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THE EFFECT OF STRESS ON PITUITARY
AND HYPOTHALAMIC METABOLISM AND
ACTH SYNTHESIS

DISSERTATION

Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

David Meyer Jacobowitz, B.S., M.Sc.

* * * * * * *

The Ohio State University
1962

Approved by

[Signature]

Adviser
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This work is dedicated to my wife Ilene and son Robert Keith who have endured the "Stress" of this dissertation.
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"A grain of slightly mad recklessness might, in this domain as in others, be the price you have to pay for great and noble findings."

- C. Levi-Strauss,
  in Diogenes.
I. INTRODUCTION AND STATEMENT OF THE PROBLEM

Observations on many animal species, including humans, indicate that in some way the central nervous system exerts a controlling influence over the discharge of ACTH from the adenohypophysis. There is also evidence that physical factors of the external environment, acting through the central nervous system, may profoundly influence the secretion of ACTH and the other pituitary tropic hormones.

The results of experiments involving stimulation and lesions in the hypothalamus, section of the pituitary stalk, and transplantation of the adenohypophysis indicate that the hypothalamus regulates the secretion of at least some of the anterior pituitary hormones. At the present time the anatomical site in the central nervous system that regulates ACTH secretion is not fully established.

A review of the literature concerning ACTH secretion reveals that many of the studies have been concerned with the mechanisms responsible for the increased secretion of ACTH from the pituitary during stress situations. However, few direct studies on the biosynthesis of ACTH have been made. Only little information is available on the possible modifications of the metabolism of the pituitary gland and the hypothalamus by conditions known to affect the secretion of anterior pituitary hormones.

Recently Vernikos-Danellis (1962) demonstrated that 2½ minutes after stress in adrenalectomized rats there was an increase in ACTH
content of the adenohypophysis and a concomitant increase in blood ACTH. In addition, Marks and Vernikos-Danellis (1962) showed that ethionine prevented the stress-induced ACTH secretion from the anterior lobe of the pituitary gland by preventing the synthesis of ACTH. These studies strongly suggest that stress caused an increased synthesis of ACTH.

Metabolic alterations have not been studied in relation to stress-induced synthesis of ACTH. Increased synthetic processes are often reflected in increased oxygen consumption of the tissue involved. In addition, several endocrine mechanisms have been shown to involve glycogenolysis which may supply the energy necessary for synthetic mechanisms.

Isolation and identification of rat ACTH have never been reported. Huge numbers of rat adenohypophyses would be necessary to isolate in pure form measureable quantities of ACTH. Studies on the electrophoretic and chromatographic properties of rat ACTH have never been performed. The in vivo incorporation of a radioactive amino acid into the ACTH molecule has never been achieved. Incorporation of a labeled amino acid into newly synthesized ACTH would greatly facilitate the task of isolation, identification and quantitation of this polypeptide. Stress-induced synthesis of ACTH could then also be measured. In addition, the stress-induced changes in uptake of a radioactive labeled amino acid in the adenohypophysis could be used to measure the synthesis and release of ACTH.

The purpose of this study is to determine whether an acute stress results in changes in metabolic properties of the pituitary
and the associated parts of the central nervous system that favor the new formation of ACTH. A secondary purpose is to characterize rat ACTH biochemically and to compare its properties with the purified hormone derived from other species.

This study attempts to answer the question - does stress activation result in an abrupt increase in ACTH synthesis in the adenohypophysis?
II. HISTORICAL REVIEW

A. Introduction

No other structure in the body is so well hidden and protected as the pituitary gland. It is a body that is concealed in a bony case, the skull, and is further protected by another bony envelope. If the acts of nature are indeed purposeful, then the importance of this centrally placed organ cannot be underestimated.

The anatomical relationship of the pituitary to the brain led ancient anatomists to suggest that the pituitary was under the influence of the brain (Cushing, 1932). Galen and Vesalius considered the pituitary to be a filter or trap through which the slime or pituita flowed. The pituitary was considered to contain waste products formed in the cerebral ventricles as a result of transformation of vital spirits into animal spirits.

These historic misinterpretations clearly show that even erroneous theories may be very valuable in directing investigations. Advances in endocrine experimentation have established the anterior-lobe of the pituitary as an important hormone-producing organ and a regulator of the activity of other endocrine glands.

Critical investigations of the function of the anterior pituitary gland was begun by Smith (1927; 1930) who perfected the technique of hypophysectomy in rats. He observed that hypophysectomy in the
rat caused an atrophy of the adrenal cortex. He showed that daily transplants of pituitary tissue could restore the adrenal cortex to a normal condition. This was therefore the first demonstration that the adrenal cortex depended upon the secretory activity of the pituitary gland. Evans (1933) and Collip et al. (1933) demonstrated that daily injections of pituitary extracts also could restore the adrenal cortex to normal. In addition, pituitary extracts injected into normal animals produced a marked hypertrophy of the adrenal cortex (Moon, 1937). Hence, the name adrenocorticotropic hormone (ACTH) has been used to designate the active principle in pituitary extracts that accomplishes repair of adrenal cortical atrophy following hypophysectomy.

Li et al. (1943) and Sayers et al. (1943) reported the isolation of an apparently homogeneous protein from the pituitary glands of the sheep and hog respectively, which was considered to represent the natural form of ACTH. In recent years adrenocorticotrophic hormones (ACTH) have been isolated in highly purified form from sheep (Li et al., 1954; 1955), pig (White, 1953; Bell, 1954; Shepherd et al., 1956; Dixon and Stack-Dunne, 1955) and beef (Li and Dixon, 1956) anterior pituitaries, and their amino acid sequences (Li et al., 1955a; 1958; Howard et al., 1955; White and Landman, 1955) have been determined.

Adrenocorticotropins from these three species are single-chain polypeptides composed of 39 amino acids with serine and phenylalanine as N- and C-terminal amino acids, respectively (Li, 1956). The only
difference in amino acid composition between the pig and sheep hormones is one more leucine in the former and one more serine in the latter. Beef and sheep have three serine molecules and the pig has two. There are no differences in amino acid content between beef and sheep ACTH, but differences in sequence exist.

It is of interest that in recent years the pituitary has generally been believed to be influenced by the brain. In 1930 Popa and Fielding demonstrated the existence of a network of fine blood vessels in the hypothalamic area at the base of the brain that coalesce into larger vessels leading down the pituitary stalk from the hypothalamus to the pituitary gland, where they once more divide into sinusoids in the adenohypophysis. This portal circulation is ideally suited to carry chemical products of the hypothalamus directly to the adenohypophysis and is the basis of the concept that neurohumoral secretions from the hypothalamus control the function of the adenohypophysis (Harris, 1955). Thus we see that modern theory is strikingly analogous to that of the early anatomists.

B. Hypothalamic Regulation of Pituitary Function

1. Anatomical Considerations

The difference in embryological origins of the two lobes of the pituitary justifies the existence of two entirely different types of connections with the hypothalamus.

The neurohypophysis is derived embryologically from ectoderm as it invaginates to form the neural tube. According to the accepted
The nomenclature for the hypophysis and its various subdivisions (Rioch et al., 1940), the neurohypophysis consists of three parts: the infundibular process (or neural lobe), the infundibular stem and the median eminence of the tuber cinereum. The median eminence is the expanded upper end of the pituitary stalk. Cytological evidence (Gersh, 1939) supports the belief that the neural lobe is composed of tissue different from that of the hypothalamus.

The neurohypophysis contains the terminations of the supraopticohypophysial tract, originating in the paraventricular and supraoptic nuclei in the hypothalamus. According to present concepts of the neurosecretory mechanism (Scharrer and Scharrer, 1954; Rinne, 1960), the hormones of the posterior pituitary gland are formed in these two nuclear groups, then transported as droplets of neurosecretory material down the axons of the tract and stored or liberated into the blood. The fibers of the supraopticohypophysial tract then are regarded as having a dual function of a transport system and a secretomotor innervation.

The adenohypophysis is derived embryologically from the endoderm of the foregut which invaginates to form Rathke's pouch. It is related to the hypothalamus through an entirely different system. The adenohypophysis is subdivided into the pars distalis, pars tuberalis, and pars intermedia. A very well-developed and unusual vascular system connects the median eminence and anterior lobe of the pituitary gland. The blood vasculature of the pars distalis may be compared with that of the liver in that it possesses a systemic arterial
supply, a portal blood supply and a systemic venous drainage. The systemic arterial supply is variable in different animal forms (Harris, 1955). In some animals (e.g., rabbit, human, dog), a definite arterial twig derived from the internal carotid artery, can be followed into the anterior pituitary, while in other forms (e.g., rat, bird) the systemic supply seems to be scanty.

In the rat, the blood supply of the anterior lobe appears to be restricted to the portal circulation (Daniel and Prichard, 1956; Goldman, 1962). The blood supply is derived from small arteries branching from the internal carotid and posterior communicating arteries which supply a plexus between the pars tuberalis and median eminence (Popa and Fielding, 1930; Green and Harris, 1947). From this plexus capillaries of varying shapes and sizes, often in the form of loops, penetrate into the median eminence where they form the primary plexus of the portal vessels. These are drained by the large trunks of the portal vessels which run down the pituitary stalk into the pars distalis. Here the portal vessels break up into sinusoids, the secondary capillary plexus of this portal system. Green and Harris (1947) suggested that this peculiar vascular formation might serve as a connecting link between the central nervous system and the adenohypophysis by means of a humoral relay through the hypophysial portal vessels.

2. Hypothalamic Control of ACTH Release

Harris (1946) recognized the importance of the vascular connection between the adenohypophysis and hypothalamus as a possible pathway for the control of the pituitary stress response. This "connecting link"
represents a "final common path" for a great variety of stimuli which induce ACTH release. The mechanism by which the portal vessels exert a controlling influence over the anterior pituitary is not certain. Harris postulated that as a result of hypothalamic activation a humoral substance or substances are released into the primary plexus of the portal vessels in the median eminence which is carried to the pars distalis of the pituitary where it exerts a controlling action.

The existence of a nerve supply to the anterior pituitary appears to be generally doubted, although the question of innervation of the adenohypophysis is still not finally settled. Metuzals (1954, 1955, 1956), using the Bielschowsky-Gros method of staining nerve fibers, found a nervous end plexus in the horse and the duck and in addition ganglion cells mainly in the capsule. Hagen (1950) observed extensive sympathetic innervation in the human hypophysis. In the cat, Metuzals (1959) found two nerve networks in the adenohypophysis - one sympathetic in origin, the other from the hypothalamus. The latter fibers degenerated after lesions in the median eminence. Recently Sigg and Schapiro (1961) described evoked potentials in the anterior pituitary following stimulation of the fornix, hippocampus and mesencephalic reticular formation, indicating that a nerve pathway from these central sites to the adenohypophysis was present.

One of the difficulties in evaluating early work on nervous innervation studies is the difficulty involved in interpreting silver staining techniques. A good deal of the experimental data indicates that nerve fibers to the adenohypophysis are not essential for
secretion of most of the tropic hormones. The fact that complete sympathectomy does not prevent normal reproduction in female rats (Cannon et al., 1929) and does not cause very significant change in the metabolic rate of cats or rats (Lee and Bacq, 1933) demonstrates that a sympathetic innervation of the pituitary plays no appreciable part in the control of the secretion of gonadotropic or thyrotropic hormones. In addition, pseudopregnancy still follows sterile coitus in the partially sympathectomized rat (Friedgood and Bevin, 1938) and ovulation still follows sterile coitus in the partially or completely sympathectomized rabbit (Brooks, 1935). The significance of a sympathetic nerve supply in relation to basal secretion of ACTH has not been demonstrated.

The rapidity with which ACTH is secreted by the pituitary gland in response to stress has supported the neurohumoral theory of secretion of ACTH. A nervous mechanism for release of ACTH due to stress would conceivably be equally or more rapid than a humoral factor released from the hypothalamus through the portal system to the anterior pituitary. Harris (1955a) states:

...the neurohumoral view as a whole will only be established if it is possible to firstly identify a particular substance which exerts a direct action on anterior pituitary cells; secondly, to show this substance is present in the blood in the hypophysial portal vessels in greater amount than in systemic blood; thirdly, to show that the concentration of this substance in the blood of the hypophysial portal vessels varies according to electrical or reflex activation of hypothalamic nerve tracts; and fourthly, to demonstrate that the activity of the adenohypophysis is correlated with this varying concentration.

Data are not yet available that fulfill these criteria.
A suggestive series of experiments support the concept of a neurohumoral mediator of hypothalamic origin involved in the release of ACTH. Porter and Jones (1956) presented strong evidence for a neurohumoral transmitter by showing that extracts from plasma, obtained from the severed portal vessels of hypophysectomized dogs, cause an adrenal ascorbic acid depletion in hydrocortisone treated rats.

 Interruption of the stalk of the pituitary gland prevents the secretion of ACTH following exposure to a variety of stress situations (Fortier and Selye, 1949; DeGroot and Harris, 1950). If the portal system regenerates after stalk section, return of normal adenohypophysial function is noted (Fortier et al., 1957). Transplants of pituitary tissue are inadequate for the maintenance of the functional activities of the adenohypophysis (Fortier and Selye, 1949; Cheng et al., 1949; Harris and Jacobsohn, 1952). Nikitovitch-Winer and Everett (1953) showed that the pituitary which had been transplanted to the kidney for a month was histologically and functionally improved when relocated under the median eminence. The cells of the transplant showed improved staining characteristics and the target organs, the ovary, thyroid and adrenal cortex, although not restored to normal, were significantly improved.

McCann (1953) showed that lesions of the median eminence completely block stress-induced increases in ACTH secretion in the rat. Similar effects were demonstrated in the rabbit (DeGroot and Harris, 1950), cat (Lacquer et al., 1955), dog (Ganong et al., 1961) and monkey (Anand and Dua, 1955).
On the other hand, in all mammalian species studied, stimulation of the median eminence and dorsally adjacent area increased ACTH secretion (Anand and Dua, 1955; DeGroot and Harris, 1950; Harris, 1955; Hume, 1952; Katsuki et al., 1956; Mason, 1959). This evidence provides strong support for a hypothalamic involvement in the control of pituitary function.

3. Corticotropin-releasing factors

It has been proposed (Harris, 1948; Hume, 1952) that neural stimulation may result in the release of a hypothalamic neurohumor which ultimately provokes the secretion of ACTH by the adenohypophysis. The term corticotropin releasing factor (CRF) was first suggested by Saffran, Schally and Benfey (1955). The term is meant to apply to a substance which has a specific action on the adenohypophysis to release only ACTH. CRF is considered to be elaborated by the hypothalamus and carried by way of the portal venous system to the anterior pituitary. By definition (Schally and Guillemi, 1960), CRF acts directly on the cells of the anterior pituitary and therefore should be active in vivo and in vitro. Saffran et al. (1955) suggested that the posterior lobe of the pituitary plays an important role in the stimulation by stress of the anterior pituitary for ACTH release. They hypothesized that the neurohypophysis is a relay between the hypothalamus and adenohypophysis for storage and secretion of the proposed neurohumor.

For the past decade attempts have been made to isolate corticotropin releasing principles. Slusher and Roberts (1954) suggested that
the posterior hypothalamus may elaborate a pituitary stimulating factor which appears to be lipid or lipoprotein in nature. Porter and Rumsfeld (1956) reported a substance found in hypophyseal portal plasma of dogs which was either a large protein molecule or is bound to a large protein. The same authors reported obtaining a substance from acetone extracts of beef hypothalamus which caused an adrenal ascorbic acid depletion (AAAD) in the cortisol-blocked but not in the hypophysectomized rat (Rumsfeld and Porter, 1959).

Fuche and Kahlson (1957) favor histamine as a CRF. They found that histamine causes a significant lymphopenic response in normal and not in hypophysectomized rabbits.

Because the hypothalamus and neurohypophysis are continuous, proposals have been made that the ACTH releasing principle would be expected to be concentrated, like vasopressin and oxytocin, in the posterior pituitary (Zuckerman, 1952). Stress stimulation has been observed to deplete the stainable neurosecretory material in the neurohypophysis, suggesting an association between the posterior pituitary hormones, vasopressin and oxytocin (thought to be associated with the neurosecretory material), and the release of ACTH (Rothbailer, 1953).

The work of Landsmeer (1951) provides an explanation for the difficulty involved in accounting for the transport of a posterior lobe ACTH releasing factor. He described in the rat extensive anastomoses between the vasculature of the two lobes of pituitary. In addition, Jewell (1956) reported that similar connections are present in the dog.
The administration of vasopressin caused secretion of ACTH in normal animals (Martini and Morpurgo, 1955; Sobel et al., 1955; McDonald et al., 1956) and in animals with hypothalamic lesions that abolished stress-induced ACTH release (McCann and Brobeck, 1954). Guillemin (1955) has experimentally demonstrated by using various pharmacological antagonists that the known neurohumors, acetylcholine, epinephrine, norepinephrine and histamine were not the agents responsible for pituitary stimulation by systemic stress.

Interest has recently been centered on peptides of hypothalamic or neurohypophysial origin. Two groups of investigators independently suggested that the ACTH-releasing activity of preparations of antidiuretic hormone was in reality ascribable to a distinct peptide present as an impurity (Saffran et al., 1955; Guillemin et al., 1957). The active "cortico-tropin-releasing factor" (CRF) was purified and found to be similar in amino acid composition to the antidiuretic hormone (Schally et al., 1958) and to possess significant antidiuretic activity itself (Guillemin et al., 1957). The CRF appears to be closely related to vasopressin judging from the difficulty involved in the chemical separation of their individual activities.

Recently two distinct active pure polypeptides with CRF activity have been isolated from hog posterior pituitary extracts (Schally et al., 1960). They have been named α- and β-CRF, and are respectively related in amino acid composition to α-MSH and to vasopressin.

The α-CRF is a peptide containing the amino acids of α-melanocyte stimulating hormone (α-MSH) plus threonine, alanine and
leucine (16 amino acids). Therefore, α-CRF and α-MSH have exactly the same first 13 amino acids as ACTH (plus an N-acetyl group).

\[
\text{CH}_2\text{CO-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Try-Gly-Lys-Pro-Val-NH}_2
\]

\[
\text{CRF} = \alpha - \text{MSH} + \text{Thr}-\text{ala}-\text{leu}
\]

Schally et al. (1960) have proposed that α-CRF is possibly a precursor in the biosynthesis of ACTH.

β-CRF, on the other hand, has a higher corticotropin releasing potency than α-CRF and has been proposed to be the true chemical mediator for release of ACTH. The exact nature of this molecule is unknown. β-CRF has 7 or 8 amino acids in common with lysine vasopressin plus "major amounts" of serine and histidine (Schally et al., 1958). β-CRF is equivalent to Guillemin's fraction D (Guillemin et al., 1957) which has CRF activity at a concentration greater than 0.5 μg, whereas Schally et al. (1958) reported that their most highly purified material had detectable activity at 1/1000 of that concentration.

Chemically related hormones with similar biological actions are not without precedence in the animal organism. The corticosteroids of the adrenal gland are outstanding in this respect. With minor differences in structure they exhibit major differences in activity, although some overlapping of activity exists. It appears that the hypothalamus-neurohypophysis may contain a family of related peptides with independent actions - vasopressin acting mainly on the kidney; oxytocin on the
mammary glands and uterus; CRF on the adenohypophysis. All have the property of releasing ACTH (McCann and Brobeck, 1954; Rinne et al., 1959). In addition, \( \alpha \)-MSH, found in the pars intermedia, and recently isolated from the hog and dog hypothalamus (Schally et al., 1962; Guillemin et al., 1962) was shown to have ACTH activity both in vivo and in vitro assays (Steelman and Guillemin, 1959).

The ACTH-releasing activity of vasopressin supports the results of those that consider it to be the CRF (Martini and Morpurgo, 1955; Sobel et al., 1955; McDonald et al., 1956). However, the antidiuretic activity of CRF may explain the increased antidiuretic titer in blood of animals exposed to stress (Mirsky et al., 1956).

The question of vasopressin being a CRF is not entirely settled. Those who favor the vasopressin-CRF theory cite experimental support demonstrating that small doses of vasopressin stimulate ACTH release (McCann and Brobeck, 1954; McDonald and Weise, 1956a; 1956b). The critics opposing this theory state that the vasopressin doses, although small, are larger than those required for maximal antidiuresis (Nichols, 1961). Nichols and Guillemin (1959) showed that exogenous \( \alpha \)-MSH was unable to stimulate the secretion of ACTH until a threshold of \( 3-7 \times 10^3 \) times the dose producing maximal physiologic antidiuresis was exceeded. On the other hand, Kwaan and Bartelstone (1959) demonstrated a definite increase in adrenal 17-hydroxycorticosteroid output in the dog by intraventricular injections of 2 milliunits (mu) of arginine vasopressin. This response closely resembled the pattern of response which followed intravenous injection of corticotropin in hypophysectomized
dogs (Nelson and Hume, 1955). In this regard, a small quantity of vasopressin liberated into the portal system would constitute a high concentration reaching the adenohypophysis. Goldman and Lindner (1962), using measurements of rat neural lobe blood flow, calculate that the concentration of ADH in the effluent of the neural lobe of the haemorrhaged rat increases by 300 fold over the resting level. Evidence that a substantial fraction of the neural lobe effluent perfuses the anterior lobe is cited by these authors. They calculate that the concentration of ADH in the neural lobe blood of the haemorrhaged rat is close to 2 units/ml, an enormously high concentration.

The close structural similarity between β-CRF and vasopressin no doubt accounts for similarity in ACTH releasing action and both indeed may physiologically share this property. It may be that the release of ACTH is a property shared by a number of substances rather than the property of a single neuchormone.

C. Effect of Stress on ACTH Release and Synthesis

It has been well established that discharge of ACTH from the adenohypophysis occurs as a result of stress and results in the activation of the adrenal cortex (Sayers, 1950). After hypophysectomy, the adrenal cortex atrophies (Smith, 1927; 1930). Stress increases the size and decreases the concentration of ascorbic acid of the adrenal cortex and fails to influence the gland in the absence of the pituitary (Ingle, 1938; Sayers et al., 1945).
There is an initial biphasic response to stress consisting of a brief elevated secretion of ACTH followed by a period of lower than normal output (Brodish and Long, 1956; Sayers, 1957; Knigge et al., 1959).

Hodges and Vernikos (1959) found that adrenalectomy resulted in an immediate and brief rise of circulating ACTH which was considered to be due to the stress of the operation. There was no detectable increase in resting blood ACTH until 10 days following adrenalectomy. After this time the blood level of ACTH became increasingly elevated, reaching its maximum at 3 weeks following adrenalectomy. In addition, adrenalectomized rats responded to a less severe stress with an equally rapid and more intense rise in blood ACTH. The time relationships of the biphasic response to stress in blood ACTH in the intact and 4-week adrenalectomized rats were similar. This rapidity of response of the pituitary in secreting ACTH in both intact and adrenalectomized rats is in agreement with the existence of a neural or neurohumoral mechanism controlling the release of ACTH in response to stress. The fact that the pituitary gland of the adrenalectomized rat is more reactive to mild stress than the pituitary gland of the intact animal may be explained by the changes in the circulating corticoid levels. Hodges and Vernikos (1959) believe that "the delay in the development of a high circulating ACTH level in the adrenalectomized rat may be due to the slow rate at which the pituitary gland adapts itself to secrete and maintain an enormously increased output of ACTH in response to the complete absence of circulating corticoids." Sayers et al., 1958, suggest
that "perhaps pituitary synthesis is unable to keep up with increased rates of release of ACTH in the early period following adrenalectomy."

Brodish and Long (1956) have proposed a dual mechanism theory for the control of ACTH secretion in response to stress. They suggested that in the biphasic pattern of ACTH secretion, the sustained slowly developing increase of ACTH secretion following the period of subnormal ACTH release was dependent on the peripheral level of corticoids.

Kitay et al. (1958) showed that the administration of exogenous ACTH to adrenalectomized rats prevented the usual fall in pituitary ACTH due to stress. They suggested that the increase in pituitary ACTH concentration produced by exogenous ACTH is due to inhibition of endogenous release of ACTH by the pituitary gland. The increased blood ACTH levels after stress could be inhibiting further release of ACTH by acting at the pituitary level.

Sydnor and Sayers (1954) suggested that the higher ACTH content of the pituitary in the rat after adrenalectomy indicates that increased synthesis as well as increased secretion of ACTH occurs following prolonged reduction of circulating adrenal corticosteroids.

Fortier (1959) demonstrated that there was an immediate rise in pituitary ACTH following bilateral adrenalectomy and a subsequent fall of ACTH content to a minimum level after 12 hours, followed by a rise to supranormal levels. First pituitary assays were performed 30 minutes after adrenalectomy. Concomitant initial peaks of pituitary and blood ACTH enabled Fortier to infer that there was a transient
burst of synthesis followed by a predominant, though gradually receding acceleration of the rate of release. This accounted for the pituitary ACTH depletion and a greater and sustained acceleration of synthesis, thereby accounting for the subsequent rise in ACTH content. Fortier further suggested that the ACTH-releasing effect of stress is markedly influenced by the circulating adrenal cortical hormones. Withdrawal of the hormones enhances both release and synthesis of ACTH with a predominant effect on synthesis. Fortier and DeGroot (1959) support these findings by experiments in which they correlated the changes in pituitary ACTH level with those in plasma corticosteroid concentration in rats with one adrenal removed and the other enucleated. The pituitary ACTH content was elevated markedly during the period of adrenal insufficiency, but then declined with cortical regeneration and increased blood corticosteroid. In order to study the degree of hypothalamic involvement in the maintenance of ACTH secretion, Fortier and DeGroot (1959a) produced electrolytic lesions in the median eminence of the rat. They were able to infer that suppression of hypothalamic influence results in marked depression of both synthesis and release of ACTH.

Fortier (1962) postulates that destruction of the median eminence may be "associated, in all likelihood, with impairment of the adenohypophysial blood supply" thereby resulting in a significant depression of both synthesis and release of ACTH. The concept of accelerated synthesis following accelerated release triggered by stress is further supported by Fortier (1959b) using spleenectomy as a stress in rats.

Vernikos-Danelis (1962) demonstrated a very rapid synthesis of ACTH in stressed adrenalectomized rats. In 2½ minutes after the
initiation of a 1 minute ether stress, both blood and pituitary ACTH were significantly increased indicating a burst of synthesis. This was followed by a decline of both pituitary and blood ACTH levels below normal for a period of about two hours and subsequently rising to supernormal levels. In normal animals, on the other hand, ether stress plus sham adrenalectomy caused a significant rise in blood ACTH levels, but the pituitary ACTH content was unaltered at this time interval. Fortier (1959a) and Kitay et al. (1958), using Saffran and Schally's technique for in vitro bioassay of ACTH, both demonstrated a rise in pituitary ACTH content in response to adrenalectomy. The lack of pituitary response due to stress in intact rats in Vernikos-Danellis' experiments as compared to the latter groups using adrenalectomy as a stress may be attributed in part to a difference in assay procedures (in vivo adrenal ascorbic acid depletion versus Saffran and Schally's in vitro technique), intensity of stress and differences in the strain of the rat studied. In addition, the unchanged content of pituitary ACTH after stress in normal rats may be a reflection of a relatively balanced synthesis and release of ACTH. That release is occurring is reflected in the increase of blood ACTH after stress.
III. MATERIALS AND METHODS

A. Animals

All experiments were performed on female albino Wistar rats. They weighed 175-200 g. unless other weights are specified. They were kept at a constant temperature at 22°C. in the animal house. Several days before experiments the animals were equilibrated with the laboratory environment. Fortier (1958) showed that a slight environmental change resulting from the transfer of rats from one room to the next resulted in a marked increase in the plasma corticosteroid concentration. Therefore, the rats were kept in the laboratory several days before the experiment.

Barret et al. (1957) demonstrated that intact female rats secreted greater amounts of ACTH than male rats after an acute stress. Kitay (1961) showed that intact female rats demonstrated higher levels of plasma corticosterone after a standard stress of ether anesthesia than male rats. He concluded that ether stress causes a greater release of ACTH in female rats than in males.

Vernikos-Danellis (1962) demonstrated an increase in concentration of pituitary ACTH 2½ minutes after ether (1 minute) stress in 30-day adrenalectomized female rats. Since this study was largely an attempt to investigate the nature of the increase in ACTH concentration in the anterior pituitary after stress, the greater responsiveness of female rats made it desirable to use females in this investigation.
B. Experimental Techniques

1. Stress

Fortier et al. (1957) have attempted to classify components of stress into two general categories --- "somatic" or "systemic" (traumatic) stress --- which act directly on the pituitary, and "neurogenic" or "psychic" stress (emotional and painful) which acts through the central nervous system. Actually, it is impossible to apply a pure somatic or a pure psychic stress. Controversy has prevailed over this division of stresses. Hypothalamic lesions have been demonstrated to block not only the response to emotional stress, but also the response to operative trauma and insulin hypoglycemia (Ganong, 1959). Miahle-Voloss (1958) has postulated a new version of this theory. She found that an emotional stimulus (loud sound) induces the discharge of ACTH from the posterior pituitary, whereas a systemic stimulus (histamine) causes the release of ACTH from the anterior lobe of the pituitary in the rat and guinea pig. Posterior lobectomized rats have depressed plasma corticosterone and adrenal ascorbic acid depletion responses to "neurogenic" stimuli (noise, strange environment) and not to "systemic" stresses (hemorrhage, nicotine, histamine) (DeWied, 1961). Treatment with pitressin for five days restores pituitary response to neurogenic stimuli in the posterior lobectomized rats. This suggests that antidiuretic hormone is involved in corticotropin release due to "neurogenic" stimuli.
Sayers et al. (1958) suggest:

...it is possible to classify stimuli by intensity rather than modality. The act of isolating the pituitary by stalk section or transplantation may damage the gland to the extent that it is capable of responding only to intense stimuli. One could then conclude that restraint and exposure to cold are less intense than epinephrine injection or laparotomy since stalk-sectioned animals only respond to the latter two stimuli.

In a review by Ganong and Forsham (1960) a listing of types of stress reported to stimulate ACTH secretion is given. They include benzene intoxication, faradic stimulation, electric shock therapy, moving an animal into an unfamiliar room, insulin hypoglycemia, crowding animals in a cage, estrogen therapy, flying airplanes, hypoxia, the injection of bacterial products, anxiety induced under hypnosis, certain flavonoids, tumbling in a drum, serotonin, lysergic acid diethylamide, morphine, and cruel and vicious moving pictures. We are cautioned to avoid generalizations from the results of one particular stress in one particular animal species.

In this experiment an attempt was made to include the so-called "neurogenic" and "systemic" components of stress in order to obtain a sufficient rise in the blood level of ACTH of normal and adrenalectomized rats (Hodges and Vernikos, 1959).

The same stress was used throughout this study --- ether and sham adrenalectomy. The animals were exposed to ether vapour for 1 minute, by the end of which time they were completely anaesthetized. They were then quickly sham adrenalectomized by making an incision of about 2 inches on the dorsal aspect of the rat and penetrating through
the musculature on one side of the animal in the region of the adrenal. This procedure took approximately 15 seconds.

2. Adrenalectomy

Bilateral adrenalectomy was performed under ether anaesthesia as described by Burn (1950). The adrenalectomized rats were maintained on a normal diet with 0.9% sodium chloride solution substituting for drinking water. The completeness of adrenalectomy was confirmed at the time of sacrifice.

3. Sacrifice and tissue removal

The animals were sacrificed by decapitation, utilizing a commercial decapitator. The head was placed on a dissecting board and a midline incision was made in order to expose the calvarium. The top of the skull was cut and removed with scissors, the brain was carefully lifted and the optic nerves were cut. The pituitary gland was then exposed. The posterior pituitary was removed first followed by removal of the anterior pituitary.

The median eminence was removed by the use of a recessed glass guide described by McIlwain (1961). It consisted of a slice 0.15x4x7 mm of the ventral hypothalamus. The hypothalamus proper was removed with a curved forceps. In this study, the tissue used as "hypothalamus" consisted of a cube approximately 5 mm in each dimension extending from the optic chiasm anteriorly to the mammillary bodies posteriorly. All tissues were obtained within $1\frac{1}{2}$ minutes following the death of the animal.
C. Metabolic Methods

1. Warburg Respirometry

All incubations were carried out in micro-Warburg flasks of 7 ml. capacity containing 1 ml. of Krebs-Ringer phosphate medium (pH 7.4). The center well of each flask contained 0.1 ml. of 10% KOH and a small cylinder of filter paper extending about 2 mm above the neck of the well. The gas phase was air. Glucose (1 mg/ml.) was utilized in some of the studies.

Preliminary experiments were run in order to ascertain the minimum amount of tissue per flask of each organ that was necessary for reproducible oxygen consumption values. The amounts of tissue required was found to be 3 anterior pituitaries, 6 median eminences, or 3 hypothalami.

Immediately after removal from the animal, tissues from all organs were placed on small weighed glass squares (about 8 x 8 mm) which were contained in Stendor dishes that were lined with moist filter paper and resting in a container of cracked ice. Wet weights of the pooled tissues were obtained.

The individual flasks were allowed to equilibrate for 10 minutes at 37°C. with constant shaking (140 times per minute). Incubations were allowed to proceed for 2 hours.

Hydrocortisone added in vitro was tipped in from the side-arm after one hour of incubation. Hydrocortisone (100 μg) was suspended in 0.5 ml. of the Krebs-Ringer medium using an ultra-sound generator.
Manometer readings were taken every 15 minutes for 2 hours. Because of initial variable readings calculations were based upon the average QO2 (µl/hr/mg) obtained during the second hour.

Statistical differences were calculated by the Student "t" test.

2. Glycogen Determination

Reports in the literature have indicated that glycogen could not be demonstrated in the rat pituitary gland (Roberts and Keller, 1953; Barondes et al., 1961). Barondes used the trichloracetic extraction method of Carroll et al. (1956). The method used by Roberts and Keller was the anthrone procedure of Seifter et al. (1950).

Goodner and Freinkel (1961) were able to demonstrate glycogenesis in the calf anterior pituitary using labeled glucose-\(^{14}\)C. Carrier glycogen was used to extract the labeled glycogen which was measured in a gas-flow counter.

A micromethod for glycogen determination was therefore indicated. Seifter et al. (1950) reported a method for analysis of small quantities of glycogen. Preliminary experiments using this method indicated that a still further modification was necessary in order to measure anterior pituitary glycogen. A modification of the Seifter method was adopted and glycogen analysis was performed on anterior pituitaries, median eminences and hypothalami under normal and stress conditions. The procedure used was as follows:

a) The tissue was quickly removed from the decapitated rat and placed on a preweighed cover slip (about 8 x 8 mm) contained in a covered Stendor dish immersed in a freezing mixture of dry ice and
acetone. The tissue immediately froze on contact with the cover slip. In this manner glycojenolysis was minimized.

Pooled anterior pituitaries, median eminences and single hypothalami were used. At first, 6 anterior pituitaries were pooled and one value obtained. Later it was found that pooling only two pituitaries was quite adequate. Three median eminences were pooled. Therefore, weights ranging between 10-70 mg. of tissue were used.

b) The tissue was quickly weighed on a torsion balance so as to prevent thawing and placed in a centrifuge tube containing 1 ml. of 30% KOH solution.

c) The tubes were placed in a boiling water-bath for 20 minutes. This reduced the non-glycogen components of the tissue to a soluble form. The tubes were then cooled.

d) 1.25 ml. of 95% ethanol was added to each tube and the contents mixed. This precipitated the glycogen.

e) The tubes were again placed in a boiling water-bath and brought to a gentle boil and immediately removed so as to avoid splattering and then cooled.

f) The tubes were centrifuged for 30 minutes at 3000 rpm.

g) The supernate was decanted and the tubes were inverted and allowed to drain on filter paper for 2 minutes.

h) The sedimented glycogen was dissolved in 0.5 ml. of water.

i) The tubes were submerged in ice-cold water and 1 ml. of anthrone reagent (0.1% anthrone in 95% H2SO4) was added to each tube. The reactants were mixed using a test tube vibrator.
j) The tubes were placed in a boiling water-bath for 10 minutes, then cooled in a bath containing ice-water. A reagent blank was run through the procedure. The color produced was measured at 620 μm in a spectrophotometer. Micro-space adapters were used in the cuvette for reduced volumes. A glucose standardization curve was employed in determining the quantity of glucose in each tube (Fig. 1). Results were expressed as μg/mg tissue. The factor used for conversion of glucose to glycogen was μg/mg

3. Phosphorylase Assay

The assay method of Cori and Illingworth (1956) for phosphorylase activity was employed with minor modifications. Two forms of phosphorylase are measured, phosphorylase a, the active form, and phosphorylase b, the inactive form. Phosphorylase a is determined in the absence of adenylic acid (5-AMP), phosphorylase a + b is determined in the presence of 5-AMP. Although physiologically, phosphorylase catalyzes the breakdown of glycogen, the phosphorylase assay is based on the ability of the enzyme to synthesize glycogen from glucose-1-phosphate. The amount of inorganic phosphate formed from glucose-1-phosphate is a measure of the phosphorylase activity. Results are expressed as μmoles of inorganic phosphate (iP) formed. The percent of the active enzyme, phosphorylase a, is obtained from the expression:

\[
\frac{\text{iP formed without 5-AMP}}{\text{iP formed with 5-AMP}} \times 100
\]
Fig. 1. Glucose standardization curve.
The anterior pituitary was analyzed for phosphorylase activity. Constant conditions were maintained. The animals were decapitated and the tissues to be analyzed were quickly chilled, weighed and placed in a Potter-Elvehjem homogenizer. The mortar was chilled in ice-water. A small amount (0.5 ml.) of 0.1M NaF-0.002M EDTA (Versene) was added to the mortar. When the weight of the last pituitary was determined, additional NaF-EDTA solution was added so that the total dilution of the extract was 1:50 when using 3 pooled anterior pituitary glands. The tissue was homogenized for 5 minutes using a chilled teflon pestle. The homogenate was poured into a lusteroid test tube and centrifuged at 4°C. for 10 minutes at 9200 rpm.

The diluted extract was warmed to 30°C. 0.1 ml. aliquots were added to test tubes containing 0.1 ml. substrate (0.016M glucose-l-phosphate, 1% glycogen, and 0.001M 5-AMP when present). Duplicate tubes were incubated with the substrate without 5-AMP. The tubes were incubated for 10 minutes at 30°C. After exactly 10 minutes of incubation the reaction was stopped by the addition of 0.2 ml. of 10% trichloracetic acid. The acidified extracts were immediately centrifuged in the cold to minimize glucose-l-phosphate hydrolysis. Duplicate blanks were prepared by adding 0.1 ml. of extract and 0.1 ml. substrate to a test tube containing 0.2 ml. 10% trichloracetic acid.

Inorganic phosphate liberated from glucose-l-phosphate was determined by the Fiske and Subbarow method (1925) using 0.2 ml. of the acidified extract.

A standard curve for inorganic phosphate was prepared using K$_2$HPO$_4$ (0.1-1μM) versus optical density (Fig. 2).
Fig. 2. Standard curve for inorganic phosphate.
Results were expressed as μM inorganic phosphate/mg tissue liberated in 10 minutes. The active phosphorylase was expressed as a percentage of the total phosphorylase. Each reported value was the mean of duplicate determinations.

D. Methods Using Radioactive Substrates

1. In vivo uptake of H\textsuperscript{3}-phenylalanine

Tritiated amino acid, DL-β-phenylalanine, uniformly labeled, was obtained from Radiochemical Centre, Amersham, England, possessing a specific activity of 1800 mc/mM (or 11.3 mc/mg). A stock solution was prepared (500 μc/ml. water) and kept frozen when not in use. Portions of the stock solution were diluted with saline before use.

Both normal (180-230 gm) and 30-day adrenalectomized (190-250 gm) rats were injected subcutaneously with 250 μc/Kg tritiated phenylalanine. Those animals receiving hydrocortisone were injected subcutaneously with hydrocortisone suspension 7.5 mg/100 g. 4 hours before receiving tritiated phenylalanine. The hydrocortisone was suspended in saline. The stressed groups of rats were stressed 10 minutes after injection of the radioactive label. The animals were sacrificed 2½ minutes after the beginning of the stress. The control group of rats were sacrificed by decapitation 12½ minutes after injection of the labeled amino acid.

The brain was removed as previously described. The anterior pituitary, posterior pituitary, median eminence, anterior hypothalamus and posterior hypothalamus were removed and weighed immediately. The
anterior pituitary was blotted with filter paper to remove adhering blood. The individual tissues were analyzed for radioactive content.

2. Determination of $H^3$ and $C^{14}$ in tissue by Schöniger combustion

Radioactivity in all tissues was measured by a procedure combining combustion of the dried tissue in a modified Schöniger flask and liquid scintillation counting as described by Kelly et al. (1961).

The principle of Schöniger combustion is the conversion of all the tissue hydrogen to water and all the carbon to CO$_2$. Tissues were put into combustible sample holders made of dialysis tubing. The bag was made from a 5 cm piece of 3/4 inch diameter Visking seamless cellulose sausage casing by folding one end and sealing with a drop of Duco cement. The bag was cut into the shape of a ladle with a 2 cm "cup" as a sample holder. The wet tissue was placed in the bag which was hung by the "handle" for air drying. Rapid drying may be accomplished by hanging the bags over a hot plate.

Combustions were carried out in heavy-walled 2 liter filter flasks. The side arm of the flask was kept closed with clamped rubber tubing for tritium analysis. For C-14 determination the side arm was closed with a rubber serum bottle stopper. Hyamine hydroxide added to the flask was injected through this cap. Commercially available ignition heads did not fit the 2 liter flasks needed for these combustions. Modified heads were designed and built (Fig. 3). The ignition head contained two 18-gauge platinum leads sealed into the bottom of glass tubing. In the glass tubing the platinum leads were
Fig. 3. Modified ignition head for Schöniger combustion.
connected by an ignition filament consisting of coiled 23 gauge platinum wire. A platinum basket sample holder was welded to one of the leads (23 gauge platinum). An F & M Remote Ignition Unit (Model 141A), delivering 12.5-25 volts, was used to trigger the ignition. The sample holder was placed in the platinum basket and the handle was wrapped around the ignition filament. In this manner the handle served as a fuse. The flask was filled with oxygen and stoppered tightly with the ignition head containing the dried tissue sample. Samples up to 200 mg. of dry weight could be combusted in this manner.

For tritium determination, the flask was placed in a dry-ice-acetone bath after combustion so that the bottom surface of the flask was in contact with the freezing mixture. The water vapor thereby condensed and froze on the bottom surface of the flask. The flask remained in the freezing mixture for ½ hour, after which time 20 ml. of toluene-phosphor solution containing 20% ethanol was added and swirled around the bottom of the flask to dissolve the ice. The stoppered flask was then placed in ice water for 15 minutes. A 15 ml. aliquot was drawn out of the flask with a pipette and added to a vial used for liquid scintillation counting. The phosphor scintillation solution was made up of 4.0 g. 2,5-diphenyloxazole (PPO) and 50 mg. 1,4-bis (5-phenyl-2-oxazolyl) benzene (POPOP) per liter of toluene. Enough absolute alcohol (redistilled) was added to make a final concentration of 20% ethanol in the phosphor.

For carbon-14 analysis, the flask was cooled after sample ignition in the dry ice-acetone freezing mixture in order to reduce the
pressure within the flask. The flask was removed from the freezing mixture and 10 ml. of 1M Hyamine hydroxide in methanol was then injected through the rubber cap enclosing the side-arm by use of a 10 ml. syringe and needle. After standing for ½ hr. at room temperature, duplicate 3 ml. aliquots of the solution were removed for counting and added to 10 ml. of phosphor solution in a vial.

a) Measurement of radioactivity by liquid scintillation counting

The assay of radioactivity was performed in a Packard Tricarb Liquid Scintillation Counter. This method has the advantage of increased sensitivity, efficiency and freedom from self-absorption effects for most tracers. The scintillation fluid used in these studies contained enough ethanol to provide effective solution of the water in the non-polar scintillator fluid medium.

In all instances in which it will be claimed in this dissertation that uptake of radioactivity occurred, sufficient counts were collected to provide a probable error of less than 5%. The graphical method of Loevinger and Berman (1951) was used to determine probable error of counting. After sufficient counting of each vial an internal standard was added. The standard for tritium was tritiated water, 1 ml. containing $1.7 \times 10^{14}$ dpm. For C-14 internal standard, benzoic acid C-14 was used, 1 ml. containing 1600 dpm. The use of internal standards corrects for quenching in the individual vials. Duplicate samples of internal standard in the scintillation fluid were run. In addition to providing quenching information, internal standards determine the
counting efficiency of the counter. In all runs a phosphor solution blank was run for the determination of background.

Results were expressed as disintegrations per minute per mg. of wet weight of tissue (dpm/mg). Calculations for obtaining dpm from observed counts/minute (cpm) follow:

\[
cpm - \text{background} \times \frac{\text{Internal Standard-background}}{\text{cpm after I.S.} - \text{cpm before I.S.}} \times \frac{1}{\text{tissue wt.}} \times \text{aliquot factor} \times 100 / \text{efficiency}
\]

The efficiency was determined by dividing the cpm obtained from the radioactive standard by the dpm added to the vial containing phosphor:

\[
E = \frac{\text{cpm observed}}{\text{dpm added}} \times 100
\]

3. In vivo uptake of radioactive amino acids and extraction of peptides

Two experiments were run, each with a different labeled amino acid, tritiated phenylalanine and C-14 serine. DL-Serine-1-\text{\textsuperscript{14}C} (\text{HOCH}_2\text{-CH(NH}_2\text{)} \text{C}\text{\textsuperscript{14}C}_2\text{OCH}), obtained from New England Nuclear Corp., with 0.039 mc/mg. of radioactivity contained in powder form, was dissolved in water to give 250 \text{mc/ml. solution.}

Each experiment contained two groups of rats, a normal and stressed group. In each group the tissue of 6 rats were pooled, except in the C-14 serine stress group where 5 rats were used. The weight of the rats in the phenylalanine group was 210-260 g. The serine group weighed 145-175 g. Identical experimental procedures were followed with each labeled amino acid.
All rats were injected subcutaneously with 250 μc/Kg of radioactive labeled amino acid. The unstressed group of rats were sacrificed by decapitation 12½ minutes after injection of the labeled amino acid. The stress group of rats were stressed 10 minutes after injection of the radioactive label. The stress was 1 minute of ether plus sham adrenalectomy as described previously. The animals were sacrificed 2½ minutes after the initiation of the stress. The brain was immediately removed in the manner noted previously. The anterior and posterior pituitary and the whole hypothalamus was removed from each rat. (The hypothalamus and median eminence were taken together.) The tissues were weighed immediately and placed in a mortar containing 2 ml. of glacial acetic acid. The pooled tissues were homogenized for 2-3 minutes. The homogenate was poured into a centrifuge tube. The mortar and teflon pestle were washed with 2-3 ml. of glacial acetic acid which was added to the homogenate (2 ml. wash for anterior and posterior pituitaries, 3 ml. wash for the hypothalami). The tubes were then heated in a 70°C. water bath for 1 hour with intermittent mixing. They were then placed in the refrigerator (4°C.) for 2 days. The tubes were centrifuged for 1 hour at 3000 rpm in the cold. An aliquot of 0.2 ml. of the measured volume of the extract was taken for radioactivity counts. The residues were washed twice with 2 ml. of glacial acetic acid and the washings were added to the original extract. The residues (KL) were saved for radioactivity counting. The extracts were lyophilized. The lyophilized material was dissolved in a small quantity of water (about 0.5 ml.). An insoluble residue
which remained after centrifugation was extracted with glacial acetic acid (R2) and a final residue remained (R3). R2 and R3 were saved for counting. The entire water solution of the lyophilized extract was applied to chromatography paper. For peptide separation, high voltage paper electrophoresis was combined with ascending chromatography.

a) Separation and identification of radioactive labeled peptides

High voltage electrophoresis

Electrophoresis was performed in a Servonuclear model ET-2⁴ high-voltage paper electrophoresis tank with an EHV 2000 high voltage power supply. Whatman No. 3 chromatography paper (18 4/" x 22 1/" ) was used. The origin was marked and the sample was spotted at the origin. The spot diameter did not exceed 1 cm. Glass rods were placed between the paper and a glass plate was 1/ inch on each side of the origin. The paper was thoroughly wetted with pH 3.5 pyridine-acetic acid-water buffer (400 ml. glacial acetic acid, 40 ml. pyridine, 3,560 ml. H₂O). The buffer was carefully applied around the spot on the origin so that it would diffuse to the center of the spot. All excess buffer was blotted from the surface with another large sheet of filter paper. The paper was placed on a rack and lowered into the tank containing the buffer (pH 3.5 pyridine-acetic acid-water). The bottom edges of the paper penetrated the surface of the buffer for 1 inch. The origin was on the side of the anode and the proteins migrated towards the cathode. The tank contained a coolant, Varsol, layered
over the buffer and completely covering the paper. Cold water circulated through cooling coils in the tank.

The papers were run at 1000 volts for 1.5 hours. The current throughout the run was 75-110 ma. The temperature range was 27-37°C. At the end of the run, the paper was dried in a chromatographic oven at a temperature of 60-90°C. The paper was next subjected to ascending chromatography.

Chromatographic separation of peptides

Ascending chromatography was used as a second dimension in the separation of peptides. The solvent system was butanol, acetic acid, and water (4:1:5). This system divides into two layers. The upper phase was used for chromatography, the lower layer was placed in the chromatocab for equilibration purposes. The time for all runs was 20 hours. The chromatograms were dried in the oven at a temperature range between 60-90°C. 1% Ninhydrin in absolute alcohol was used for detection of spots.

Chromatograms containing extracts of tissues of rats that received radioactive amino acids were not developed with ninhydrin. These chromatograms were subjected to the technique of autoradiography.

The term "chromatogram" will be used to indicate the paper following 2-dimensional separation by chromatography and electrophoresis.
Paper autoradiography

Paper autoradiography is a method of detecting radioactivity on a chromatogram by the use of an X-ray film that is firmly pressed against the chromatogram for a period of time.

Ansco "No-Screen" X-ray film 14 x 17 inches with emulsion on both sides was placed on top of the chromatogram and held securely by weights in complete darkness for 1-2 months. The paper was stapled to the film in several places along the edge. Identifying holes were punched through the paper and film. After exposure the film was developed in Kodak D-19 developer for 5 minutes and fixed in hypo until clear.

The developed autoradiogram provides a permanent record of the relative amounts of radioactivity distributed over the original chromatogram.

Spots on the paper chromatograms, corresponding to exposed spots on X-ray film, were cut out and combusted by the method previously described and the radioactivity counted in the liquid scintillation counter.

b) Peptide identification - ACTH assay
of peptides separated on paper

An attempt was made to establish the identity of radioactive labeled peptides located by paper autoradiography.

Five rats (235-305 g.) that had been adrenalectomized for 7½ months were utilized in this experiment. They were stressed by the use of ether for 1 minute and sham adrenalectomy and sacrificed 2½
minutes after the onset of the ether. The anterior and posterior pituitaries and hypothalami (with median eminences) were removed, weighed, and each of the 3 organs were pooled in individual homogenizers containing 2.5 ml. of 0.1N HCl ($\frac{1}{2}$ ml. HCl per gland). The tissue was homogenized for 5 minutes and allowed to stand in the refrigerator overnight. The extract was lyophilized and dissolved in enough water for application to paper chromatograms. Electrophoresis and chromatography were run as described previously. The temperature in the drying oven was between 50-60°C.

The areas on the paper corresponding with the radioactive spots found in the pituitary gland and hypothalamus as indicated by the X-ray plates were cut out and extracted with $2\frac{1}{2}$ ml. 0.1 N HCl for 24 hours. An equivalent section of paper was cut out and used as a control.

The HCl extracts were assayed for ACTH. The extracts were diluted with normal saline and were immediately assayed for ACTH content using cortisol-blocked male rats, as described by Hodges and Vernikos (1959). In order to differentiate between ACTH and CRF, assays were also carried out on male hypophysectomized rats, using Munson's modification of the method of Sayers et al. (1948). A lyophilized preparation of ACTH (Armour), dissolved in normal saline, was used as a standard. The results were expressed as milliunits (mu) ACTH/mg or as adrenal ascorbic acid depletion (AAAD) in mg/100 g.

The assays were kindly performed by Dr. J. Vernikos-Danellis.
Purified pituitary hormones, for use as standards in the separation of peptides were supplied by the following people: Dr. C. H. Li - ovine ACTH; Dr. J. D. Fisher of Armour - porcine ACTH; Dr. A. V. Schally -α-MSH; Dr. A. E. Wilhelmi supplied LH, FSH, GH, prolactin, and vasopressin through the courtesy of the Endocrinology Study Section, National Institutes of Health.

4. Tissue Autoradiography

Autoradiography is a technique used to demonstrate the localization of radioactive isotopes in tissue sections.

The tissue of 4 rats (185-245 g.) were utilized for this study. All rats were injected with 50 µc H3-phenylalanine subcutaneously.

Two normal and two adrenalectomized rats were used. One of each was stressed 10 minutes after injection by use of ether (1 min.) and sham adrenalectomy and decapitated 2½ minutes after the initiation of stress. The other two rats were controls (unstressed) and were sacrificed 12½ minutes after the injection of the labeled amino acid.

The whole pituitary and hypothalamus (including the median eminence) were removed and fixed in 10% neutral formalin. The tissues were processed in an Autotechnicon through various gradations of alcohol (70-100%), xylene, and embedded in paraffin.

Sections 5µ thick were cut on the microtome. Deparaffinized sections were taken to the darkroom for application of photographic strips of film. Kodak AR-10 stripping film was employed. Strips of film were floated on the slides containing the tissues. The dried
slides were placed in light-tight boxes containing silica gel (drying agent) and stored in the refrigerator for one month, after which time they were brought to the darkroom and developed. The slides were air-dried and stained with 0.025% Azure A in MacIlvaines Buffer pH 4 for about one minute, rinsed in water (few dips), and dried in air. These preparations were permanent.

All tissues were microscopically observed for deposition of silver grains indicating sites of radioactive isotopes.
IV. RESULTS

A. Metabolic Studies

1. Oxygen Consumption

Experiment 1: The effect of glucose on oxygen consumption of anterior pituitary, median eminence and hypothalamus of the rat. Oxygen consumption studies were performed by the method described in the section on Materials and Methods. The effect of glucose, 1 mg/ml., on the oxygen consumption of the 3 organs studied is shown in Table 1.

Table 1

The effect of glucose on the oxygen consumption of anterior pituitary, median eminence, and hypothalamus of the rat.

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Anterior Pituitary</th>
<th>Median Eminence</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1.09 ± .04 (6)</td>
<td>0.78 ± .07 (6)</td>
<td>0.90 ± .01 (7)</td>
</tr>
<tr>
<td>+ (1 mg/ml.)</td>
<td>1.00 ± .05 (5)</td>
<td>1.31 ± .16 (5)*</td>
<td>0.99 ± .07 (11)</td>
</tr>
</tbody>
</table>

* p<.01 (student t)
Number of observations in parenthesis.
Values are the average QO2 between 60 and 120 minutes of incubation.
The oxygen consumption of the median eminence was significantly elevated in the presence of glucose. Anterior pituitary and hypothalamus were not significantly different.

Experiment 2: The effect of stress on the oxygen consumption of anterior pituitary, median eminence, and hypothalamus.

a) The effect of stress on oxygen consumption without glucose in the medium.

Oxygen consumption of the tissue of stressed rats was measured as described in the section on Materials and Methods. One group of rats was sacrificed 2½ minutes after the beginning of stress, another group was sacrificed 5 minutes after the initiation of stress. The results are summarized in Table 2.

---

Table 2

The effect of stress on oxygen consumption of the anterior pituitary, median eminence and hypothalamus of the rat.

$Q_{O_2} (\mu l/mg/hr) \pm S.E.$

<table>
<thead>
<tr>
<th>Time after Stress</th>
<th>Anterior Pituitary</th>
<th>Median Eminence</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress</td>
<td>1.09 ± .04 (6)</td>
<td>0.78 ± .07 (6)</td>
<td>0.90 ± .01 (?)</td>
</tr>
<tr>
<td>2½ min.</td>
<td>1.13 ± .07 (6)</td>
<td>0.90 ± .07 (5)</td>
<td>0.96 ± .03 (11)</td>
</tr>
<tr>
<td>5 min.</td>
<td>1.17 ± .03 (6)</td>
<td>0.71 ± .09 (5)</td>
<td>0.91 ± .03 (12)</td>
</tr>
</tbody>
</table>

Number of observations in parenthesis. Values are the average $Q_{O_2}$ between 60 and 120 minutes of incubation. Glucose was excluded from the Krebs phosphate medium.
The oxygen consumption of the median eminence was increased 2½ minutes after the onset of stress, although this was not statistically significant. There was no change in oxygen consumption 5 minutes after the beginning of stress.

Oxygen consumption in the anterior pituitary and hypothalamus was not affected by the stress procedure.

b) The effect of stress on oxygen consumption with glucose in the medium.

Oxygen consumptions were measured as indicated above, except glucose was included in the medium. The rats were decapitated 2½ minutes after the initiation of stress. The results are given in Table 3.

Table 3:
The effect of stress on oxygen consumption of the anterior pituitary, median eminence and hypothalamus of the rat.

<table>
<thead>
<tr>
<th>Time after Stress</th>
<th>Anterior Pituitary</th>
<th>Median Eminence</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress</td>
<td>1.00 ± .05 (5)</td>
<td>1.31 ± .16 (5)</td>
<td>0.99 ± .07 (11)</td>
</tr>
<tr>
<td>2½ min.</td>
<td>0.98 ± .16 (4)</td>
<td>1.14 ± .08 (2)</td>
<td>0.97 ± .02 (4)</td>
</tr>
</tbody>
</table>

Number of observations in parenthesis.
Values are the average QO₂ between 60 and 120 minutes of incubation. Glucose was included in the Krebs phosphate medium.
There were no statistically significant differences in the tissues studied as a result of stress when glucose was included in the medium.

Experiment 3: Oxygen consumption studies on 30-day adrenalectomized rats.

Oxygen consumption of the anterior pituitary, median eminence and hypothalamus of 30-day adrenalectomized rats were measured. Glucose was excluded from the incubation medium. The results are summarized in Table 4.

<table>
<thead>
<tr>
<th></th>
<th>Anterior Pituitary</th>
<th>Median Eminence</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.09 ± .04 (6)</td>
<td>0.78 ± .07 (6)</td>
<td>0.90 ± .01 (7)</td>
</tr>
<tr>
<td>30-day adrenalectomy</td>
<td>1.11 ± .00 (2)</td>
<td>0.59 ± .03 (2)</td>
<td>0.93 ± .07 (4)</td>
</tr>
</tbody>
</table>

Number of observations in parenthesis.
Values are the average QO₂ between 60 and 120 minutes of incubation. Glucose was excluded from the incubation medium.

There was no statistically significant difference in oxygen consumption between the tissues of normal and adrenalectomized rats. A fall in the QO₂ of median eminence is apparent, but the number of runs was not sufficient to establish statistical significance.
The effect of adrenalectomy on the oxygen consumption in the 3 tissues studied was observed with glucose in the incubation medium. This experiment was the same as the experiment above, except glucose was included in the incubation medium. The results are given in Table 5.

<table>
<thead>
<tr>
<th></th>
<th>Anterior Pituitary</th>
<th>Median Eminence</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.00 ± .05 (5)</td>
<td>1.31 ± .16 (5)</td>
<td>0.99 ± .07 (11)</td>
</tr>
<tr>
<td>30-day adrenalectomy</td>
<td>1.06 ± .05 (2)</td>
<td>1.55 ± .06 (2)</td>
<td>1.13 ± .04 (4)</td>
</tr>
</tbody>
</table>

Number of observations in parenthesis. Values are the average QO₂ between 60 and 120 minutes of incubation. Glucose was included in the incubation medium.

As in the previous experiment comparing the oxygen consumption of the tissues of normal and adrenalectomized rats, there were no statistically significant differences between the two groups of tissues studied, although it appears that the addition of glucose increases the oxygen consumption of median eminence tissue.

The oxygen consumption of the anterior pituitary, median eminence and hypothalamus from adrenalectomized rats were compared with and without glucose in the medium. The results are summarized in Table 6.
Table 6

The effect of glucose in the incubation medium on the oxygen consumption of the anterior pituitary, median eminence and hypothalamus of the adrenalectomized rat.

\[ Q_02 (\mu l/mg/hr) \pm S.E. \]

<table>
<thead>
<tr>
<th>Glucose</th>
<th>Anterior Pituitary</th>
<th>Median Eminence</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>1.11 ± .00 (2)</td>
<td>0.59 ± .08 (2)</td>
<td>0.93 ± .07 (4)</td>
</tr>
<tr>
<td>+ (1 mg/ml.)</td>
<td>1.06 ± .05 (2)</td>
<td>1.55 ± .06 (2)*</td>
<td>1.13 ± .04 (4)</td>
</tr>
</tbody>
</table>

* p < .02 (student t)
Number of observations in parenthesis.
Values are the average QO2 between 60 and 120 minutes of incubation.

The oxygen consumption of the median eminence was significantly increased when glucose was included. The anterior pituitary and hypothalamus were not significantly different. Even though the number of observations is small, the effect of glucose addition upon the oxygen consumption of the median eminence tissue is striking.

The effect of stress on the oxygen consumption of the anterior pituitary, median eminence and hypothalamus of the adrenalectomized rat was measured.

Rats adrenalectomized for 30 days were utilized in this study. The animals were stressed in the manner indicated previously and decapitated 2½ and 5 minutes after the onset of stress. The respiratory activity of the anterior pituitary, median eminence and hypothalamus
was measured with glucose in the incubation medium. The results are
given in Table 7.

Table 7
The effect of stress on the oxygen consumption of the anterior pitui-
tary, median eminence and hypothalamus of the adrenalectomized rat.

<table>
<thead>
<tr>
<th>Time after Stress</th>
<th>Anterior Pituitary</th>
<th>Median Eminence</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Stress</td>
<td>1.06 ± .05 (2)</td>
<td>1.55 ± .06 (2)</td>
<td>1.13 ± .04 (4)</td>
</tr>
<tr>
<td>2½ min.</td>
<td>0.98 ± .04 (3)</td>
<td>1.18 ± .51 (4)</td>
<td>0.97 ± .04 (8)</td>
</tr>
<tr>
<td>5 min.</td>
<td>0.97 ± .05 (2)</td>
<td>1.67 ± .00 (1)</td>
<td>1.19 ± .00 (2)</td>
</tr>
</tbody>
</table>

Number of observations in parenthesis.
Values are the average QO₂ between 60 and 120 minutes of incubation.
Glucose was included in the incubation medium.

There were no statistically significant differences in oxygen
consumption between the normal and stressed tissues of the adrenalecto-
tomized rats, although it appears that 2½ minutes after stress the
oxygen consumption of the median eminence was decreased.

Experiment 4: The effect of hydrocortisone on the oxygen
consumption of anterior pituitary, median eminence and hypothalamus.

Hydrocortisone suspension in saline was injected subcutaneously
at a dose of 7.5 mg/100 g. The animals were decapitated four hours
after administration of the hydrocortisone. Oxygen consumption of the
anterior pituitary, median eminence and hypothalamus was measured. Glucose was excluded from the incubation medium. The results are summarized in Table 3.

Table 3

The effect of an injection of hydrocortisone (7.5 mg/100 g.) on the oxygen consumption of the anterior pituitary, median eminence and hypothalamus.

<table>
<thead>
<tr>
<th></th>
<th>Anterior Pituitary</th>
<th>Median Eminence</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.09 ± .04 (6)</td>
<td>0.78 ± .07 (6)</td>
<td>0.90 ± .01 (7)</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1.26 ± .04 (5)*</td>
<td>1.04 ± .03 (5)*</td>
<td>0.96 ± .02 (12)</td>
</tr>
</tbody>
</table>

* $p < .01$ (student t)
Values are the average QO$_2$ between 60 and 120 minutes of incubation.
Glucose was excluded from the incubation medium.
Number of observations in parenthesis.

Hydrocortisone administered to the rat four hours before sacrifice caused a significant increase in oxygen consumption in the anterior pituitary and the median eminence. No significant change was observed in the hypothalamus.

Hydrocortisone suspension, 100 µg in 0.5 ml. of incubation medium was added to Warburg flasks through the side-arm one hour after incubation. The results for oxygen consumption of the anterior pituitary and hypothalamus are given in Table 9.
Table 9
The effect of in vitro addition of 100 μg. hydrocortisone on the oxygen consumption of the anterior pituitary and hypothalamus.

<table>
<thead>
<tr>
<th></th>
<th>Anterior Pituitary</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>1.09 ± .04 (6)</td>
<td>0.90 ± .02 (7)</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1.12 ± .03 (3)</td>
<td>0.73 ± .01 (6)*</td>
</tr>
</tbody>
</table>

* p < .02 (student t)
Values are the average QO₂ between 60 and 120 minutes of incubation.
Glucose was excluded from the incubation medium.
Number of observations in parenthesis.

In vitro addition of 100 μg. hydrocortisone to the incubation medium caused a significant fall in the oxygen consumption of the hypothalamus. No change was observed in the anterior pituitary.
2. Glycogen Content

Glycogen determinations were performed on the tissues of rats that were stressed in the manner described in the section on Materials and Methods. The animals were sacrificed 2.5 minutes after the onset of stress. The results are expressed in Fig. 4 and Table 10.

<table>
<thead>
<tr>
<th>Glycogen ± S.E. (\mu g/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
</tr>
<tr>
<td>Anterior Pituitary</td>
</tr>
<tr>
<td>Median Eminence</td>
</tr>
<tr>
<td>Hypothalamus</td>
</tr>
<tr>
<td>Stress</td>
</tr>
<tr>
<td>Anterior Pituitary</td>
</tr>
<tr>
<td>Median Eminence</td>
</tr>
<tr>
<td>Hypothalamus</td>
</tr>
</tbody>
</table>

Number of observations in parenthesis.

Glycogen was found to be present in the anterior pituitary, median eminence and hypothalamus by the modified method described previously.

The content of glycogen in the anterior pituitary and median eminence were significantly decreased as a result of stress. No significant change was observed in the hypothalamus as a result of stress.
Fig. 4. The effect of stress on the glycogen content of the anterior pituitary, median eminence and hypothalamus.
GLYCOGEN (>μg/mg)

NORMAL

2.5 min. STRESS

PK < 0.05

PK < 0.01

0.3

0.4

0.5

0.6

0.7

0.8

0.9

1.0

1.1

1.2

Glycogen (μg/mg)

ANTERIOR PITUITARY

MEDIAN EMINENCE

HYPOTHALAMUS

(8)

(10)

(7)

(20)

(14)
3. Phosphorylase activity in the adenohypophysis

The percent phosphorylase a in the anterior pituitary was determined by the method described in the section on Materials and Methods. The effect of a 2.5 min. stress on the percent phosphorylase a content of the rat is given in Table 11.

---

**Table 11**

The effect of stress on the percent phosphorylase a content of the anterior pituitary.

<table>
<thead>
<tr>
<th></th>
<th>Mean Inorganic P ± S.E. (µM/mg/10 min.)</th>
<th>Phosphorylase a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Without AMP</td>
</tr>
<tr>
<td>Normal</td>
<td>4</td>
<td>0.075 ± 0.003</td>
</tr>
<tr>
<td>2.5 min. Stress</td>
<td>2</td>
<td>0.063 ± 0.004</td>
</tr>
</tbody>
</table>

1. Number of determinations.

There appears to be no difference in inorganic P in the presence or absence of AMP in this assay. It appears that all the phosphorylase exists in the active form in both normal and stressed rats.
B. Uptake of Labeled Amino Acids

1. In vivo uptake of $H^3$-phenylalanine

The following experiments compare the uptake of $H^3$-phenylalanine by the adenohypophysis, posterior pituitary, median eminence, and anterior and posterior hypothalamus in normal, adrenalectomized and hydrocortisone treated rats.

The rats were injected subcutaneously with $H^3$-phenylalanine at a dose of 250 $\mu$g/Kg. All animals were sacrificed 12$\frac{1}{2}$ minutes after injection of the phenylalanine.

a) Resting animals

The uptake of $H^3$-phenylalanine in tissues from unstressed rats is summarized in Figure 5. The greatest uptake per unit weight of tissue is observed in the normal posterior pituitary gland. The normal anterior pituitary and median eminence have a slightly lower uptake. The uptake in the hypothalamus is considerably less than both lobes of the pituitary gland. The posterior pituitary had 39% greater uptake than the anterior pituitary gland and 150% greater uptake than the median eminence.

Compared to intact rats, adrenalectomy resulted in marked reduction in uptake of phenylalanine in the posterior pituitary and median eminence. A somewhat smaller reduction in uptake was evident in the anterior pituitary and the posterior hypothalamus.

Administration of a large blocking dose of hydrocortisone also produced its most profound depression of phenylalanine uptake in the
Fig. 5. The uptake of $\text{H}^3$-phenylalanine in tissues of unstressed rats.
H\textsuperscript{3}-PHENYLALANINE
250 \(\mu\)c/kg

- INTACT
- ADRENALECTOMY
- HYDROCORTISONE 7.5 mg/100 gm.

DPM/mg TISSUE

ANT. PIT. POST. PIT. MEDIAN EMINENCE ANT. HYPOTHAL. POST. HYPOTHAL.
posterior pituitary and median eminence. The uptake in the posterior hypothalamus was also reduced. Other tissues were not markedly effected.

b) Effect of hydrocortisone pretreatment

Figure 6 compares the effect of hydrocortisone on uptake of phenylalanine in normal and adrenalectomized unstressed rats. These are expressed as the percent of control uptakes. Absolute values for controls have been given in Fig. 5.

Except for the anterior pituitary, which remained unchanged, hydrocortisone produced a distinct decrease in uptake in all the organs of intact animals. The fall that occurred in the median eminence, posterior pituitary and posterior hypothalamus was particularly striking.

Hydrocortisone given to adrenalectomized animals produced an increase in uptake in all organs. Except for the minor rise in the anterior hypothalamus, a 34-76% increase was noted in the remaining tissues.

c) Effect of stress

Figure 7 shows the effect of stress on the content of H$_2$-phenylalanine in the tissues studied. Each value given in Fig. 7 is the percent change from its control value given in Fig. 5. The control value was set at 100%.
Fig. 6. The effect of hydrocortisone on the uptake of $\text{H}^3$-phenylalanine in normal and adrenalectomized rats. Absolute values for the controls were those given in Fig. 5.
INTACT HYDROCORTISONE

ADRX. HYDROCORTISONE

H$^3$-PHENYLALANINE
250 $\mu$g/kg

ANT. POST. MEDIAN ANT. POST.
PIT. PIT. EMINENCE HYPOTH. HYPOTH.

PERCENT OF CONTROL

175
150
125
100
75
50

25
An acute stress in intact animals decreased the uptake of radioactivity. The changes in the anterior pituitary and anterior hypothalamus were minor. The most profound changes were noted in the median eminence (-66\%), posterior pituitary (-39\%) and posterior hypothalamus (-43\%).

The effect of stress in adrenalectomized animals was generally not striking. A small rise in uptake was evident in the anterior pituitary (+9\%) and the most profound change occurred in the posterior hypothalamus (+65\%).

In hydrocortisone treated animals, the same stress resulted in a uniformly increased uptake in H\textsuperscript{3}-phenylalanine except for the anterior pituitary, which was unchanged.

In summary, 2 minutes after the onset of stress, there was a diminished number of counts in all organs (Fig. 7). Hydrocortisone reversed this trend and caused an increased number of counts in all organs except the anterior pituitary. In resting intact rats, hydrocortisone caused a decreased content of radioactivity in all organs except the anterior pituitary (Fig. 6). In adrenalectomized rats, hydrocortisone produced a substantial increase in H\textsuperscript{3}-phenylalanine uptake in all organs.

Whether or not the increased or decreased content of radioactivity is a reflection of changes in release or synthesis of ACTH will be considered in the discussion. In these experiments there is no way of knowing whether the content of radioactivity is in the form of the free H\textsuperscript{3}-phenylalanine or whether the labeled amino acid has
Fig. 7. The effect of stress on the uptake of $^3$H-phenylalanine in intact, adrenalectomized and hydrocortisone treated rats. Absolute values for the controls were those given in Fig. 5. Animals were sacrificed $2\frac{1}{2}$ min. after the onset of an ether-laparotomy stress.
STRESS

- INTACT
- ADRENALECTOMIZED
- HYDROCORTISONE 7.5mg/100gm
- H³-PHENYLALANINE 250µc/kg

PERCENT OF CONTROL

0  50  100  150

ANT. PIT.  POST. PIT.  MEDIAN EMINENCE  ANT. HYPOTHAL.  POST. HYPOTHAL.
been incorporated into one or more peptide hormones. The following experiments, using both H\(^3\)-phenylalanine and C-14 serine, will attempt to resolve this problem.

2. In vivo uptake of radioactive amino acids and extraction of peptides
   
a) Separation of radioactive labeled peptides
   
i. Radioactivity of glacial acetic acid extracts

Experiments were performed with two different labeled amino acids, phenylalanine-\(H^3\) and serine-C\(^{14}\). Each experiment contained a control and a stressed group of rats. All rats were injected subcutaneously with 250 μc/Kg of radioactive labeled amino acid. Ten minutes after injection a group of rats was given the standard ether-laparotomy stress. The rats were decapitated 2.5 minutes after the onset of stress. The controls were unstressed. The anterior and posterior pituitary and the hypothalamus were removed from each rat and the peptides were extracted with glacial acetic acid by the procedure described in the section on Materials and Methods. An aliquot of each glacial acetic acid extract was counted for radioactivity. The effect of stress on the uptake of the radioactive labeled amino acids in the tissues studied is given in Tables 12 and 13.
### Table 12

The effect of stress on the glacial acetic acid extracts of \(^3\)H-phenylalanine injected rats.

<table>
<thead>
<tr>
<th></th>
<th>DPM/mg. (wet)</th>
<th>DPM/Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Stress</td>
</tr>
<tr>
<td>AP</td>
<td>444</td>
<td>536</td>
</tr>
<tr>
<td>PP</td>
<td>3563</td>
<td>766</td>
</tr>
<tr>
<td>Hyp</td>
<td>111</td>
<td>122</td>
</tr>
</tbody>
</table>

1. Each value was obtained from the pooled tissue of 6 rats.

### Table 13

The effect of stress on glacial acetic acid extracts of C-14 Serine injected rats.

<table>
<thead>
<tr>
<th></th>
<th>DPM/mg. (wet)</th>
<th>DPM/Organ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Stress</td>
</tr>
<tr>
<td>AP</td>
<td>1,066</td>
<td>1,531</td>
</tr>
<tr>
<td>PP</td>
<td>1,030</td>
<td>1,032</td>
</tr>
<tr>
<td>Hyp</td>
<td>108</td>
<td>108</td>
</tr>
</tbody>
</table>

1. Each value was obtained from the pooled tissue of 6 rats.
2. Each value was obtained from the pooled tissue of 5 rats.
In both experiments stress induced an increased incorporation of the label in the glacial acetic acid extracts of the anterior pituitary. The increase was 44% in the serine experiment and 21% in the phenylalanine experiment. Stress resulted in a marked decrease of 79% in radioactivity in the posterior pituitary extract of the phenylalanine treated rats. No change in radioactivity in the posterior pituitary extracts was noted in the C-14 experiment. Changes in radioactivity of the hypothalamus as a result of stress were not remarkable.

It should be noted that approximately 8 times as much phenylalanine was taken up by the posterior than by the anterior lobe, while in the serine experiment the uptake in the two lobes of the pituitary was essentially equal. Reasons for this difference will be considered in the discussion. With both amino acids, the uptake into the extractable portion of hypothalamus was quite low, on a weight basis.

ii. Radioactive incorporation into non-extractable fraction

The residue (RI) remaining after glacial acetic acid extraction was counted for radioactivity after Schöniger combustion. The effect of stress on the incorporation of the labeled amino acids into the non-extractable portion of the tissue is summarized in Table 14.
Table 14

The effect of stress on the incorporation of radioactive amino acids into the Residue (Rl) from the glacial acetic acid extracts of the anterior and posterior pituitary and the hypothalamus.

<table>
<thead>
<tr>
<th></th>
<th>H³-Phenylalanine</th>
<th>C-¹⁴ Serine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPM/mg (wet)</td>
<td>DPM/Organ</td>
</tr>
<tr>
<td></td>
<td>Normal Stress</td>
<td>Normal Stress</td>
</tr>
<tr>
<td>AP</td>
<td>92</td>
<td>92</td>
</tr>
<tr>
<td>PP</td>
<td>141</td>
<td>37</td>
</tr>
<tr>
<td>Hyp</td>
<td>17</td>
<td>18</td>
</tr>
</tbody>
</table>

1. Each value was obtained from the pooled tissue of 5 or 6 rats.

After stress there was 62% less radioactivity in the phenylalanine residue of the posterior pituitary. No other striking changes were observed in either of the amino acid groups as a result of stress.

There appeared to be a greater incorporation of tritiated phenylalanine than of serine into the residues of all three organs. Total incorporation into the anterior pituitary of the phenylalanine experiment was 154% greater than the serine experiment. Total incorporation in posterior pituitary was 520% greater in the phenylalanine experiment than in the serine experiment. It is also obvious that in various tissues up to 30 times as much radioactivity is found in the glacial acetic acid extracts as is present in Rl.
iii. Radioactive incorporation into the insoluble residue (R2) obtained from the lyophilized glacial acetic acid extract

The glacial acetic acid extracts were lyophilized and dry powder was dissolved in a small quantity of water for chromatographic spotting. A small insoluble residue remained in each case. This residue was subjected to a second extraction with glacial acetic acid. The final residue (R3) and the glacial acetic acid extracts (R2) were counted separately. Results are given in Tables 15 and 16.

<table>
<thead>
<tr>
<th></th>
<th>$^3$H-Phenylalanine</th>
<th></th>
<th>C-14 Serine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Stress</td>
<td>Normal</td>
<td>Stress</td>
</tr>
<tr>
<td>Anterior Pituitary</td>
<td>41</td>
<td>25</td>
<td>86</td>
<td>53</td>
</tr>
<tr>
<td>Posterior Pituitary</td>
<td>102</td>
<td>189</td>
<td>16</td>
<td>58</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>9.6</td>
<td>12</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

1. Radioactivity expressed as DPM/mg wet weight.
2. Each value was obtained from the pooled tissue of 5 or 6 rats.
Radioactivity in all tissues in the R2 and R3 fractions was minor compared to that in the soluble extracts. No radioactive phenylalanine in the R3 fraction of the posterior pituitary was found in the stressed group. No other marked changes resulted from stress in either of the amino acid groups.

A consideration of the total amount of \( \text{H}^3 \)-phenylalanine uptake per anterior pituitary (Fig. 5) demonstrates fairly good agreement between the total amount of radioactivity and the sum of the radioactivity found in the glacial acetic acid extract, R1, R2, and R3.

Of the total phenylalanine radioactivity, the glacial acetic acid extracts of the anterior pituitary amounted to 77\%, the remaining radioactivity existing in the insoluble fractions. The C-14 serine uptake

---

Table 16

Radioactivity\(^1\) found in Fraction R3.\(^2\)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>( \text{H}^3 )-Phenylalanine</th>
<th>( \text{C}-14 ) Serine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Stress</td>
</tr>
<tr>
<td>Anterior Pituitary</td>
<td>50</td>
<td>62</td>
</tr>
<tr>
<td>Posterior Pituitary</td>
<td>293</td>
<td>0</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

1. DPM/mg wet weight.
2. Each value was obtained from the pooled tissue of 5 or 6 rats.
in the glacial acetic acid extracts of the anterior pituitary amounted to 91% of the total radioactivity, the remainder existing in the insoluble residue.

iv. Peptide separation and localization

The water soluble lyophilized material was spotted on chromatography paper and separation was performed by the electrophoretic and chromatographic 2-dimensional system described in the section on Materials and Methods.

The chromatograms were subjected to autoradiography by the use of X-ray film. The tritiated phenylalanine chromatograms were developed after one month, the C-14 serine chromatograms were developed after two months. Autoradiograms are reproduced in Figures 8 to 17.

Radioactive spots were found on all of the films. Every film in both amino acid experiments contained a particular radioactive spot (spot 1) whose \( R_f \) value and electrophoretic migration was considered identical within the reproducible limits of the 2-dimensional procedure (Table 1). The radioactive spots were generally denser in the C-14 serine autoradiograms. The C-14 anterior pituitary spots were particularly dense (Figs. 8 and 9). In addition, the stressed anterior pituitary spot 1 is clearly denser than the unstressed control anterior pituitary spot.
Fig 8. Autoradiogram of the anterior pituitary extract of unstressed rats treated with Serine-1-$^{14}$C. Radioactive spots are numbered.
Fig. 9. Autoradiogram of the anterior pituitary extract of stressed rats treated with Serine-1-C$^{14}$. Radioactive spots are numbered.
Fig. 10. Autoradiogram of the posterior pituitary extract of un-stressed rats treated with Serine-1-C$^{14}$. Radioactive spots are numbered.
Fig. 11. Autoradiogram of the posterior pituitary extract of stressed rats treated with Serine-1-C¹⁴. Radioactive spots are numbered.
Fig. 12. Autoradiogram of the hypothalamus extract of unstressed rats treated with Serine-1-O$^{14}$. Radioactive spots are numbered.
Fig. 13. Autoradiogram of the hypothalamus extract of stressed rats treated with Serine-1-Cl\textsuperscript{14}. Radioactive spots are numbered.
Fig. 14. Autoradiogram of the anterior pituitary extract of unstressed rats treated with H\(^3\)-phenylalanine. Radioactive spot is numbered.
Fig. 15. Autoradiogram of the anterior pituitary extract of stressed rats treated with H\textsuperscript{3}-phenylalanine. Radioactive spot is numbered.
Fig. 16. Autoradiogram of the hypothalamus extract of unstressed rats treated with $^3$H-phenylalanine. Radioactive spot is numbered.
Fig. 17. Autoradiogram of the hypothalamus extract of stressed rats treated with H$^3$-phenylalanine. Radioactive spot is numbered.
Table 17

The \( R_f \) values and electrophoretic migrations (\( E_m \)) of radioactive spot 1 in the C-14 Serine and \( H^3 \)-phenylalanine chromatograms of normal (N) and stressed (S) rats.

<table>
<thead>
<tr>
<th></th>
<th>C-14 Serine</th>
<th>( H^3 )-phenylalanine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anterior Pituitary</td>
<td>Posterior Pituitary</td>
</tr>
<tr>
<td></td>
<td>N S</td>
<td>N S</td>
</tr>
<tr>
<td>( E_m ) (cm.)</td>
<td>2.5 3.0</td>
<td>1.0 2.0</td>
</tr>
</tbody>
</table>

The average \( R_f \) values for the serine and phenylalanine group are .137 and .130 respectively. The average electrophoretic migration of spot 1 for the serine and phenylalanine groups are 1.9 and 1.7 cm. respectively. These very similar coordinate values strongly suggest that the radioactive labeled spot 1 in both the serine and phenylalanine chromatograms are identical.

Unlabeled phenylalanine and serine were subjected to the 2-dimen- 
sional procedure in order to rule out identity with the radioactive labeled spot 1. The chromatograms are shown in Figures 18 and 19. The \( R_f \) values for serine and phenylalanine were .12 and .43 respectively. The electrophoretic migrations for serine and phenylalanine were 6.2 and 7.0 cm. respectively. The difference in electrophoretic migration of the serine and spot 1 indicates that spot 1 is not serine. The marked difference in both \( R_f \) values and electrophoretic migration
of phenylalanine and spot 1 rules out the possibility that this amino acid is spot 1. The fact that both the labeled serine and phenylalanine spot 1 fall in the same position indicates that both of these labeled amino acids incorporate into the same peptide.

In addition to spot 1, several other labeled spots were found. Two radioactive labeled spots adjacent to spot 1 can be observed in the C-14 serine X-ray films (Figs. 8 and 9). Both of these spots (spot 2 and spot 3) are more intense in the stressed group. Spot 3, found extending from the origin along the electrophoretic path has a zero R_f value and electrophoretic migration of 2.5 and 3 cm. in the normal and stressed groups respectively. Spot 2, between spots 1 and 3, has R_f values of .06 and .07 and electrophoretic migrations of 2 and 2.6 cm. in the normal and stressed groups respectively. Spot 3 was also found in the posterior pituitary stress group (Fig. 11) and normal hypothalamus of the C-14 serine X-rays (Fig. 12).

An additional spot (spot 4) appeared on the C-14 serine normal posterior pituitary X-ray film (Fig. 10). The R_f value was .16, electrophoretic migration was 5.2 cm. This spot did not correspond exactly with any of the other labeled spots, but it corresponded approximately with the position of serine itself.

Another spot (spot 5) appeared in both the normal and stressed hypothalamus chromatogram of the C-14 serine group (Figs. 12 and 13). This spot appeared close to the solvent front with R_f values of .78 and .74 in the normal and stressed hypothalamus respectively and electrophoretic migration of 0.
Fig. 18. Paper electrophoresis and chromatography of serine.
SERINE

ELECTROPHORESIS 1000 V. 1 hr.

CHROMATOGRAPHY
Bu/Ac/H₂O 4:1:5 20 hr.
Fig. 19. Paper electrophoresis and chromatography of phenylalanine.
PHENYLALANINE

ELECTROPHORESIS

1000 V. 1 1/2 hr.

CHROMATOGRAPHY

Bu/Ac/H₂O 4:1:5 20 hr.
b) Quantitation of radioactivity in the radioactive spots

The spots on the paper chromatograms, corresponding to the dense areas on the X-ray films, were cut out and ignited by the Schöniger combustion method. Radioactivity was counted in the liquid scintillation counter. The results are given in Table 18.

<table>
<thead>
<tr>
<th></th>
<th>C-14 Serine</th>
<th></th>
<th>H(^3)-phenylalanine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Stress</td>
<td>Normal</td>
<td>Stress</td>
</tr>
<tr>
<td>Anterior Pituitary</td>
<td>263</td>
<td>511</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td>Posterior Pituitary</td>
<td>5.5</td>
<td>288</td>
<td>13</td>
<td>8</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>13</td>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

1. Expressed as DPM/mg wet weight of original tissue.
2. Each value was obtained from the pooled tissue of 5 or 6 rats.

A 94% increase in the DPM/mg of the anterior pituitary spot containing C-14 serine was observed after stress. An additional striking increase of C-14 serine incorporation was observed in the posterior pituitary after stress.

An extremely small number of counts were retrieved from the tritiated phenylalanine spots. This has not yet been explained. A considerable number of counts were obtained in the glacial acetic acid
extracts (Table 12). It is questionable whether a proper appraisal of the effect of stress could be made on as small a number of counts as this, especially when 75-90% of the counts applied to the chromatography paper cannot be accounted for.

Spots 2 and 3 were also processed for radioactivity counting. The results in Table 19 show that almost a four fold increase occurred in spot 2 and almost a five fold increase in spot 3 after stress.

Table 19
Radioactivity$^1$ in the labeled spots 2 and 3 in the anterior pituitary of the C-14 serine chromatograms.$^2$

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spot 2</td>
<td>5.7</td>
<td>21</td>
</tr>
<tr>
<td>Spot 3</td>
<td>4.1</td>
<td>19</td>
</tr>
</tbody>
</table>

1. DPM/mg wet weight of tissue.
2. Each value was obtained from the pooled tissue of 5 or 6 rats.

c) Identification of radioactive labeled peptides

1. Chromatography and electrophoresis of glacial acetic acid extracts of the anterior and posterior pituitary and hypothalamus of rats

An attempt was made to see if spots corresponding to radioactive labeled areas could be visualized by developing a color with ninhydrin in chromatograms of extracts of normal rats.
The tissue of 6 rats was pooled and extracted with glacial acetic acid by the method described previously. 300 µg of the water soluble lyophilized material was spotted on paper sheets. Electrophoresis and chromatography were run in the usual manner and ninhydrin was applied. Tracings of the chromatograms are given in Figures 20, 21, 22. In all three tracings (anterior and posterior pituitary and hypothalamus) there appeared a spot corresponding to spot 1. The Rf value of this spot was .13 in both the anterior and posterior pituitary and .15 in the hypothalamus. The electrophoretic migration in the anterior and posterior pituitary and hypothalamus were .5, 1.5 and 2 cm, respectively.

Since the pituitary of adrenalectomized rats is known to have a high ACTH content, the anterior pituitaries of two normal and two stressed adrenalectomized rats were each extracted with 1 ml of 0.1N HCl and were allowed to stand overnight. The extracts were lyophilized. All of the water soluble lyophilized material was spotted on paper chromatograms. Electrophoresis and chromatography were run as usual and ninhydrin was applied. Tracings of the chromatograms are given in Figures 23 and 24.

In the extract of unstressed adrenalectomized rats there were two faint spots that approximated the area of spot 1. One spot had an Rf value of .11 and an electrophoretic migration of 4.2 cm. The other spot has an Rf value of .17 and electrophoretic migration of 2.0 cm. The extract of adrenalectomized rats' pituitary 2½ minutes after stress demonstrated a larger spot 1 that was more intense in its ninhydrin staining characteristic than the previous spots from unstressed
Fig. 20. Tracing of the ninhydrin positive spots following electrophoretic and chromatographic separation of an extract of rat anterior pituitaries.
ELECTROPHORESIS

RAT ANTERIOR PITUITARY

CHROMATOGRAPHY
Bu/Ac/H_2O  4:1:5  16 hr.
Fig. 21. Tracing of the ninhydrin positive spots following electrophoretic and chromatographic separation of an extract of rat posterior pituitaries.
RAT POSTERIOR PITUITARY

ELECTROPHORESIS

1000 V. 1/4 hr.

CHROMATOGRAPHY

Bu/Ac/H2O 4:1:5 20 hr.
Fig. 22. Tracing of the ninhydrin positive spots following electrophoretic and chromatographic separation of an extract of rat hypothalami.
ELECTROPHORESIS IOOOV.

RAT HYPOTHALAMUS

CHROMATOGRAPHY
Bu/Ac/H2O 4:1:5 20 hr.
Fig. 23. Tracing of the ninhydrin positive spots following electro-
phoretic and chromatographic separation of an extract of the anterior
pituitary of 30-day adrenalectomized rats.
ELECTROPHORESIS

ANTERIOR PITUITARY

30 DAY ADRENALECTOMIZED RATS

CHROMATOGRAPHY

Bu/Ac/H2O 4:1:5 20 hr.
Fig. 24. Tracing of the ninhydrin positive spots following electrophoretic and chromatographic separation of an extract of the anterior pituitary of 30-day adrenalectomized stressed rats.
ANTERIOR PITUITARY
30 DAY ADRENALECTOMIZED RATS
STRESSED

ELECTROPHORESIS 1000 V. 1 hr.

CHROMATOGRAPHY
Bu/Ac/H2O 4:1:5 20 hr.
adrenalectomized rats. The $R_f$ was .12 and the electrophoretic migration was 2.5 cm.

ii. Chromatography and electrophoresis of standard hormone preparations

Since there are no available pure rat pituitary hormones to be used as standards, hormones of other species were used in an attempt to identify the radioactive labeled spots (especially spot 1). The 2-dimensional patterns of ACTH (sheep and pig)$\alpha$-MSH, LH (sheep), oxytocin (synthetic) are shown in Figures 25 to 29. The $R_f$ values and electrophoretic migrations are given in Table 20. None of these hormones correspond with any of the radioactive spots (1, 2 or 3). The Armour ACTH was not pure, although the major spot corresponded with the pure ACTH obtained from C. H. Li. It is clear that spot 1 does not correspond with the known ACTH. The lysine vasopressin, FSH, growth hormone and prolactin preparations used were found not to be pure materials. None of them had a major component corresponding to spot 1. With growth hormone, there is a strong absorption of the material to the origin. FSH had two major components that differed from Spot 1.
Fig. 25. Tracing of the ninhydrin positive spot following electrophoretic and chromatographic separation of pure sheep ACTH.
ACTH
(sheep) (C.H. Li)

CHROMATOGRAPHY
Bu/Ac/H2O 4:1.5 20 hr.
Fig. 26. Tracing of the ninhydrin positive spots following electrophoretic and chromatographic separation of pig ACTH.
ELECTROPHORESIS

1000 V. 1 1/2 hr.

CHROMATOGRAPHY

Bu/Ac/H2O 4:1.5 20 hr.

ACTH
(Pig) (Armour)
Fig. 27. Tracing of the ninhydrin positive spot following electrophoretic and chromatographic separation of \( \alpha \)-MSH.
ELECTROPHORESIS

CHROMATOGRAPHY

Bu/Ac/H$_2$O 4:1:5  20 hr.

$\alpha$-MSH
(AV Schally)
Fig. 28. Tracing of the ninhydrin positive spot following electro-
phoretic and chromatographic separation of sheep LH.
BU/Ac/HtO 4:1:5 20 hr.

ELECTROPHORESIS 1000 V.

CHROMATOGRAPHY

SF

ELECTROPHORESIS 1000 V.

14 hr.

lH

(Shagg)
Fig. 29. Tracing of the ninhydrin positive spot following electrophoretic and chromatographic separation of synthetic oxytocin.
ELECTROPHORESIS

OXYTOCIN (Synthetic)

CHROMATOGRAPHY
Bu/Ac/H2O 4:1.5 20 hr
Table 20
The $R_f$ values and electrophoretic migration ($Em$) of purified pituitary hormones used as standards.

<table>
<thead>
<tr>
<th></th>
<th>ACTH Sheep (C.H.Li)</th>
<th>ACTH (Armour) $\alpha$-MSH</th>
<th>LH Sheep</th>
<th>Oxytocin</th>
<th>Lysine vasoressin</th>
<th>Pro-lactin</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_f$</td>
<td>0</td>
<td>0</td>
<td>0.09</td>
<td>0</td>
<td>0.22</td>
<td>0.06</td>
</tr>
<tr>
<td>$Em$ (cm.)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>8.5</td>
<td>8</td>
<td>9.8</td>
</tr>
</tbody>
</table>

iii. ACTH assay of peptides separated on paper

An attempt was made to establish the identity of the radioactive labeled spot 1 by bioassay. Extracts of the pooled pituitaries and hypothalami of stressed chronically adrenalectomized rats were prepared as described in Materials and Methods. The extracts were spotted on paper chromatograms. The papers were run in the 2-dimensional system in the usual manner. The areas on the papers corresponding to spot 1 were cut out and extracted overnight with HCl. The HCl extracts were assayed for ACTH using both cortisol-blocked male rats and hypophysectomized rats. Table 21 summarizes the results obtained.

A considerable concentration of ACTH was found in the spot 1 from anterior pituitary. The posterior pituitary and hypothalamus also contained small amounts of adrenocorticotropic activity in spot 1 areas. This is the first identification of a rat ACTH separated on paper chromatograms.
That a large amount of ACTH activity is lost in the electrophoretic and chromatographic procedure is demonstrated in Table 22. ACTH that was applied to the chromatography paper and not processed through electrophoresis and chromatography and kept at -10°C for 48 hours, showed a recovery of 48% when assayed in hypophysectomized animals. When electrophoresis and chromatography was performed, a recovery of approximately 5-10% was obtained in two experiments. Therefore, corrected values for those given in Table 21 are 10 to 20 times greater.

Since the spot obtained by chromatography and electrophoresis of standard ACTH (Figs. 25 and 26) differs markedly from spot 1, an attempt was made to assay the area equivalent to spot 1 on the chromatograms of ACTH standards (C.H. Li and Armour). The actual ACTH spot (fluoresces) was also assayed. Table 23 shows that there is no ACTH activity in the spot 1 area on standard ACTH chromatograms, in contrast to considerable activity obtained in the actual ACTH spots.

Table 23 also shows that an area equivalent to the ACTH spot (C.H. Li) on the chromatogram of the extract of the anterior pituitaries of stressed adrenalectomized rats (Table 21) contained no ACTH activity. Spot 1, on the other hand, contained considerable activity. Extracts from neutral areas of ACTH chromatograms did not show ascorbic acid depleting activity.

The area equivalent to spot 5 (Figs. 12 and 13) from a chromatogram of the extract of the hypothalami of stressed adrenalectomized rats was assayed for ACTH activity. No activity was observed (AAAD was 1 ± 6).
<table>
<thead>
<tr>
<th>Organ</th>
<th>Number of Animals</th>
<th>Mean body weight (mg.)</th>
<th>Mean wet weight (mg.)</th>
<th>Number of assay rats</th>
<th>Assay animals</th>
<th>Corticotropin Concentration mu/organ</th>
<th>Corticotropin Concentration mu/mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior</td>
<td>5</td>
<td>270</td>
<td>12.34</td>
<td>12</td>
<td>Hydrocortisone</td>
<td>43.24</td>
<td>3.5</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hypox.</td>
<td>69.21</td>
<td>5.6</td>
</tr>
<tr>
<td>Posterior</td>
<td>5</td>
<td>270</td>
<td>2.03</td>
<td>6</td>
<td>Hydrocortisone</td>
<td>15</td>
<td>7.4</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hypox.</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>2</td>
<td>6</td>
<td>Hydrocortisone</td>
<td>approx 4-5</td>
<td>Hypox.</td>
<td>approx 4-5</td>
<td>---</td>
</tr>
</tbody>
</table>

1. Values below the lower limit of the standard curve.
2. Hypothalamus includes the median eminence.
Table 22

The recovery of ACTH activity from chromatographic paper.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose ACTH injected (mu/100 gm)</th>
<th>AAAD ± S.E. (mg/100 gm adrenal weight)</th>
<th>ACTH found (mu/100 gm)</th>
<th>% ACTH recovered from paper</th>
<th>Assay animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH (C.H. Li)</td>
<td>1.2</td>
<td>112 ± 15</td>
<td>---</td>
<td>---</td>
<td>Hypox.</td>
</tr>
<tr>
<td>ACTH (C.H. Li) on chromatographic paper</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No processing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kept at -10°C. 48 hrs.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ACTH (C.H. Li) extracted from paper after Electrophoresis and Chromatography</td>
<td>12</td>
<td>74 ± 14</td>
<td>.64</td>
<td>5</td>
<td>Hydrocortisone</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>39 ± 10</td>
<td>.28</td>
<td>9</td>
<td>Hypox.</td>
</tr>
</tbody>
</table>
Table 23

Comparison of ACTH activity in spot 1 area and standard ACTH spot area from chromatograms of ACTH standards and a chromatogram of an extract from stressed adrenalectomized rats.

<table>
<thead>
<tr>
<th>Extract Injected</th>
<th>Dose ACTH injected (mu/100 gm)</th>
<th>AAAD$^{1,2}$ ± S.E. (mg/100 gm adrenal weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH spot (C.H. Li)</td>
<td>12</td>
<td>74 ± 14 (6)</td>
</tr>
<tr>
<td>Spot 1 Area from C.H. Li ACTH Chromatogram</td>
<td>12</td>
<td>5 ± 16 (5)</td>
</tr>
<tr>
<td>ACTH Spot (Armour)</td>
<td>12</td>
<td>131 ± 10 (5)</td>
</tr>
<tr>
<td>Spot 1 Area from Armour ACTH Chromatogram</td>
<td>12</td>
<td>-21 ± 6 (4)</td>
</tr>
<tr>
<td>Standard ACTH spot from Chromatogram of rat pituitary extract</td>
<td>1 pit. : 100 2 ml./100 gm</td>
<td>1 ± 9 (6)</td>
</tr>
<tr>
<td>Spot 1 from Chromatogram of rat pituitary extract</td>
<td>1 pit. : 100 2 ml./100 gm</td>
<td>98 ± 9 (6)</td>
</tr>
</tbody>
</table>

1. Cortisol-blocked assay animals.
2. Control adrenal ascorbic acid content was 552 mg/100 g.
3. Number of assay animals in parenthesis.
d) Tissue Autoradiography

Tissue autoradiographs of the whole pituitaries and hypothalami of 4 rats were examined for incorporation of $^3$H-phenylalanine into cellular elements. Two normal and two adrenalectomized rats were used. One of each group was stressed for 2½ minutes. All were sacrificed 12½ minutes after injection of 50 μc $^3$H-phenylalanine.

No unusual localization of radioactivity was noted in the anterior, posterior, and intermediate lobes of the pituitary. Likewise, hypothalami and median eminences were without distinct localization of silver grains. However, an unusual structure, located on the rostral wall of the third ventricle, was found to contain intense localization of radioactivity. This structure has been tentatively identified as the subfornical organ (Fig. 30). It is a relatively vascular structure, situated in the midline, and enveloped by the choroid plexus. The cells in this organ are round, oval and polygonal shaped with varying intensity of staining.

Radioactive incorporation was observed in this structure in an intact and an adrenalectomized rat (Fig. 31). Sections including the region of the subfornical organ were not obtained in the other two rats.
Fig. 30. Tissue autoradiogram of the rat subformical organ. Section obtained from the brain of a rat treated with $\text{H}^3$-phenylalanine. (40 X)
Fig. 31a. Tissue autoradiographic localization of $^3$H-phenylalanine in the subfornical organ. Oil-immersion. (640 X)

Fig. 31b. Tissue autoradiographic localization of $^3$H-phenylalanine in the subfornical organ. (205 X)
V. DISCUSSION

The discussion of the results presented will proceed with an emphasis on the major purpose of this thesis - to study the increase in ACTH biosynthesis resulting from stress. Stress-induced metabolic alterations will be considered in this context.

A. Metabolic Studies

1. Oxygen Consumption

Roberts and Keller (1953) showed that in the absence of glucose in the incubation medium the anterior pituitary retained its initial respiratory activity for at least 3 hours. These investigators used male rats sacrificed under pentobarbital anesthesia. Levey and Roberts (1957) sacrificed their rats under ether anesthesia. In the studies cited above and those of Roberts and Keller (1955) the QO₂ values for the anterior pituitary gland ranged from 0.79 to 1.19.

In this study, the addition of glucose to the incubation medium did not alter the oxygen consumption of the anterior pituitary gland, thus confirming the finding by Roberts and Keller (1953). Although the sex of the animals and the method of sacrifice differs in this study compared to that of Roberts, a QO₂ of 1.00 for the anterior pituitary (with glucose in the medium) falls within the range determined by the above workers. Roberts and Keller (1953) also studied the oxygen consumption of the anterior and posterior hypothalamus. They found that...
the oxygen utilization in the hypothalamus declined rapidly when glucose was omitted from the incubation medium. In addition, the QO2 values obtained by them for the hypothalamus was approximately twice the value obtained in this study (with glucose in the medium). These differences can be explained by the presence of the median eminence which at that time was not considered separately.

Oxygen consumption studies on the median eminence have not been performed previously. The results here are indeed striking. In the absence of glucose, the oxygen consumption is significantly lowered (Table 1). That glucose is utilized in the median eminence is further supported by the observation that there is a pronounced fall in the glycogen content after stress (Fig. 4). The importance of the median eminence in the stress-induced increase in ACTH secretion has been demonstrated by lesions in this tissue. Destruction of the median eminence was shown to suppress the stress-induced increase in ACTH secretion in several species (McCann, 1953; DeGroot and Harris, 1950; Lacquer et al., 1955; Ganong et al., 1961; Anand and Dua, 1955). In addition, stimulation of the median eminence increased ACTH secretion (DeGroot and Harris, 1950; Anand and Dua, 1955; Harris, 1955; Hume, 1952; Kutsuki et al., 1956; Mason, 1959).

An increased oxygen consumption was observed in the median eminence 2.5 minutes after the onset of stress when glucose was excluded from the medium (Table 2), although this was not statistically significant. Five minutes after the onset of stress the QO2 returned to pre-stress level. When glucose was included in the medium, the
stress had no effect (Table 3). This can be explained by the fact that apparently maximal \( QO_2 \) is achieved by the supplying glucose in the medium. Consequently, no effect of stress in increasing the \( QO_2 \) can be seen.

It is interesting that 5 minutes after the initiation of stress the oxygen consumption in the median eminence had returned to normal indicating that any metabolic alterations occurring 2½ minutes after stress had returned to the pre-stressed level. Vernikos-Danellis (1962) found an increased ACTH content in the adenohypophysis of the adrenalectomized rat 2½ minutes after the onset of stress. Five minutes after stress the corticotropin concentration in the pituitary was normal. In addition, Goldman (1962) showed that the anterior pituitary blood flow 2½ minutes after the onset of stress increased by about 40% and is again normal by 5 minutes after the beginning of stress. It is tempting to suggest a relationship of the median eminence to the stress-induced synthesis of ACTH, although no cause and effect relationship has been proved.

Stress did not alter the oxygen consumption of the anterior pituitary or hypothalamus. It is to be expected that an increased synthesis of ACTH and perhaps other polypeptides (see Figs. 8 and 9) would result in increased metabolic demands in the adenohypophysis. Several reasons could explain the apparent resistance of the anterior pituitary seen in these studies and those of Roberts and Keller (1953, 1955):

(a) It has been suggested (Levey and Roberts, 1957) that an
increased elaboration of the adenohypophyseal hormones need not be accompanied by corresponding increases in the total metabolism of this tissue. It was shown that castration or adrenalectomy in the male rat produced no significant changes in the respiration of the anterior pituitary gland, although castration resulted in marked hypertrophy of this gland. However, thyroidectomy produced an adenohypophyseal hypertrophy and an elevated oxygen consumption by this structure in vitro. The authors were tempted to assume that the increased respiration and enhanced elaboration of thyrotropin were related, although they considered that the inhibitory influence which the thyroid hormone exerted on the synthesis of thyrotropin might extend to other unrelated metabolic activities in the adenohypophysis.

(b) It is conceivable that a change in oxygen consumption resulting from stress may not have been recorded in these studies. The pituitary glands are placed in the Warburg apparatus approximately 20 minutes after removal from the rat. An equilibrium period of 10 minutes exists before the first manometric reading. In this period of about 30 minutes the adenohypophysis may have recovered from any stress-induced change in oxygen consumption so that what in effect is observed appears to be a metabolically unaltered gland. In addition, the values are the average QO₂ between 60 and 120 minutes of incubation. The first few readings during the first hour were too variable. Only after the first hour were the results consistent. There were several indications of initial higher readings that subsequently fell to steady
levels during the second hour, thereby suggesting a leveling off period and a return to normal $QO_2$ levels.

(c) It has been shown (Nukariya, 1926; Severinghaus, 1933) that the basophils of the anterior pituitary of the rat constitute the smallest number of the cell types and the acidophils and chromophobes comprise the bulk of the anterior pituitary gland. The female rat adenohypophysis contained 15.5% basophils, 52.9 chromophobes and 31.6% acidophils. These figures are very similar to the adult human male (Rasmussen, 1929). Numerous investigators (Smelser, 1944; Giroud and Martinet, 1948; Halmi, 1950) presented evidence that the basophils are responsible for the elaboration of ACTH, although this is still an unsettled problem.

The basophils have been differentiated into at least two types, the beta and delta cells (Romeis, 1940). Halmi (1950) showed correlations between the variations of the beta cells and the fluctuations in ACTH activity in the adenohypophysis. Long exposure to cold of unilateral adrenalectomized rats caused a significantly increased number of beta cells, many of which were enlarged and more or less degranulated. Formalin (0.5cc of 4%) given for 3 days constituted a stress that caused a drop in the number of beta cells and a rise in the percentage of delta cells.

If the effect of stress is confined to metabolic alteration of the basophils, then considering that the basophils constitute the smallest part of the adenohypophysis, any alteration in oxygen consumption may have been missed by the method employed in this study.
It can therefore be concluded that either (1) stress causes no respiratory responses in the adenohypophysis or (2) alterations in oxygen consumption due to stress could not be measured under the condition of this experiment.

No significant difference in oxygen consumption between the tissues of normal and adrenalectomized rats was observed, thus confirming the observations of Roberts and Keller (1955) and Levey and Roberts (1957). The lack of a significant difference was observed whether or not glucose was included in the medium (Tables 4 and 5).

Of the 3 tissues studied in the adrenalectomized group, only the median eminence showed a significantly increased oxygen consumption when glucose was added to the medium (Table 6). In this respect the median eminences of intact and adrenalectomized rats are similar.

A statistically significant difference was not observed in median eminences obtained from adrenalectomized rats 2½ minutes after stress (Table 7). The QO2 observed after stress appeared to diminish, but a large standard error gave no justification for significance. Again, this picture was similar to that observed in the median eminence of intact stressed rats (Table 3). Similarly, pituitary respiration was unaltered by stress in adrenalectomized animals.

Hydrocortisone administered to rats four hours previous to sacrifice caused a significant increase in oxygen consumption in both the anterior pituitary and the median eminence. However, Roberts and Keller (1955) found that the intravenous administration of cortisone (2.5 mg/100 g. rat) produced a decline in oxygen consumption of the anterior
pituitary. It is pertinent to consider that they sacrificed their animals under pentobarbital anesthesia, and, in addition, glucose was included in the incubation medium. The above differences in experimental procedure hardly allows for a valid comparison between the two studies.

When hydrocortisone (100 µg) was added to the incubation medium, no change in oxygen consumption was observed in the anterior pituitary, although a significant decline occurred in the hypothalamus (Table 9). Roberts and Keller (1955) found that moderate amounts of cortisone acetate (10-25 µg) had no discernable effect on oxygen consumption. Larger amounts (250 µg) produced a depression of the QO₂. This in vitro inhibitory action was considered by them to be of a non-specific toxic nature. Eisenberg et al. (1950) compared the effect of varying concentrations of desoxycorticosterone upon the oxygen consumption of rat brain homogenate. They found that increasing amounts of the steroid produced increasing amounts of inhibition of QO₂, the relationship being linear when the logarithm of the dose was plotted against percent inhibition produced. It is interesting that the inhibition produced by 100 µg of steroid was almost identical to the percent inhibition observed in the hypothalamus in this study.

2. Glycogen Content

Previous attempts to measure the glycogen content of the anterior pituitary gland have been unsuccessful (Roberts and Keller, 1953; Barondes et al., 1961).
Barondes et al. (1961) used the trichloracetic acid extraction method of Carroll et al. (1956). However, Roe et al. (1961) demonstrated that complete removal of glycogen from tissues by this method of trichloracetic acid extraction was not accomplished due to inadequate homogenization.

Roberts and Keller (1953) used the method of Seifter et al. (1950) for their glycogen determinations. They did not report taking precautions to prevent post-mortem glycogen breakdown. Kerr (1936) emphasized that quick freezing of brain tissue was necessary to avoid post-mortem change in glycogen content. In addition, the effect of pentobarbital anesthesia on the rat pituitary glycogen content is unknown, and cannot now be evaluated, although Carter and Stone (1961) showed that the glycogen content in mouse brain was unaffected by pentobarbital anaesthesia.

The minimum sensitivity of the Seifter method for glycogen concentration is 15 µg. Lower values would therefore not be determined.

In the modified method of Seifter et al. (1950) used in this study, it was possible to detect glycogen in the adenohypophysis, median eminence and hypothalamus (Fig. 4, Table 10). No glycogen could be detected in six pooled posterior pituitary glands. As few as two anterior pituitaries, 3 median eminences or 1 hypothalamus were sufficient for glycogen detection. The lower limit of sensitivity for glycogen in this modified method is less than 5 µg.

It is interesting that Templeton (1961) recently modified the method of Seifter for micro-determination of glycogen with the anthrone
reagent. He was able to detect glycogen in 20-40 μg of skeletal muscle. Measurement of 0.05 μg of glycogen within ± 10% was possible. Interference by proteins was less than 0.06% and was not significant.

Carter and Stone (1961) used the glucose oxidase method for glucose formed from acid hydrolyzed glycogen isolated by alcoholic KOH precipitation. They found a mean glycogen value of 0.7 μg/mg rat brain. The mean value of brain glycogen in mice was 0.53 μg/mg brain. Chance and Walker (1954) estimated the cortical glycogen of mouse brain to be 0.74 μg/mg cerebral cortex.

The above values are fairly consistent with brain and pituitary values found in this study - median eminence (1.03 μg/mg), hypothalamus (0.39 μg/mg), and anterior pituitary (0.69 μg/mg).

Kerr (1936) pointed out that brain glycogen precipitates contain small amounts of cerebrosides. Correction for cerebroside content was not made in this study. The cerebroside content of the anterior pituitary, median eminence and hypothalamus cannot be estimated at this time. An important factor to consider in these results, however, is the fact that the cerebroside content would not be expected to change in response to an acute stress, since they are believed to be structural rather than metabolically active components of tissue.

Stress caused a 38% decline in anterior pituitary and a 49% decrease in median eminence glycogen. No change was observed in the hypothalamus.

Goodner and Freinkel (1961) working with slices of anterior pituitary of rat and calf were able to demonstrate the incorporation
of C-14 glucose into glycogen, thereby demonstrating the capability of
the pituitary to turn over glycogen. They considered that "... the
mobilization of glycogen, and the oxidation of glucose-6-phosphate
within the pituitary could subserve important functions in selective
or generalized hormonogenesis."

Barondes et al. (1961) showed that glucose-1-C-14 oxidation by
rat and beef anterior pituitary slices was stimulated by epinephrine,
norepinephrine and serotonin. They considered that if neurohumoral
agents are important in regulating anterior pituitary function, they
might mediate this effect by altering the glucose metabolism of this
gland.

Recent histochemical studies (Balogh and Cohn, 1962) on the
localization of oxidative enzyme systems in the human anterior pi-
utitary demonstrated that the basophils exhibited strong DPNH and TPNH
diaphorase, succinic, lactic, malic and glucose-6-phosphate dehydro-
genase activities. TPNH is generated when glucose is metabolized via
the hexose monophosphate shunt. Since TPNH has also been found to
stimulate protein synthesis (Wilson and Siperstein, 1959), Field et
al. (1960) suggested that this cofactor could be important in pitui-
tary production of the various tropic hormones. The pentose shunt
also appears to be important in other endocrine organs (Field et al.,

In view of the above considerations of glycogen mobilization and
glucose metabolism in the anterior pituitary and other endocrine or-
gans, the effect of stress on glycogen content can be considered in the
light of increased metabolic demands on the tissue. This would, there-
fore, be consistent with the view that an acute stress causes an
increased synthesis of ACTH.

3. Phosphorylase Activity

In view of the fact that stress has been shown to cause glyco-
genolysis in the anterior pituitary and median eminence, it was con-
sidered of interest to study the nature of the phosphorylase response
to stress. Active phosphorylase is known to catalyze the breakdown of
glycogen to glucose-1-PO₄. Phosphorylase determinations have not pre-
viously been reported in the pituitary gland.

The results in Table 11 indicate that all of the phosphorylase
exists in the active form in both the normal and stressed rat pitui-
taries. This phenomenon is not without precedence. Rabbit liver
slices were shown to contain all the phosphorylase in the active form
(Sutherland and Ball, 1960). However, the existence of this enzyme
entirely in the active form in the intact animal is not known. It
must be seriously considered that immediately before and after sacri-
fice, release of catecholamines causes activation of inactive phos-
phorylase to the active form. A prominent metabolic effect of cate-
cholamines is the increased glycogenolysis observed in liver and
skeletal muscle. The anterior pituitary has been shown to take up
large quantities of H⁵⁻epinephrine and norepinephrine (Axelrod et al.,
1961; Weil-Malherbe et al., 1961; Samorajski and Marks, 1962). It is,
therefore, possible that brief handling of the animal will release
catecholamines and cause activation of the phosphorylase present in the
tissue. In addition, decapitation may very well cause central sympa­
thetic discharge of catecholamines. On the other hand, the nature
of the enzyme may differ from that known in liver and muscle. Suther­
land and Rall have pointed out that phosphorylases from different tis­
sues show physical and kinetic differences. Phosphorylase provides an
example of an enzyme which varies considerably in properties from tis­sue to tissue while being a catalyst for an identical reaction in these
tissues (Sutherland and Rall, 1960).

A final possibility in evaluating the present results must be
considered. A basic assumption made in this study was that the in­
active phosphorylase could be activated by 5-AMP in the assay procedure.
This is consistent with assay procedures demonstrating cardiac or
skeletal muscle phosphorylase. However, in the liver, adenosine-3',
5'-phosphate (cyclic 3', 5'-AMP), is the only cofactor capable of acti­
vating phosphorylase in the assay system (Sutherland and Rall, 1960).
Physiologically, cyclic 3', 5'-AMP is the cofactor responsible for acti­
vation of phosphorylase. In muscle and liver, epinephrine causes an
increased formation of cyclic 3', 5'-AMP. Norepinephrine does this to
a smaller extent. In the adrenals, however, only ACTH causes an accumu­
lation of cyclic 3', 5'-AMP. Williams et al. (1961) demonstrated that
in bovine corpus luteum slices, phosphorylase could be activated by the
addition of anterior pituitary extract, chorionic gonadotropin, and
growth hormone. It will obviously be desirable to test the activating
effect of cyclic 3', 5'-AMP in the pituitary.
It can be concluded that total activation of phosphorylase as observed in this study can be due to one of several reasons:

(a) Phosphorylase exists in the pituitary only in the active form.

(b) Activation of phosphorylase has not actually been observed because cyclic 3',5'-AMP may be the only possible cofactor that can demonstrate activation in the assay system.

(c) Discharge of a catecholamine, or another neurohumor (serotonin, histamine), as a result of handling and decapitation may cause total activation of the phosphorylase present.
B. Uptake of Labeled Amino Acids

1. In vivo uptake of \(^{3}\text{H}\)-phenylalanine

Experiments were performed to compare the uptake of \(^{3}\text{H}\)-phenylalanine by the adenohypophysis, posterior pituitary, median eminence, anterior and posterior hypothalami in normal, adrenalectomized and hydrocortisone treated rats.

The animals were sacrificed at 12\(\frac{1}{2}\) minutes after the injection of the phenylalanine. Roberts et al. (1959) studied the rates at which phenylalanine incorporated into the rat cortex. They showed maximum concentration of the label in the plasma and tissue pool in 10 minutes, whereas the tissue protein concentration was relatively low at that time. Therefore, in this study the rats were stressed 10 minutes after injection of \(^{3}\text{H}\)-phenylalanine and sacrificed 2\(\frac{1}{2}\) minutes after the onset of stress. Recently, Lajtha and Toth (1962) showed that following subarachnoidal administration of labeled phenylalanine, the maximum cerebral content of the amino acid was reached 5 minutes after administration and was maintained at this level for 20 minutes and then subsided.

In the following discussion it must be clearly understood that the results are given in terms of radioactive content of the tritium label in the tissue representing the combined incorporated and free phenylalanine. In these experiments there is no way of knowing if the phenylalanine has been incorporated into one or more peptide hormones. It is, therefore, hazardous to attempt to relate changes in uptake of the labeled amino acid under the various conditions of the experiment.
with increased incorporation of the phenylalanine or increased release of peptides containing the radioactive labeled amino acid.

Minor changes cannot be readily interpreted as changes of significant value. On the other hand, in comparing changes between experimental procedures (adrenalectomy, hydrocortisone), the direction of change can be of value if the changes are considerable.

The posterior pituitary demonstrates a great avidity for labeled phenylalanine (Fig. 5). This may be a reflection of a greater flow of blood through the posterior pituitary gland per mg. of tissue. Goldman (1962) has demonstrated a greater flow of blood per unit of neurohypophysial tissue than that of the adenohypophysis of the rat. Since there is no equivalent concentration of serine in the posterior lobe, the differences in blood flow cannot be a major factor in determining the uptake.

The existence of a partial blood brain barrier for amino acids probably explains the comparatively smaller uptake of phenylalanine in the hypothalamus (Waelisch and Lajtha, 1961). Greater synthetic activity and polypeptide content of the anterior and posterior pituitary might also be responsible for greater uptake of the amino acid in these tissues.

The significance of the reduced content of \( H^2 \)-phenylalanine in the adrenalectomized posterior pituitary and median eminence (Fig. 5) is difficult to explain at this time. It is possible that in the time selected for measurement, there is a greater turnover of the labeled amino acid in adrenalectomized rats. Goldman (1962) showed that in
30-day adrenalectomized female rats, the anterior pituitary blood flow in the unstressed rat was significantly higher than normal. However, posterior lobe blood flow in the adrenalectomized rat did not appear to be altered in comparison to intact rats. The increased rate of ACTH secretion at this time also supports the idea that increased turnover is occurring in the anterior pituitary gland. Hoberman (1950) showed that biochemical reactions leading to the formation of body protein were accelerated in 6-day adrenalectomized rats. He observed that the utilization of $N^{15}$-glycine for protein synthesis is greater than normal in adrenalectomized rats.

The decreased uptake of $H^3$-phenylalanine in adrenalectomized rats observed in the present studies may, therefore, be a reflection of the increased uptake of this amino acid by other tissues of the body, with a consequent reduction of the amino acid available to the pituitary.

The most profound effect of adrenalectomy is clearly manifested in the neurohypophysis rather than the adenohypophysis (Fig. 5). It is interesting that both portions of the neurohypophysis, the neural lobe and the median eminence, appear to respond in identical ways. A possible explanation for the fall in neurohypophysial uptake of amino acid after adrenalectomy could be in terms of an ACTH feedback mechanism causing an inhibition of CRF production in the neurohypophysis. Long term adrenalectomized rats contain large quantities of ACTH, both in the circulation and the adenohypophysis. Considering that part of the amino acid sequence in CRF (both α and β) and MSH are identical with ACTH, an inhibitory feedback on the enzymatic production of these smaller
peptides by ACTH is conceivable. Evidence that favors or opposes this hypothesis is not available, since no studies have been performed showing changes in CRF content resulting from experimental alterations in blood ACTH content.

In addition, the lack of circulating catecholamines manufactured in the adrenal medulla can be another explanation for the fall in adenohypophysial and neurohypophysial uptake of amino acid after adrenalectomy. There is evidence that suggests that the movements of the amino acids between blood and brain are part of an active transport system (Waelsch and Lajtha, 1961). Guroff et al. (1961) have shown that glucose is directly involved in the cellular system responsible for concentrating amino acids. Rall et al. (1957) showed that the lack of epinephrine in the adrenalectomized rat slows down glycogenolysis. The reduction of circulating epinephrine, therefore, might possibly affect the uptake of $H^3$-phenylalanine in the tissues noted in this experiment. The fact that circulating norepinephrine is concentrated in the adenohypophysis and neurohypophysis is further supported by autoradiographic studies demonstrating the uptake of $H^3$-norepinephrine in this tissue (Samorajski and Marks, 1962).

Administration of a large blocking dose of hydrocortisone also produced its most profound depression of $H^3$-phenylalanine uptake in the posterior pituitary and median eminence (Fig. 5). There was also a reduction in uptake in the posterior hypothalamus. The other tissues were not markedly affected. It is noteworthy that hydrocortisone had no effect on uptake in the anterior pituitary gland. There is evidence
that suggests that large doses of corticoids act on the hypothalamus rather than the adenohypophysis itself (Endroczí et al., 1961; Davidson and Feldman, 1962). It has also been suggested that large doses of hydrocortisone act by blocking the secretion of a corticotropin releasing factor (McCann et al., 1958; Schapiro et al., 1958; Brodish and Long, 1957).

It is therefore tempting to suggest that the lack of hydrocortisone effect on the anterior pituitary and the reduction of uptake observed in the posterior pituitary, median eminence and posterior hypothalamus is a reflection of this site of action of the corticosteroid.

The mechanism whereby the corticoid inhibits ACTH synthesis by acting on neural tissue is a matter of conjecture. Clark (1953) presents evidence indicating that one of the actions of cortisone in the body is to regulate protein synthesis by interfering with the formation of a new protein. An antianabolic effect of hydrocortisone on the brain tissue could explain the diminished uptake of H³-phenylalanine observed in this tissue.

Long et al. (1940) showed that adrenal steroids participate in the regulation of protein metabolism, the predominant effect being the mobilization of endogenous protein. Considerable supporting evidence has since accumulated and has been reviewed by Engel (1951).

Hydrocortisone given to adrenalectomized animals produced an increase in uptake in all organs (Fig. 6). Except for the minor rise in the anterior hypothalamus, a 34-76% increase was noted in the remaining tissues.
The cause for this very striking phenomenon is not obvious. Increase in uptake observed in the hydrocortisone adrenalectomized group may be a reflection of a reduction of release of the label or an increased uptake of the amino acid or both of these possibilities. Adrenalectomy has been shown to increase incorporation of amino acids into body protein (Hoberman, 1950; Roberts, 1953; Wool and Weinshelbaum, 1959). These studies were on animals adrenalectomized for less than one week. Uptake studies in this experiment were performed on 30-day adrenalectomized rats. The difference in time of adrenalectomy may be very significant.

Korner (1960) has performed experiments on the incorporation of radioactive amino acids into protein by rat liver microsomes. He found that adrenalectomy caused an immediate increase in the ability of microsomes to incorporate amino acids into protein. This occurs to a greater extent in males. Females show a rise of about 50% in their incorporating ability. This increased incorporation in microsomes from female rats decreases rapidly and vanishes about 1 week after adrenalectomy, and subsequently they become less able to incorporate amino acids into protein than normal rat liver microsomes. Microsomes from male rats show increased incorporating ability for more than 2 weeks after adrenalectomy, after which there is an opposite effect. When normal rats are treated with small doses of cortisol acetate (0.25-5 mg/day) for 7 days there is a rise in the ability of their liver microsomes to incorporate amino acid into protein in vitro. On the other hand, 7-day adrenalectomized rats given cortisol for 1 week
clearly show a reduction in incorporation of amino acids into protein in vitro. "It appears that removal of the adrenal glands results in the immediate release of some restraint which has checked the capacity of the liver microsomes to incorporate amino acids, but that from the long-term view the adrenal glands appear to be needed, at least to some extent, to prevent amino acid incorporation from occurring at sub-optimal rates" (Korner, 1960).

Therefore, in long-term adrenalectomized animals, corticosteroids are needed to increase amino acid incorporation into liver microsomes. If this is a reflection of what occurs in the pituitary of 30-day adrenalectomized rats used in this study, an increase in uptake of phenylalanine due to hydrocortisone would be understandable.

Figure 7 shows the effect of stress on the content of H\(^2\)-phenylalanine in the tissues studied. The new dimension added here, stress, complicates the interpretation of these findings considerably.

Stress reduced the content of the radioactive label in all organs of intact rats. Whether this is a reflection of increased release or decreased uptake of amino acid is not certain.

It is again interesting that the posterior hypothalamus and the neurohypophysis show the greatest changes and that they appear to respond similarly. This helps to confirm the idea that these structures interact as a single functional unit in regard to processes related to stress, ACTH formation and metabolic alterations produced by endocrine agents.
In order to determine whether in fact any differences were produced by stress in the metabolic fate of the labeled amino acids, further studies were performed to fractionate the radioactivity. These will be discussed in the next section.

2. Separation of Radioactive Labeled Peptides

The content of H\(^3\)-phenylalanine and C-14 serine in glacial acetic acid extracts of pituitary and hypothalamus is shown in Tables 12 and 13. Several interesting points are to be noted here. The posterior pituitary extract contains approximately 8 times as much H\(^3\)-phenylalanine as the anterior pituitary extract. This increased uptake is supported by the studies discussed above (Fig. 5) which showed that the uptake of labeled phenylalanine was greater in the posterior pituitary. On the other hand, uptake of C-14 serine by both lobes of the pituitary were similar per unit weight of tissue. The adenohypophysis appeared to have a much greater affinity for serine than for phenylalanine. This is shown by the fact that the adenohypophysis took up more serine counts than phenylalanine counts even though the serine had a much lower specific activity. Uptake by the hypothalamus of both amino acids was remarkably similar.

Stress induced an increased uptake of both amino acids in the extracts of the anterior pituitary. The increase was greater in the serine group. However, stress resulted in a marked decrease of H\(^3\)-phenylalanine content in the posterior pituitary extract, again supporting the in vivo incorporation studies cited above (Fig. 7). The reason for this phenomenon is not known. In the posterior pituitary,
phenylalanine appears to be incorporated into one or more polypeptides that are labile to stress. This does not appear to occur with serine. It is, therefore, tempting to suggest that phenylalanine incorporates into one or more polypeptides that do not contain serine. Vasopressin and oxytocin are the only posterior pituitary peptides containing phenylalanine that are known not to contain serine. The other known peptides, CRF and MSH, contain serine as well as phenylalanine.

The incorporation of the labeled amino acids into the non-extractable residue (RL) is given in Table 14. It is clear that greater incorporation into this residue is accomplished by phenylalanine. What is most significant here is that stress does not have an effect on the content of either amino acid in the anterior pituitary gland. However, stress reduced the H\textsuperscript{3}-phenylalanine content of the residue of the posterior pituitary by 62\%. This reduction is of similar magnitude to that observed in the glacial acetic acid extracts above (Table 12). Compared to the uptake of amino acids in the extracts, the amount taken up by the residue is considerably smaller. The composition of the RL fraction of tissues is not known, but it undoubtedly consists mostly of structural and enzyme proteins, and, in the case of hypothalamus, a large proportion of lipid.

An insoluble residue, remaining after lyophilized glacial acetic acid extracts were taken up in water, was examined for its content of radioactivity. The content of radioactivity in the R2 and R3 fractions is given in Tables 15 and 16. The insoluble residue (R3) is probably denatured protein. Considering the effect of stress on the acid extract and the insoluble residue in the posterior pituitary, labilization
of the structural protein as a result of stress is suggested. The question therefore arises, does stress cause protein breakdown to smaller peptides? This indeed is an interesting consideration. Specific peptidase activation as a result of stress could be responsible for this phenomenon. This problem could be approached through protein and enzymatic analytical procedures. In addition, histochemical localization of activated peptidases could be very revealing. Ellis (1961) has demonstrated the existence of proteinases in anterior pituitary extracts. Existence of these enzymes in the posterior pituitary is therefore very possible.

A point of some interest that might be considered from Table 16 is that phenylalanine and serine almost surely must be in different chemical substances in the posterior pituitary. Evidence for this is the markedly different response to stress of these two labels.

The counts in the R2 fraction are not striking. In both amino acid groups there is a fall in the number of extractable counts in the anterior pituitary and a rise in the posterior pituitary as a result of stress. Since the distribution of counts in R2 is generally similar to those in the original glacial acetic acid extract, R2 is probably essentially the unseparated portion of the original lyophilized extract.

The lyophilized glacial acetic acid extracts were taken up in water and were submitted to electrophoresis and chromatography. The chromatograms were autoradiographed. Radioactive spots were found on all of the chromatograms (Figures 3 to 17). Every film in both amino
acid experiments contained the same radioactive spot (spot 1). The $R_f$ value and electrophoretic migration of these spots from each chromatogram is given in Table 17. The mean $R_f$ value ranges between .13 and .14 and the mean electrophoretic migration is 1.8 cm. The very similar coordinate values found in both the phenylalanine and serine experiments strongly suggests that the radioactive labeled spot 1 is identical in both the serine and phenylalanine chromatograms. The fact that the coordinate values for the free amino acids serine and phenylalanine differ from that of spot 1 rules out the possibility that these amino acids are spot 1. The fact that both the labeled serine and phenylalanine spot 1 fall in the same position indicates that both of these labeled amino acids are incorporated into the same peptide. The most striking result of the autoradiographic study is the marked increase in intensity of spot 1 in the stressed adenohypophysis extract.

In addition to spot 1, several other much less intensely labeled spots were found. Two radioactive labeled spots adjacent to spot 1 was observed in the C-14 serine autoradiograms (Figs. 8 and 9). Both these spots (spot 2 and spot 3) are more intense in the stressed group. Spot 3 was also found in the posterior pituitary stress group (Fig. 11) and normal hypothalamus of the C-14 serine X-rays (Fig. 3).

An additional spot (spot 4) appeared on the C-14 serine normal posterior pituitary X-ray film (Fig. 10). This spot corresponded approximately with the position of serine itself.

Another spot (spot 5) appeared in both the normal and stressed hypothalamus chromatogram of the C-14 serine group (Figs. 12 and 13).
This spot appeared close to the solvent front with a mean $R_f$ value of .76. No electrophoretic movement was noted. An acidic lipid compound containing serine having a zero net change at pH 3.5 would fit the characteristics of this spot in that it would show no electrophoretic movement, but considerable chromatographic movement. One possibility in the identification of spot 5 is phosphatidylserine. Verification of this can only come when a known sample of phosphatidylserine is studied in the system used here.

3. Quantitation of Radioactivity in the Radioactive Spots

Table 18 shows the recovery of the radioactivity in all paper chromatograms of spot 1 corresponding to the dense areas on the X-ray films. It appears that approximately 25% of the glacial acetic acid extracts of the serine injected rats was recovered in spot 1 of the unstressed anterior pituitary gland; 35% was recovered in the stressed anterior pituitary. An extremely small number of counts were retrieved from the $^3H$-phenylalanine spots. The reason for this is unknown.

Stress resulted in a 94% increase in the content of radioactive serine in spot 1. A marked increase of C-14 serine incorporation was also observed in the posterior pituitary after stress. Considering that there was no change in the glacial acetic acid extracts (Table 13) of the posterior pituitary after stress, most of the radioactivity that is unaccounted for probably lies in spot 4, which is considered to be C-14 serine itself. Stress had no effect on the radioactive content of spot 1 in the hypothalamus.
The results of the autoradiographic study of glacial acetic acid extracts of pituitary and hypothalamus make several things very clear. Most important is the observation that a single major radioactive compound (tentatively a polypeptide) is found in the anterior pituitary, posterior pituitary and hypothalamus of rats 12\frac{1}{2} minutes after injecting labeled amino acids. It follows, then, that this must be the most rapidly synthesized peptide in these organs. The content of radioactivity is much higher in the adenohypophysis than in other structures noted, so that one must consider the possibility that this radioactive peptide originates in this organ and may be distributed to the other sites either before or after decapitation.

The second important conclusion is that the synthesis of this major component is markedly stimulated by a brief stress. This confirms the results of the radioactivity counting of the glacial acetic acid extracts, and directs our attention to the possibility that this major component that is so rapidly synthesized may be ACTH.

4. Identification of Radioactive Labeled Peptides

Ninhydrin-stained 2-dimensional patterns of tissue extracts from 6 pooled rats showed a spot that closely approximated the coordinate values of spot 1 (Figs. 20-22). Chromatographic separation of extracts from pooled tissue of only 2 adrenalectomized rats showed a faint indication of a peptide in approximately the same region as spot 1 (Fig. 23). The chromatogram of extracts from a stressed group of adrenalectomized rats contained a larger and denser spot that very closely resembled
spot 1 in its location (Fig. 24). If spot 1 is ACTH, the relationship observed between Figs. 23 and 24 resembles the increased ACTH content of pituitary after stress as reported by Vernikos-Danelis (1962).

Hormone standards from various species run in this chromatographic system did not correspond to any of the radioactive spots (Figs. 25 to 29). The R_f values and electrophoretic migrations are given in Table 20. It is pertinent for this study to emphasize the fact that standard ACTH, obtained from two sources, did not at all correspond to spot 1 (Figs. 25 and 26).

Chromatograms of stressed adrenalectomized rats were prepared in order to assay spot 1 for ACTH. Table 21 summarizes the results obtained. Extracts of spot 1 were assayed both in cortisol-blocked and hypophysectomized rats in order to rule out the presence of CRF in spot 1.

It is clear that a considerable concentration of ACTH was found in spot 1 of the anterior pituitary gland. The posterior pituitary and hypothalamus also contained small amounts of adrenocorticotropic activity. The results in Table 22 demonstrates that a large amount of ACTH activity is lost in the electrophoretic and chromatographic procedure. Considering that a loss of over 90% ACTH activity has occurred in the isolation procedure utilized in this study, correction for this loss indicates that approximately 45-90 μg/mg ACTH had been isolated in spot 1. This includes the reported ACTH content of these glands (Vernikos-Danelis, 1962).
Since the spot obtained by chromatography and electrophoresis of standard ACTH (Figs. 25 and 26) differs markedly from spot 1, it was considered that ACTH may have an additional component that separates into the spot 1 region and cannot be detected with ninhydrin. That this consideration was not correct was shown in Table 23. No ACTH activity existed in the spot 1 area in either the pure ACTH obtained from C. H. Li or the ACTH obtained from Armour. All the activity existed in the spot indicated in Figures 25 and 26. In addition, an area equivalent to the standard ACTH spot on the chromatogram obtained from the extract of the anterior pituitaries of stressed adrenalectomized rats contained no ACTH activity. This indicates that in the rat ACTH is found in spot 1, while in other species ACTH is found in another area.

Is it possible that rat ACTH differs markedly from species whose ACTH has already been characterized (sheep, pig, beef, human)? Certainly, both the electrophoretic and chromatographic behavior of rat ACTH is markedly different from that of ACTH from other species. There are other possibilities that must be ruled out before spot 1 can be finally considered to be rat ACTH.

(a) α-CRF, containing exactly the same sequence of the first 13 amino acids contained in ACTH plus threonine, alanine and leucine, has some inherent adrenocorticotropin hormone activity, equal to or less than 0.2 u./mg (Schally et al., 1960). It is felt that for several reasons spot 1 is not α-CRF. (1) Reports of the presence of α-CRF in the anterior pituitary have not appeared in the literature. (2) ACTH
activity was evident in hypophysectomized assay rats in which CRF is inactive. (3) Only recently has \( \alpha \)-MSH, vasopressin, and \( \alpha \)-CRF been separated by column chromatography (Schally et al., 1962a). \( \alpha \)-MSH and lysine vasopressin were very close in the chromatographic system used in this study. \( \alpha \)-CRF would be expected to have similar electrophoretic and chromatographic properties possessed by \( \alpha \)-MSH and vasopressin. In addition, \( \alpha \)-MSH and \( \alpha \)-CRF closely resemble each other in amino acid content. It would therefore be expected that the \( \alpha \)-CRF would migrate to a position other than that of spot 1.

(b) An ACTH-like peptide was isolated recently from pig and dog hypothalami by column chromatographic procedures (Guillou et al., 1962; Schally et al., 1962). It differs from ACTH in its elution pattern. It is very similar in this respect to \( \alpha \)-MSH, vasopressin and \( \alpha \)-CRF. This substance has not been further characterized, and has not been reported in the anterior pituitary. As considered above, the similarity in column chromatographic elution patterns would rule out its migration to the region occupied by spot 1.

Considering that ACTH activity was found in spot 1 in both cortisol-blocked and hypophysectomized rats it is concluded that spot 1 is rat adrenocorticotropic. This is therefore the first demonstration of isolation of rat ACTH by the use of electrophoretic and chromatographic separation. In addition, it is the first time a radioactive labeled amino acid has been incorporated into ACTH in vivo. Wool et al. (1961) reported the in vitro incorporation of labeled phenylalanine into rat ACTH within anterior pituitaries contained in an incubation
medium. Holub (1962) used the method of Wool to confirm incorporation of labeled amino acids into ACTH. Both groups of investigators showed that adrenalectomy caused a greater incorporation of the labeled amino acid.

It is concluded from this study that stress causes an increased synthesis of new ACTH in intact rats. Increased pituitary ACTH content as a result of stress has not been measured before in intact animals, although increased blood levels are detected after stress. Sydnor and Sayers (1954) contend that stress may have produced a loss of ACTH from the pituitary which could not be detected by the assay procedure. Using this same line of reasoning it is conceivable that stress produced an increase in pituitary ACTH content which could not be measured by the assay method. Considering that stress in intact rats causes increased blood ACTH without a detectable change in ACTH content in the pituitary, it is possible that an increased synthesis of ACTH may have occurred. That this is the case is borne out by the results in this study.

How can a long chain polypeptide such as ACTH be formed within 2½ minutes? Two possibilities exist that attempt to explain this remarkable phenomenon. (1) The existence of a polypeptide precursor could facilitate rapid synthesis of ACTH. α-CRF is one such precursor that has been postulated (Schally et al., 1961). That α-CRF is a precursor has not been established. Dasgupta and Young (1958) have proposed an inactive corticotropin, "precorticotropin", that is activated by acid. This is considered unlikely because serine, or any
amino acid, would not incorporate by this activation process. (2) New synthesis of ACTH is the other possibility. A recent report demonstrates that a complex polypeptide molecule, hemoglobin, is synthesized at the rate of 2 amino acids per second (Dintzis, 1961). Peptide synthesis at this rate could adequately account for new ACTH synthesis in a small fraction of the $2\frac{1}{2}$ minutes used in this study. Actually an earlier time of sampling might reveal even greater stress effects.

New synthesis of ACTH in response to stress is compatible with recent studies of the effect of ethionine on stress-induced ACTH secretion (Marks and Vernikos-Danellis, 1962).

The increased synthesis of ACTH observed after stress is of physiological significance. An increased synthesis and release of ACTH (above the basal level) as a result of stress would cause an increased secretion of corticoadrenal hormones and a subsequent attempt by the body to resist further damage from stress.

The answer to the original question proposed in this thesis -- does stress cause an increased synthesis of new ACTH? -- is clearly demonstrated in this dissertation. Stress does cause an increase in synthesis of ACTH with remarkable speed!
VI. SUMMARY AND CONCLUSIONS

Metabolic and radioactive amino acid uptake studies were performed in order to study the effect of stress on the activation of ACTH synthesis.

1. Stress did not produce any changes in the respiration of the anterior pituitary or the hypothalamus, but under some conditions it produced an increase in the oxygen consumption of the median eminence. Respiration of the median eminence had not previously been studied.

2. A new modification of the anthrone method for glycogen determination enabled measurement of glycogen in the anterior pituitary and median eminence for the first time. Stress caused a significant decrease in the glycogen content of both the anterior pituitary and median eminence. No change was observed in the hypothalamus. The effect of stress in these tissues was considered in the light of increased metabolic demands.

3. Phosphorylase determinations have been performed in the anterior pituitary for the first time. The results indicate that all of the phosphorylase exists in the active form in rat pituitaries. Possible explanations for this phenomenon have been discussed.

4. Experiments were performed to compare the uptake of $^3$H-phenylalanine by the adenohypophysis, posterior pituitary, median eminence, anterior and posterior hypothalami in normal, adrenalectomized, hydrocortisone treated and stressed rats.
The greatest uptake of radioactive phenylalanine per unit weight of tissue was observed in the normal posterior pituitary gland. Adrenalectomy resulted in a marked reduction in uptake of phenylalanine in the posterior pituitary and median eminence. The reasons for this are discussed. A blocking dose of hydrocortisone produced its most profound depression of amino acid uptake in the posterior pituitary and median eminence. No effect was noted in the anterior pituitary gland. Hydrocortisone given to adrenalectomized animals produced an increase in uptake in all organs. The cause for this striking phenomenon was not established.

Stress reduced the content of the radioactive label in the neurohypophysis and posterior hypothalamus of normal rats. The posterior hypothalamus and the neurohypophysis show the greatest changes and appear to respond similarly in these experiments. This supports the idea that these structures interact as a single functional unit in regard to processes related to stress, ACTH formation and metabolic alterations produced by endocrine agents.

5. Experiments were performed with $^3$H-phenylalanine and $^{14}$C-serine in order to study the incorporation of these amino acids into peptides of the anterior pituitary and hypothalamus. The adenohypophysis appeared to have a greater affinity for serine and the posterior pituitary had a greater affinity for $^3$H-phenylalanine.

Stress induced an increased uptake of both amino acids in peptide extracts of the anterior pituitary. The increase was greater in the serine group. Stress resulted in a marked decrease of $^3$H-
phenylalanine content in the posterior pituitary extract, with no change in serine content. It is suggested that phenylalanine and serine are incorporated into different chemical substances in the posterior pituitary.

6. Radioactive peptides were separated by 2-dimensional electrophoresis and chromatography. In all instances, the same spot was detected on autoradiograms. Stress resulted in an increased incorporation of radioactivity in this spot. Comparison with known hormone standards was not successful in identifying this peptide. By bioassay in cortisol-blocked and hypophysectomized rats, this peptide was identified as ACTH. The characteristics of this peptide differ from those of ovine and porcine ACTH.

Other radioactive spots were also observed and their significance was discussed.

7. The results of this study indicate that stress causes an increased synthesis of new ACTH. The mechanism of rapid new synthesis of ACTH may be metabolically related to the simultaneous glycogenolysis which was demonstrated to occur in the pituitary and median eminence.
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