 3.4	8	32,42	<0.01	.....
2. Phleb-otomy	M	5 days	3.4± 0.3	5	51	<0.01	vs.resting level >0.50
3. Pitressin 0.5-1.0 units	M	4 hours	12.0± 3.0	4	72	....	vs.resting level 0.13
	M	48 hours	5.9± 1.3	8	47,48	<0.01	vs.resting level 0.22
							vs.phlebotomy 0.11

Table 11

Plasma Corticosteroid Stress Responses
of Normal Animals

Description	Protocol Number	µg. % B M ± SE	Significance of Difference	AAA mgm.% M ± SE Left	Right	Significance of Difference
1. Electrical Shock 15min.	64,66 67	74.5 ± 10.3(9)*	vs. resting levels <0.01	419±37(6*).....		vs. resting <0.01 vs. splenectomy 0.18
2. High Freq. Sound 15min.	53	14.4 ± 6.7(4)	.....	.....		.....
3. Unilateral Adrenal-ectomy 20min.	68,69 70	37.0 ± 5.1(9)	vs. resting levels <0.01 vs. shocking 0.01 vs. splenectomy <0.01	483 (3)	437±25(6)	.....
60 min.	68,69 70	40.0 ± 4.0(9)	vs. 20 min. >0.50	506 (3)	388±25(6)	.....
4. Sham Adrenal-ectomy 20min.	69	40.3 ± 6.5(3)	vs. resting levels 0.01 vs. shocking >0.05	.....		.....
60 min.	69	42.0 ± 11.6(3)	.....	.....		.....
5. Splenectomy 20 min.	70	68.6 ± 7.3(4)	vs. resting levels <0.01 vs. shocking >0.50	.....465 (3)		vs. resting 0.02
60 min.	70	59.2 ± 8.7(3)	vs. 20 min >0.50	.....328 (3)		.....

* Number of animals

1. Effects of Electrical Shock

Under controlled environmental circumstances unanesthetized animals were subjected to electrical shock. Their clinical response was an immediate squealing accompanied by tetanic rigors of the extremities. It was noted in earlier experiments that continual electrical shock produced unconsciousness; therefore the current was momentarily broken when tetany occurred. The vigorous response of the animals to this terrorizing procedure led to a superficial attempt to measure any possible effect on their neighboring rats. In two rats exposed to the cries of their neighbors the plasma levels were 14.5 and 19 µg.%B. This is comparable to the levels of two controls decapitated before the same experiment which were 15.0 and 19 µg.%B (exp. 64). The plasma B response of the stressed animals at 15 minutes had a mean of 74.5 ± 10.3. The mean ascorbic acid content of the left adrenal of six of these rats was 419 ± 15 mg.%.

2. Effects of High Frequency Sound

Under identical experimental conditions to those used above rats were exposed to a 5 minute

interval of high frequency, high intensity sound from an electronic tone generator. Of the four animals so exposed the plasma corticosterone levels at 15 minutes were 10.0, 34.5, 9.0 and 24.0 µg.%. The mean of these values is 14.4 ± 6.7, not significantly different from the control values in the same environment.

3. Effects of Unilateral Adrenalectomy

Under nembutal anesthesia the left adrenals of normal rats were removed. The animals were allowed to recover from the anesthesia and blood samples taken following decapitation. At 20 minutes the mean plasma B level was 37.0 ± 5.1 µg.% and at 60 minutes 40.2 ± 4.0 µg.%. There was no statistical difference between these values. The right adrenal was removed at decapitation and a fall of ascorbic acid content of 11.9% was seen at 20 minutes and 19.5% at 60 minutes.

4. Effects of Sham Operation

In this group of animals under nembutal anesthesia a flank incision identical to that used for adrenalectomy was developed and the periadrenal tissue manipulated. The mean plasma B response was 40.2 ± 6.5 µg.% at 20 minutes and 42.0 ± 11.6 µg.% at

60 minutes. The small population of the two groups prevented statistical comparison. In the 60 minute response the spread of values is attributed to the low plasma B response of 24.7 µg.% seen in one of the animals which received only a skin incision because of poor anesthesia.

5. Effects of Splenectomy

Using the same operative technique as that for adrenalectomy the spleen was removed under nembutal anesthesia. The mean plasma B level following this procedure was 68.6 ± 7.3 µg.% at 20 minutes and 59.2 ± 8.7 µg.% at 60 minutes. The corticosterone difference at these two time intervals is insignificant. The mean ascorbic acid content of the right adrenals was 465 mg.% at 20 minutes and 328 mg.% at 60 minutes.

6. Analysis of Differences

Each of the above stressing procedures produced significant elevations in the plasma corticoids as compared with the resting levels in the same environment. There was no difference between the responses following electric shock and splenectomy. The response to unilateral adrenalectomy was significantly less

than that to electrical shock or splenectomy. Sham operation and splenectomy were statistically identical in their response to that seen in electical shock. In no case was the 60 minute response different from the 20 minute response (Table 11).

Studies of Adrenal Stress Response in Operated Rats

The possible role of the posterior pituitary in the release of ACTH is discussed at length in the Introduction. The decisive evidence for a role of this gland should be demonstrated by the loss of ACTH releasing function in the neurohypophysectomized animal. Heretofore only equivocal results have resulted from this approach. The use of direct steroid measurement offers hope of clearly uncovering any such anterior-posterior lobe relationship.

In the group of animals used in this study the neurohypophysis was removed by the parapharyngeal approach. Clinical records were kept in the post-operative period to determine the physiologic status of the animals. These records are summarized in Table 12. The animals fell into three fairly clearly separated groups according to anterior and posterior lobe function.

Table 12

Clinical Notes on Neurohypophysectomized Rats

Description	Animal Number	µg.% B	AAA mgm.%	Adr.Wt. mgm.	Maximum daily H_2O	Wt.Cain gm/8days	% Gland Present post.	intermed.	ant.
Successful Neurohypophysectomies (Posterior lobe insufficiency)									
Shock	N- 4	69.0	452	17.7(L)*	120cc.	18	0	0	25
Shock	N- 7	103.5	444	10.5(L)	100	24	0	0	25
Anesth.	N-17	26.25	607	10.8(R)	130	18	100	50	20
Anesth.	N-23	28.25	553	18.6(R)	187	20	0	0	40
Splenx.	N-16	85.75	464	21.2(R)	150	42	0	0	100
Splenx.	N-21	46.25	500	15.8(R)	130	22	0	0	90
Splenx.	N-24	64.0	388	17.1(R)	187	34	0	0	50
Adrex.	N-25	56.75	534	12.8(R)	187	32	0	10	90
Adrex.	N-28	46.25	568	9.7(R)	187	28	100	100	100
Incomplete Neurohypophysectomies (euglandular or sham op.)									
Control	N- 1	31.5	534	19.7(L)	75	22	100	100	80
Control	N- 2	21.5	561	18.2(L)	75	32	100	50	100
Control	N- 3	35.0	490	20.7(L)	57	26	100	100	60
Control	N-10	35.0	548	22.1(L)	95	34	20	10	50
Shock	N-11	45.0	458	15.3(L)	45	54	100	100	60
Shock	N-12	50.5	446	16.8(L)	35	48	100	100	100
Shock	N-13	94.5	462	11.4(L)	55	46	100	100	100
Anesth.	N-14	17.75	614	15.2(R)	57	48	80	100	50
Splenx.	N-26	64.0	497	14.7(R)	75	22	0	0	50
Hypophysectomized (anterior lobe insufficiency)									
Control	N- 9	17.0	310	6.7(L)	65	-6	0	0	0
Shock	N- 8	84.5	472	17.7(L)	75	6	100	100	40
Anesth.	N-20	21.5	432	6.9(R)	110	12	0	0	40
Splenx.	N-18	28.25	503	9.0(R)	57	8	60	60	0
Adrex.	N-15	35.5	492	9.4(R)	75	10	0	20	25

*L - left adrenal; R - right adrenal

Diabetes insipidus was used as an index of posterior pituitary insufficiency and growth failure or adrenal atrophy was the criteria for anterior lobe insufficiency. The animals demonstrating euglandular function according to these indices were designated "sham operated" animals.

The experiments were conducted in the special room and blood samples taken after decapitation. The operative stresses are those described in the previous section. Resting levels were determined in both anesthetized and unanesthetized animals. The stress response was determined in unanesthetized electrically shocked rats and nembutal anesthetized splenectomized or unilaterally adrenalectomized animals.

The raw data from these observations is presented in Table 12. As is apparent from examination of this table the division of data in eight subgroups composed of one to four members renders analysis of this data impossible. The data becomes intelligible and of significant dimensions if the smaller subgroups are combined. In the subsequent reporting of data this regrouping of results has been directed first toward the differences in control, operative control and neurohypophysectomized response to stress, assuming

that there is no difference between the various resting conditions or the response to various stressors. The second approach was to assume that there was no difference in the response of neurohypophysectomized and operative controls and to direct interest toward the effects of the various resting conditions and the various stresses. In each animal adrenal ascorbic acid levels and plasma B levels were determined 20 minutes after stress.

1. Results Classified According to Physiologic Status

The compiled data from the experimental results when sorted as to the effect of neurohypophysectomy, operative control and normal control are presented in the first part of Tables 12, 13 and 14.

a. Corticosterone. - From the statistical comparison it is apparent that the corticosterone response to stress was not inhibited in any of the operated animals regardless of posterior lobe function. There was no difference in the degree of response. The resting levels of the operative controls (sham op.) were significantly elevated above those of the normal controls and the resting levels of the neurohypophysectomized

Table 13

Cumulative Data on Neurohypophysectomized and Control Rats

Description	No. of Animals	µg.% B M±SE	Significance of Data (P)	No. of Animals	mgm.% AAA M±SE	Significance of Data (P)
Results Classified According to Physiologic Status						
Normal Controls						
Resting	32	13.7 ± 0.86	<0.00	6	492±11.9	0.00
Stressed	22	52.8 ± 6.5	<0.00	15	431±14.3	0.00
Operated Controls						
Resting	5	28.2± 3.5	<0.01	5	549±28.3	<0.01
Stressed	4	63.5±11.1	<0.01	4	464± 9.5	0.00
Neurohypophysectomy						
Resting	2	27.3	....	2	580	....
Stressed.	7	67.5± 8.9	<0.01	7	480±22.7	<0.01
Results Classified According to Type of Procedure						
Normal Controls						
Shocking	9	74.5±10.3	< 0.01	6	419±15	<0.01
Splenx.	4	68.6± 7.8	<0.01	3	465±36	<0.01
Operated						
Resting*	7	27.8± 2.37	<0.01	7	558±24.7	<0.01
Shocking	5	72.4±11.3	<0.01	5	452± 3.45	0.00
Splenx.	4	65.0± 7.9	<0.01	4	462±25.7	<0.01
*Results Classified According to Anesthesia						
Anesthetized	3	24.1± 3.5	<0.03	3	591±23.0	<0.03
Unanesth.	4	30.7± 3.4	<0.01	4	533±15.5	<0.01

Table 14

Statistical Correlations of Studies
in Neurohypophysectomized Rats

Experimental Groups Compared vs.		Significance of Differences (P) B	AAA
Results Classified According to Physiologic Status			
Stress Neurohypox.	Stress Controls	0.17	0.05
Stress Neurohypox.	Stress Sham Op.	0.77	0.56
Stress Sham Op.	Stress Controls	0.43	0.21
Resting Controls	Stress Controls	<0.01	<0.01
Resting Sham Op.	Stress Sham Op.	0.01	<0.01
Resting Exper.*	Stress Neurohypox.	<0.01	<0.03
Resting Sham Op.	Resting Controls	<0.01	0.08
Results Classified According to Type of Procedure			
Resting Exper.*	Resting Controls	<0.01	0.01
Shocked Exper.	Shocked Controls	0.77	0.04
Splenx. Exper.	Splenx. Controls	0.92	0.82
Resting Exper.*	Shocked Exper.	<0.01	0.00
Resting Exper.	Splenx. Exper.	<0.01	<0.01
Shocked Exper.	Splenx. Exper.	0.50	0.50
Anesthetized Resting Exper.	Unanesthetized Resting Exper.	<0.05	<0.01

* Combined neurohypophysectomized and sham operated resting levels.

animals were elevated although analysis of this difference was impossible.

b. Adrenal ascorbic acid. - All three groups responded to stress with a significant ascorbic acid depletion at 15-20 minutes if compared with respective resting levels. When compared with control resting levels neither the sham op. (P 0.07) nor the neurohypophysectomized (P 0.62) responded in a significant manner. This discrepancy is explained by the significant difference between the resting levels of the control and experimental animals (P $<$ 0.01).

2. Results Classified According to Type of Procedure

After rearranging the data according to the procedure used these observations are presented in the second portion of Tables 12, 13 and 14.

a. Corticosterone. - Here again it is apparent that the resting plasma B levels were higher in the operated animals than in the normal controls. The response to stress was significant in each of these groups and the quantitative response did not differ for electrically shocked or surgically stressed animals.

b. Adrenal ascorbic acid. - The AAA content of the resting operated animals was higher than that of the controls. The response to electrical shocking of the operated animals was diminished; however both groups responded equally well to splenectomy as a stress.

3. Effect of Operative Procedures upon Adrenal Weight

This data is admittedly skewed because all animals with atrophic adrenals are eliminated from the series of operated animals under consideration. The mean weight of control adrenals (both right and left) were 15.8 mg. in 12 normal controls of similar body weight. Under identical experimental circumstances the mean adrenal weight was respectively 15.4 and 17.1 mg. in neurohypophysectomized and sham operated groups of nine animals each.

4. Correlation of Anatomical and Physiological Observations

At autopsy all operated animals had the pituitary remnants removed and fixed in formalin. Serial sections were studied after staining with hematoxylin and eosin. The gross estimates of the

percentage of the various parts of the pituitary appear in Table 12. These estimates are based upon the amount of intact tissue present as compared with the normal gland. There is general correlation between the presence or absence of the pars neuralis and the incidence of diabetes insipidus. Of the 18 animals studied only 3 showed the absence of one in the presence of the other.

There seems to be no correlation between the percentage of surviving pars anterior tissue and the adrenal weight, growth or diabetes insipidus. It is noteworthy that the only animal with neural lobe in the absence of anterior lobe failed to respond to stress. The presence of pars intermedia parallels the presence of the neural lobe but does not seem to be of significance other than as an index of neuro-hypophysectomy.

PART VI

Discussion

The development of the concept of neural control of ACTH release was reviewed along historic lines in the opening sections of this thesis. The development of this concept has been the product of many theories each contributing to the stimulation of new inquiry and eventually new theories. It is interesting to note that the brave investigator who advanced the new hypothesis was often the worker who later proved it insufficient.

One of the most recent theories to explain the strange union of the nervous system with the endocrine release of ACTH is that the antidiuretic hormone is the agent secreted by the neural tissue which stimulates the anterior pituitary to increase ACTH secretion. In accordance with the classic modes of endocrine investigation the activity of ADH or vasopressin has been studied in additive experiments (normal animals); in replacement experiments (blocked animals) and in

experimentally defective animals (diabetes insipidus). The results of these studies are discussed in Part II of this paper. Another classic technique has been the surgical exterpation of the gland in order to determine its essential role in the body economy: if we remove the posterior pituitary we should be able to demonstrate the necessity of this gland in the control of ACTH release.

The neurohypophyseoprival animal has been studied by several investigators but the results of these experiments are contradictory and difficult to interpret. This discord encouraged us to renew the study of the effects of neurohypophysectomy upon ACTH release. They also gave us the burden of explaining the existing discrepancies in the literature.

In the studies reported here we have used both adrenal ascorbic acid depletion and plasma corticosterone increases as indices of ACTH release. In as much as the latter technique is a new method and few quantitative studies of adrenal steroid secretion have been conducted in the rat,preliminary studies of the normal and hypophysectomized rat were undertaken. Because these are new observations they are included in the thesis.

Corticosterone Levels in the Resting Rat

Careful control of the experimental environment is necessary for valid studies of plasma corticosterone responses to stress. The sensitivity of the rat to changes in environment was noted by Guillemin *et al.* and Fortier *et al.* who found that the removal of rats from the rat quarters to the laboratory was sufficient to cause elevations in plasma B. They noted elevations of 40.6 ± 2.5 μg.% and 33.4 ± 3.5 μg.% within 12 to 20 minutes.[152,160] In our observation on rats quartered in the laboratory we observed a mean of 42.2 ± 10.0 μg.% B which agrees closely with their observations.

When animals were kept in the special room where noise, temperature and light were strictly controlled we observed resting levels of 13.7 ± 0.8 μg.% B. This is in accord with the values of 12.8 ± 1.5 μg.% and 12.2 ± 0.3 μg.% reported by the above investigators. Although there was no significant difference between these values and those observed in our regular rat room there was a closer uniformity and better reproducability under these circumstances. This led to our use of the special environment for the subsequent studies.

Corticosterone Levels in the Nembutal Anesthetized Rat

The use of nembutal as an anesthetic agent was studied by Guillemin et al. in their classic investigation of corticosterone levels in the rat. It was observed that nembutal although causing a transient rise in plasma B levels permitted the observation of resting levels after 35 minutes.[152] We also observed the significant rise in corticosterone following nembutal administration and the subsequent return to the initial levels. However in the animals with high initial levels this return toward true resting levels was never complete. The rats housed in the laboratory showed a mean plasma concentration of 35.5 µg.% B after forty minutes. This was not statistically different from the elevated initial levels of these animals and both were significantly elevated above the absolute resting levels observed in the special room. It is apparent from this study that nembutal anesthesia is not a substitute for the careful environmental controls used in the special room.

The Plasma Corticosterone Levels of the Hypophysectomized Rats

The observed plasma B levels in hypophysectomized male rats 48 hours after operation are in general agreement with the value of 5 µg.% observed by Silber and Guillemin.[158,152] These levels have been attributed by Fortier, Guillemin and Silber to a residual fluorescence of rat plasma and indeed Fortier in the reporting of his observations subtracts a value of 5 µg.% from the observed plasma concentration of normal rats in order to speak in absolute corticosterone concentrations.[160]

An unusual observation was the measurement of significantly elevated levels in long term hypophysectomized female rats. Whether this represents an increase in residual fluorescence or a compensatory increase in the production of corticoid hormones in the presence of chronic ACTH deficiency is speculative at this time. Personal communication with Guillemin reveals that this has been observed in long term hypophysectomized female rats in his laboratory.[64] Toeppel has told us of observed increases in the adrenal effluent plasma

corticoids in chronically hypophysectomized rats.[161] This subject certainly is one of the more stimulating leads gained during the present study.

The reported direct effect of Pitressin upon the adrenal was studied. As will be recalled from the Introduction Nelson, Hume[140] and Hilton[125] attribute such an effect to direct adrenal activation and Sayers[121] has attributed such activity to the release of "bound" ACTH. The possibility that such effects might arise *via* vasoconstrictive tissue ischemia was studied by phlebotomizing hypophysectomized rats. No change in corticosterone levels was observed in these animals despite the removal of sufficient blood volume to induce shock. Pitressin was given to 48 hour post-operative rats without an increase in plasma corticosterone.

The failure of this posterior lobe pressor substance to elicit corticogenesis is therefore in conflict with the studies of Nelson and Hilton. It will be recalled that these observers worked with adrenal effluent corticoids in the dog. The discrepancy in observations may be due to species variation or it may be due to the inability of peripheral levels of B

to mirror the type of stimulating effect of Pitressin they have observed. A third possibility is that the 48 hour hypophysectomized animal may have lost sensitivity to Pitressin. Sayers had found that Pitressin caused ascorbic acid depletion in 24 but not 48 hour hypophysectomized rats. In our study with four hour hypophysectomized rats no corticogenic increase was observed in response to Pitressin. This observation would seem to eliminate this third possibility. Of the other two explanations possible species variation is elusive of discussion. The possible discrepancy between adrenal effluent and peripheral corticoid levels is a subject of practical importance. The kinetics of adrenal effluent response are much accelerated over the peripheral response and the peripheral levels reflect both increased synthesis and decreased utilization of adrenal corticoids. There is no available evidence, however, that a rapid rise in corticogenesis such as that seen in the normal stress response is not paralleled by increases in peripheral corticoid levels.

From the teleologic point of view it would seem that any increase in steroid production failing to increase peripheral corticoid levels would be of no

physiologic significance. It is important to recall that of the two indices of adrenal corticogenesis the measurement of effluent steroid is complicated by manipulation of adrenal blood supply. This is particularly true in Hilton's perfused adrenal gland. One cannot but wonder whether this activation of the isolated adrenal by a pharmacologically potent agent may not be due to technique in a way that parallels the Uncertainity Relations of the physicists. These speculations are of less significance, however, than the observed failure of Pitressin to activate the adrenal of hypophysectomized rats. The conclusion seems justified that Pitressin plays no role in the activation of the rat adrenal gland per se.

The Response of the Normal Rat to Stress

Although the activation of the rat adrenal by stress is one of the most widely studied phenomena in endocrinology very few direct measurements of the adrenal cortical response have been made. Fortier has reported that unanesthetized rats respond to electric shock with a rise to 30 µg. % B at 15 minutes and a return to normal at 60 minutes.[162] He has also observed

increased plasma B levels of about 62 µg. % one and two hours after laparotomy which returned to normal at 24 hours.[163] Guillemin observed rises to 46.5 µg.% and 48.1 µg. % 15 minutes after cannulation of the carotid and after bilateral fractures of tibia respectively. Although the response after cannulation was falling at 45 minutes the response to fracture remained elevated at 60 minutes.[152]

In our laboratory increases in plasma B levels of comparable character were observed following electric shock, unilateral adrenalectomy, sham unilateral adrenalectomy and splenectomy. The responses to shock and splenectomy (74.5 and 68.6 µg. % B) are not significantly different however the response to unilateral adrenalectomy was statistically reduced (37.0 µg. % B).

The observation of a reduced response in the unilateral adrenalectomized animal led to the speculation that the peripheral corticosterone response was handicapped by the presence of only one adrenal. This has been confirmed subsequently by Brodish who found that there is significant diminution of the peripheral response to stress when only one adrenal is secreting.[139]

In the animals subjected to operative stress there was no difference between the level of response at 20 and 60 minutes indicating a prolonged response to these stresses.

The frequently observed and reported adrenal ascorbic acid depletion after stress was seen following electric shock, unilateral adrenalectomy and splenectomy. This was significant at fifteen to twenty minutes after stress.

The Response of the Neurohypophysectomized Rat to Stress

In our studies we found no inhibition of the pituitary-adrenal response to stress after neurohypophysectomy. The ability of these animals to release ACTH following stress was demonstrated with both plasma corticosterone and adrenal ascorbic acid indices of ACTH release.

The previous studies of ACTH release in the neurohypophyseoprival rat have reported that the stress blockade is specific for only certain stresses. Nowell felt that only "systemic" stresses were inhibited in this animal[115] whereas Smelik and DeWeid felt that only "psychic" stresses were inhibited.[142,143] There was

agreement however that epinephrine failed to elicit ACTH release in the rats without the neurohypophysis when ascorbic acid depletion was used as an index.[108,142,144] The classification of stresses into neurologic and metabolic stresses dates from Fortier's theory proposed in 1951 that the mechanism for stimulation of ACTH in the pituitary was mediated by factors divisable into these two modes.[41] In personal conversation Fortier admits that such classification is highly artificial and that it is doubtful if either type of stress may exist in the total absence of the other. Although our investigations do not confirm or deny the theory of Fortier they do indicate that the posterior lobe is not essential for ACTH release to either category of stress. In our observations neither neurologic stress (electric shock of the unanesthetized animal) or metabolic stress (surgical manipulation) was inhibited.

In the following paragraphs we will discuss the previous studies of the neurohypophysectomized animal and attempt to demonstrate that the observed inhibitions are factitious results related to experimental design rather than posterior pituitary function. A review of the results with the neurohypophysectomized rat in

our experiments reveals that in both the animal without the posterior pituitary and the sham operated controls significantly elevated resting levels of adrenal ascorbic acid were observed. This high level of ascorbic acid is a variable finding among the experiments reported by others and may provide the key to understanding their results. For example, in Nowell's study, the resting ascorbic acid level in the adrenals of operated animals ranged from 322 to 516 mgm.%.[115] With this variation a depletion to 365 mgm.% by a stress may be interpreted as either a complete inhibition or a 29.3 % depletion. In this regard it is enlightening to note that his reported inhibitions by neurohypophysectomy are in each case dependent upon low resting ascorbic acid levels. The successful stimuli depend upon higher control values.

In the study by Fisher and De Salva a similar variation in the ascorbic acid concentration of the control adrenals is observed.[144] These workers reported the values of 418 and 550 mgm.% as resting levels. There were two animals in each of their control groups. When the lower value was used no ascorbic acid depletion was observed following epinephrine administration. If the higher control value is used the depletions

become apparent at 30 and 120 minutes after epinephrine. It is interesting to note that these workers also report increased resting plasma B levels in the neurohypophysectomized rats.

Smelik's study of the neurohypophysectomized rat reveals similar variations in initial adrenal ascorbic acid concentrations.[142] The control adrenals in his series of animals ranged from 405 to 502mgm.% with the reported responses to epinephrine and environmental stress ranging from 372 to 454 mgm.% of ascorbic acid. We are inclined to interpret their results as we have above, however, his observation of a depletion to 279 mgm.% after unilateral adrenalectomy strains this hypothesis. The control adrenal concentration was 424 mgm.% in these animals. This is the lowest post-stress ascorbic acid concentration ever reported in neurohypophysectomized rats. It is of interest to note a variation in his technique which might account for this extreme depletion: these animals had their control adrenal removed under ether anesthesia and were sacrificed after 60 minutes by ether. In the other experiments the animals were unanesthetized or nembutalized. The cumulative effect of ether and

adrenalectomy may have caused the severe ascorbic acid depletion reported. The ability of ether to release ACTH in the rat has been demonstrated by many investigators.

Arimura studied both ascorbic acid depletion and blood ACTH activity in the neurohypophysectomized rat. He noted an increase in the resting ACTH activity in the blood of these rats and found that further elevation did not follow electric shock. He suggests that the apparent inhibition to the stress may be a nonspecific phenomenon because it was also observed after chronic dehydration and carotid cannulation. The failure of response, however, is difficult to interpret in view of our unequivocal responses to electric shock. The difference may lie in technical factors such as strength of stimuli, however, the chronic elevation of ACTH activity is certainly in keeping with our observations of chronic elevation of plasma B in these rats. Arimura also reports the failure of the neurohypophysectomized animal to respond to epinpehrine with ascorbic acid depletion. His paper does not state the ascorbic acid concentrations of his control adrenals.[108]

As noted earlier we observed significant

elevations in the plasma corticosterone and adrenal ascorbic acid levels of the resting neurohypophysectomized rat. This was not a property of the posterior lobe status because this was also observed in the sham operated rats. When the stress response of the adrenal ascorbic acid is related to the elevated levels observed in these animals significant depletion occurs. However if one compares the adrenal ascorbic acid concentrations after stress with normal control animals significant inhibition appears to be demonstrated. In the previous paragraphs we reviewed the reported inhibitions noted by other investigators. Wide variations in the concentration of control adrenal ascorbic acid were noted. Moreover the presence or absence of inhibition seemed related to the resting level used for comparison. Inhibition occurred only when the control adrenal ascorbic acid was lower than the levels reported here. Depletion occurred when the control adrenal ascorbic acid agreed with our observations. What the previous investigators have noted has been a fluctuation in resting adrenal ascorbic acid concentration rather than inhibition of the stress response. The use of ascorbic acid depletion as an index of ACTH release is a valuable tool in the

hypophysectomized animal but may be misleading in the euglandular animal subject to chronic stress.

The corticosterone increases following stress are statistically identical to the responses of the normal animal. In the neurohypophysectomized and sham operated animals a two fold increase in resting corticosterone levels also was observed. Such elevations make the use of percentage response a misleading technique for the expression of data. As an example the stress responses of the normal rats were 400 to 445 % and the stress responses of the operated animals 134 to 160%. In reality the responses of the two groups when expressed in terms of plasma corticosterone concentrations are statistically identical (P: 0.77 to 0.92). The fact that elevated resting levels were observed in neurohypophysectomized and operated controls indicates that this phenomenon is not a function of the loss of the posterior pituitary.

Several recent investigators have reported on ACTH-like activity which is present in the posterior lobe and is depleted by stress. The significance of this activity is completely obscure despite suggestions that it may be the mechanism whereby neural and systemic

stresses are separated. This postulate states that the posterior lobe might be the gland active in either the neural or the systemic ACTH stress response. Mialhe-Voloss, however, reports that the presence of only the posterior lobe is insufficient to produce adrenal ascorbic acid depletion to either stress modalities.[112] In one of our animals (N-18) in which the pars anterior was fortuitously removed instead of the posterior lobe there was no significant increase in plasma B after splenectomy despite relative maintenance of adrenal weight. This would tend to confirm Mialhe-Voloss's observation.

Contribution of These Results to the Physiology of ACTH Release

The anatomic proximity of the neural and glandular lobes of the hypophysis is very suggestive of some type of interrelationship between these diverse tissues. The repeated demonstration of neural control of ACTH release has led a number of investigators to study the posterior pituitary hoping to demonstrate that this organ or one of its hormones may be the hypothetical link with the nervous system. The early

students of this relationship saw two major areas of evidence to support their hypothesis: the ability of vasopressin in large doses to activate the pituitary-adrenal axis; and a parallelism between stimulation and inhibition of vasopressin and ACTH release. More recently evidence was advanced that was said to demonstrate the essential role of the pars neuralis *per se* in the ACTH response to stress.

It is an unfortunate circumstance that in experimental medicine the emphasis on publishing only positive findings has thrust the burden of proof upon the critics of an hypothesis rather than its advocates. In recent years we have turned our interest toward the unrewarding task of disproving this hypothesis. Along with many other investigators we have observed the failure of parallelism between ACTH and ADH release; finding that either can be released independently or inhibited independently.[101,91,164] We also studied the pharmacology of the ACTH stimulation by vasopressin observing that doses far in excess of those physiologically operative in the body were necessary to produce this effect.[101] In this thesis we advance evidence that the neural lobe *per se* is not essential for ACTH release.

These negative findings all argue against a physiologic role of vasopressin stimulation of ACTH release.

What then is the significance of all the studies on ADH and ACTH interrelationships? The evidence at hand does not exclude vasopressin from the cast of supporting players. Just as the theories of epinephrine release and peripheral utilization of corticoids have been demonstrated to be insufficient in their attempts to explain the full phehomena of ACTH release, the ADH theory cannot account for the observed phenomena when rigorous criteria are used. Just as epinephrine and push-pull theories have remained part of a background of well documented factors regulating the pituitary-adrenal axis, the "vasopressin theory" will form part of our understanding of ACTH physiology.

Several facts remain unquestioned about the relationship of the antidiuretic hormone to ACTH release. The first is the ACTH releasing activity of a large variety of preparations of the pressor material in a variety of animal preparations as observed by all of the available criteria for ACTH release (Table 3). The further observations that the ACTH releasing activity to pressor activity ratio is constant regardless

of purification[51,97] the studies with purified or synthetic vasopressin; and the parallel inactivation of these activities[109] indicate that the activity is not due to a contaminant. The action of vasopressin in animals blocked to stress indicates a specificity of the effect on ACTH release (Table 4). Furthermore, the activity of this material is directly upon the pituitary (Table 5). These phenomena are valuable contributions to an expanding field of pharmacology of peptides.

Another contribution of these studies is that they may give insight to the nature of the physiologic corticotrophin releasing factor. It is tempting to speculate, as has Guillemin, that the activity of exogenous vasopressin may be due to a structural relationship to the physiologic mediator of ACTH release.[165,56,166] Indeed, Schally et al. have found that their ACTH releasing factor is closely related in size and amino acid composition but lacking in vasopressin activity.[62]

Although the evidence seems conclusive that ADH is not essential for the stress response of ACTH there are several indications that it may play a more

subtle role in regulating this anterior lobe hormone. That there is a relationship between the pituitary-adrenal system and ADH is supported by the excellent work of Gaunt et al.who found that hypothalamic antidiuretic activity varied inversely with plasma cortical hormone content.[146] The studies of pituitary ACTH content by Fortier[167,168] reveal that changes in ACTH concentration parallel the hypothalamic antidiuretic activity observed by Gaunt.

The inhibitory effect of ADH on ACTH release as demonstrated by Arimura and other Japanese investigators may be another factor in the background of the ACTH release story. The fact that this phenomenon is demonstrated with near-physiologic doses and seems to be quite specific in activity supports such a concept.

With these qualifications in mind it seems justified to relegate the ADH theory of ACTH release to the emeritus role enjoyed by previous attempts to explain this neural-glandular link in ACTH release. The contributions of this theory have been both material and intellectual for it has stimulated much of the present work in the field. Is not this sufficient justification for these studies?

PART VII

Conclusion

The possibility that the posterior pituitary may be essential for the release of ACTH by the anterior pituitary is of current interest largely because of the ACTH releasing activity of one of its hormones, vasopressin. Theoretical and experimental evidence against a role of vasopressin in the homeostatic control of the adrenal cortex was reviewed in the introductory sections of this thesis. The ACTH activating role of the posterior pituitary per se, however, has not been studied satisfactorily.

In an experimental approach to this relationship between the two parts of the pituitary the ACTH stress response was evaluated in neurohypophysectomized rats. These animals which demonstrated clinical and anatomical evidences of loss of the posterior pituitary and maintenance of the anterior pituitary were subjected to "neurologic" and"metabolic" stresses. ACTH release was measured by quantitative increases in peripheral plasma corticosterone and adrenal ascorbic acid depletion.

The neurohypophysectomized animal responds to either mode of stress with a quantitative elevation of plasma corticosterone statistically identical to the response of the normal rat. In addition, both the neurohypophysectomized and sham operated rats demonstrate a chronic increase in the resting levels of corticosterone. Ascorbic acid depletion revealed no inhibition of ACTH release when the experimental adrenals were compared to the significantly elevated control adrenal concentrations found in the neurohypophysectomized and sham operated animals.

There was no quantitative difference in the corticosterone elevations following neurologic or metabolic stresses in either normal, sham operated or neurohypophysectomized animals. These observations stand in sharp contrast to those who argue that the posterior lobe is necessary for either neural or metabolic stress induced ACTH release. Using corticosterone as an index of ACTH response we found no difference between the types of stress; both were equally effective in the neurohypophysectomized as well as the sham operated and normal rat. An apparent inhibition to both was noted with the adrenal ascorbic acid depletion

test for ACTH, however, this was a nonspecific effect also seen in the sham operated animals. When the ascorbic acid response is compared to the significantly elevated resting adrenal ascorbic acid concentrations seen in the neurohypophysectomized and sham operated animals significant depletion occurs. It is believed that the inhibitions of ACTH release reported by other investigators are based upon their failure to recognize that this elevation occurs in operated animals regardless of posterior pituitary function and that their use of lower control values has masked the actual ascorbic acid depletion of the neurohypophysectomized animal.

These evidences lead us to conclude that the presence of the posterior pituitary <u>per se</u> is not essential for ACTH release. The significance of this conclusion is far reaching. It indicates that the presence of ACTH releasing factors in the neural lobe is not essential for ACTH release. When taken in conjunction with our earlier studies in hypothalamic lesioned rats with diabetes insipidus but no inhibition of ACTH release, this stands as evidence against the necessity of vasopressin in ACTH release. It can also be interpreted as excluding the supraoptico-

hypophyseal tract, essential for ADH release, from playing a major role in ACTH release.

In view of the previous investigations which have demonstrated the physiologic dissociation between ACTH release and ADH release and in view of the excessive doses of exogenous vasopressin necessary for the release of ACTH, the conclusion that ADH does not play an essential role in ACTH release seems inescapable.

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ABSTRACT

The neural control of ACTH release has been well documented,however,the mechanism of this neuroglandular relationship remains unknown. One of the current theories explaining this neural-glandular link is that a substance might be released by the posterior pituitary which would act as a humoral stimulus of ACTH release by the anterior lobe. It is also postulated that vasopressin is this neurohumoral mediator.

In support of this theory are the observations by several investigators that neurohypophysectomy blocks part of the ACTH release following stress. The results of these investigations are discordant and do not adequately support their thesis. This was re-investigated with plasma corticosterone as the index of ACTH release and it was observed that there was no quantitative inhibition of ACTH release in the neuro-hypophysectomized rat. Concomitant observations of adrenal ascorbic acid depletion in these animals demonstrated apparent blockade of this response to

ACTH however close scrutiny reveals that this inhibition, reported by other investigators, is only apparent and has no relationship to the type of stress, absence or presence of the posterior lobe and is an artifact based upon defective experimental design.

The significance of these observations is far reaching for they provide evidence that the posterior lobe is not the site of the ACTH stimulating neuro-humoral release; they demonstrate that ACTH release is possible in the absence of vasopressin (diabetes insipidus) and they also give insight into the discrepancies between corticosterone and ascorbic acid depletion indices of ACTH release which are being reported in the contemporary literature.

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