CATECHOLAMINES AND CORTICOSTERONE IN ADRENAL GLANDS AND PLASMA FOLLOWING RESTRAINT IN CHICKENS

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CHAPTER I

INTRODUCTION

Most of the work concerning the response to stress has been performed with the use of mammals as experimental animals. Soon after the discovery that epinephrine initiated a response similar to that observed after subjecting an animal to any non-specific stressor, many workers attempted to fit epinephrine into the mechanism of the stress response. Three sites, the adrenal cortex, anterior pituitary, and hypothalamus, were closely investigated in the mammal as being possible areas of action of epinephrine in bringing about mobilization of the adrenal cortex. To date, no conclusive evidence has come to light to indicate whether epinephrine has a role in the general stress response and more specifically in causing activation of the adrenal cortex and a subsequent release of cortical hormones. As a consequence of most work having been done in mammals, the role of endogenous epinephrine or norepinephrine in the reaction mechanism to stress in chickens is unknown.

This investigation was conducted in order to characterize the response pattern of epinephrine, norepinephrine, and corticosterone in both plasma and adrenal glands of birds following acute restraint. A number of drugs were employed in an attempt to block the release of the two catecholamines from the adrenal gland during stress with the hope of obtaining more evidence concerning the necessity of epinephrine and/or norepinephrine release in the normal stress response in chickens.

CHAPTER II

REVIEW OF LITERATURE

It has been shown that intravenous infusion of physiologic doses of epinephrine caused an immediate and long-lasting stimulation of the adrenal cortex, the increased secretion being several times the basal output of cortical hormones (3, 4, 80, 102). A great many experiments have been carried out in an effort to evaluate the effects of epinephrine (adrenalin, sympathin) on the adrenal cortex, the anterior pituitary, and the hypothalamus, the three most likely sites of action of epinephrine in mobilizing the adrenal cortex.

ADRENAL GLAND

Adrenomedullary Histology: In the mammal, norepinephrine (noradrenalin, arterenol) is stored in specific cells within the adrenal medulla which also possesses cells that contain principally epinephrine (50). The cells that contain only norepinephrine lack the methylating enzymes necessary to convert norepinephrine to epinephrine. It has been suggested that both types of cells are selectively innervated, which allows for the release of the relative and absolute amounts of the two hormones to be controlled by the central nervous system (22, 50).

The existence of two specific, catecholamine-secreting cellular types in the adrenal medulla of the rat, mouse, cat, and hamster has been verified by Eranko (26, 27) who also found that cells that formed epinephrine

or norepinephrine, in the intact adrenal, retained their ability to produce the same specific hormones when transplanted to another site (28).

In contrast to the familiar arrangement of the discrete outer cortex and inner medulla in mammalian species, the avian adrenal gland has no definite cellular distribution into cortex and medulla. The cords of "medullary" cells are arranged in a uniform network with the anastomosing cortical strands and are not aggregated into a central mass (24). The norepinephrine-secreting cells are randomly distributed, in a solitary manner, throughout the islets and tongues of chromaffin tissue, with each cell being surrounded by cortical cells or chromaffin cells that contain only epinephrine (12). Independent epinephrine and norepinephrine secretory granules have been located within discrete cells of the chromaffin tissue of the chicken (93).

Elliot and Tuckett (24) and Shepherd and West (94) recognized that medullary tissue formed at least 50% of the adrenal gland in chickens. Unlike the adrenal glands of mammals, those of fowl do not increase in weight after the third month of life (24).

Eranko (29), using a fluorometric technique, found that norepinephrine made up only one-third of the total catecholamine content of the adrenal glands from adult, laying hens. This investigation failed to support an earlier report that 85% of the total adrenal catecholamine content was norepinephrine (94).

Vascularization: Intramuscular injection of epinephrine caused an increased filling of the adrenocortical vessels in the rabbit, as shown by Harrison in 1957 (46). The increased filling of cortical capillaries is brought about by the constriction of the arteriae medullae of Flint (34) which normally carry blood directly to the medulla from the arterial

plexus in the capsule of the gland. This work appears to agree with the earlier report of Gersh and Grollman (40) who noted that the adrenal glands of rats and mice, subjected to 10° C. for 7 hours, showed a more pronounced filling of the cortical capillaries, which resulted in an immediate blood supply for a greater number of cortical cells. This reaction, which appears about 33 minutes after the beginning of epinephrine infusion, is reported to be independent of the hypothalamus and adenohypophysis (46).

Harrison and Hoey (47) demonstrated in the rat that the increased filling of cortical capillaries, induced by epinephrine, was also produced by ACTH, however, the latter hormone required a longer period of time to produce the effect even when administered intra-arterially. Stimulation of the femoral nerve by crushing or by electrical means resulted in an increased filling of cortical vessels within 30 minutes following the initial stimulus. When rats were acutely subjected to extremes of heat or cold, a considerable increase in filling of the cortical capillaries, as well as hemorrhages in the zona fasciculata, were noted in the adrenal glands. It was suggested that epinephrine might assist in causing the release of cortical hormones by increasing the blood supply to the cortical cells.

Hechter (48), using perfused bovine adrenals, demonstrated that ACTH in the perfusion medium caused an increased release of cortical hormones, but wide variations in the concentration of epinephrine failed consistently to elicit cortical hormone release. In contrast to this report, it was found that epinephrine caused eosinopenia in hypophysectomized rats, but failed to bring about the eosinopenic response in hypophysectomized, adrenalectomized rats (63). Histological evidence indicated that epinephrine directly stimulated the zona glomerulosa in order the bring about the condition of eosinopenia.

Walker et al. (103, 104, 105) demonstrated that increased activity of the adrenal cortex in dogs, in response to hemorrhage or tissue trauma, was also associated with increased adrenomedullary activity. The medullary activity was more immediate and subsided more quickly on reinfusion of lost blood or cessation of stimuli. The medulla also responded to a greater degree to other factors, such as pain or apprehension.

Synthesis of cortical hormones: In vitro experiments by Cooper et al. (14) have shown that the addition of small amounts of bovine adrenal medullary homogenates to bovine cortical homogenates markedly stimulated the conversion of progesterone to 17α, 21-dihydroxycorticosteroids. Since the secretory granules of adrenomedullary cells are associated with a high content of ATP (6, 19), one might expect a stimulation of the rate of any enzyme-catalyzed reaction on the addition of medullary homogenate, but the same response was produced with 100 μg., or less, of crystalline epinephrine, norepinephrine, or isopropyl-norepinephrine.

Schonbaum (92), in an earlier, contrasting report, stated that epinephrine had no effect on the formation of corticoids in rat adrenals, however this failure may have been the result of using different assay techniques for the steroids.

Corticosterone (compound B) is the major secretory product of the avian adrenocortical cells according to the reports of Chester Jones et al. (11), deRoos (20, 21), and Nagra et al. (74). Small amounts of hydrocortisone and/or aldosterone have also been identified.

ANTERIOR PITUITARY

Much evidence has accumulated which indicates that epinephrine brings about the release of adrenocortical hormones by directly stimulating the

anterior pituitary to release ACTH. In 1945, Long and Fry (59) reported that hypophysectomy abolished the cortical mobilization usually seen following injection of epinephrine (32). Farrell and McCann (32) demonstrated an increased blood ACTH titer within one minute after intravenous injection of epinephrine and Kitay et al. (55) reported that the acute release of ACTH was brought about by a single, subcutaneous injection of epinephrine, the minimal dose (20 μg.) in rats being well within physiologic limits. Since epinephrine failed to cause histological changes in the adrenal cortex similar to those caused by ACTH, Coutinko (17) stated that ACTH release was not brought about by epinephrine. The work of Guillemin (42) would seem to eliminate epinephrine as the sole humoral agent responsible for stimulating the pituitary to release ACTH following systemic stress since the same response can be brought about by many other agents (Pitressin, histamine, lysine-vasopressin).

Newman (79) was unable to alter the output of aldosterone or hydrocortisone following norepinephrine infusion; even though Whitby (109) found that norepinephrine was bound by blood and tissues more strongly than was epinephrine, and therefore remained in the body for a longer period of time.

Recant et al. (86) reported that a normal stress response was observed after epinephrine injections in dogs with sectioned pituitary stalks, even though a thin piece of polyethylene had been placed in the section to prevent regeneration of the blood vessels. Contrary to this report, McCann (68) stated that stalk section, with interrupted hypophysial portal system, prevented the stress response to epinephrine injections in the rat.

By transplanting the anterior pituitary to the anterior chamber of the eye, Gershberg et al. (41) and McDermott et al. (72, 73) reported that epinephrine had a direct effect on the pituitary in causing the release of

ACTH. The rats bearing the transplants showed the typical stress response following injections of saline or epinephrine. The minimal amount of epinephrine necessary to cause an activation of the adrenal cortex when injected directly into the eye containing the functional pituitary transplant, did not produce a response when injected into the opposite eye. In a contradictory report, Casentini et al. (10) found that rats, with anterior pituitary transplants in the anterior chamber of the eye, did not respond to injections of epinephrine or other substances known to cause the release of adrenocortical hormones.

Fortier (35) also made pituitary transplants and reported that epinephrine (0.02 mg./100 gm.), cold, and histamine brought about eosinopenia in the rat with the translocated pituitary, but that sensory and emotional stimuli (sound and immobilization, respectively) had no effect.

Colfer et al. (12) stated that epinephrine did not consistently activate the adrenal cortex in rabbits, nor did denervation of the adrenal glands alter the normal response to stress. Adrenal demedullated rats responded to cold exposure in a manner similar to that of the normal rats, which indicated that the adrenal medulla was not a necessary component for activation of the adrenal cortex and the subsequent release of the cortical hormones (73). Pfeiffer et al. (82), using parabiotic rats, one hypophysectomized and the other adrenalectomized, showed that the presence of the adrenal medulla was necessary in the hypophysectomized partner in order for eosinopenia to be produced in both parabionts after injection of formalin.

HYPOTHALAMUS

Electrical Stimulation: DeGroot and Harris (18) demonstrated that electrical stimulation of the posterior region of the tuber cinereum or of the

mammillary bodies in unanesthetized, unrestrained rabbits resulted in a pronounced lymphopenia which was similar in time relations and magnitude to that following a stimulus of emotional stress. Stimulation of other areas in the hypothalamus and pituitary did not elicit the response to stress.

The tuberal and mammillary areas also appear to be the hypothalamic regions, in the cat, that are responsible for the release of cortical hormones following electrical stimulation (84). An increase in cortical hormones in the adrenal effluent blood, as well as a decrease in cortical hormones in the adrenal cortex of the cat, was noted by Katsuki (53) following electrical stimulation of the posterior portion of the hypothalamus.

The content of catecholamines in the adrenal venous blood was increased by the stimulation of the middle portion of the hypothalamus, while direct stimulation of other areas gave no response (53). Magoun et al. (60) showed that stimulation of anterior and lateral portions of the hypothalamus resulted in the release of epinephrine within 22 seconds after the stimulus was applied.

Two specific sites in the hypothalamus, which appear to be responsible for the specific neural control of the release of epinephrine and norepinephrine, have been located by Redgate et al. (87). The center for epinephrine release is located near the mammillary bodies; the norepinephrine control center is situated just posterior to the optic chiasma. The work of Mirkin (65) appears to substantiate the dual control of the release of the adrenomedullary hormones.

Hypothalamic Lesions: Porter (84) reported that the eosinopenic response to epinephrine injections was abolished by lesions in the posterior hypothalamus, but not by anterior lesions in the hypothalamus. Bilateral

lesions of the tuber cinereum and anterior border of the mammillary bodies completely disrupted the adrenocortical response to stress (96). The hypothalamic-hypophysial portal system remained intact and no diminution of secretion of gonadotrophins was observed. McCann (68) pointed out that partial lesions of the median eminence did not prevent adrenocortical activation in response to epinephrine injections. However, the adrenocortical response was not evident in rats bearing complete lesions of the median eminence as evidenced by the absence of compensatory adrenal hypertrophy following the surgical removal of one adrenal, or by the abolition of the eosinopenic response to epinephrine injections. Subcutaneous injections of epinephrine failed to bring about a stress response in cats with lesions in the median eminence (58).

Destruction of at least 50% of the median eminence resulted in the loss of compensatory adrenal hypertrophy and normal stress response in dogs (37, 51) and in rats (36, 71). Following lesions of the median eminence, adrenal atrophy was not apparent in dogs, even after long periods of time (37). In rats, the ACTH content of the pituitary was maintained at a level of 50% of that found in normal controls (71).

Epinephrine injections did not produce significant ACTH secretion in rats bearing hypothalamic lesions that destroyed a large fraction of the supraopticohypophysial tract, along with much of the median eminence (69).

Electrical Activity: With the aid of electrodes positioned with a stereotaxic instrument, Porter (83) established that stressful stimuli led to a marked increase in the electrical activity of the posterior hypothalamus. Approximately 30 seconds following intravenous injection of epinephrine, an increased activity of this area was observed, while doses 10 times greater failed to increase the activity elsewhere in the hypothalamus.

Exogenous ACTH decreased the activity induced by epinephrine. It is interesting to note that this area coincides with the lesioned regions that abolished the pituitary response to epinephrine injections (18).

Sutin (100) found an area in the hypothalamus of the cat, lateral and slightly rostral to the mammillary bodies, that responded with an increased electrical activity following intravenous injections of Pitressin, but not after injections of epinephrine. The latent period following Pitressin injection was much longer than that observed after epinephrine injection, the period being 4-8 minutes for Pitressin (100) and 30 seconds for epinephrine (83).

Since it has been shown that epinephrine and the hypothalamus are quite closely associated in bringing about adrenocortical mobilization, the reports of Weil-Malherbe (106, 107) take on considerable importance. Tritium-labelled epinephrine and norepinephrine were found in different brain regions of the cat, and with the exception of the hypothalamus, only in amounts to be expected from their blood content. The larger amounts found in the hypothalamus were attributed to a stronger retention of epinephrine and norepinephrine by this structure than by plasma or other parts of the brain. These results, coupled with those already reviewed, strongly support the hypothesis that epinephrine acts on the hypothalamus to bring about mobilization of the adrenal cortex.

Corticotrophin Releasing Factor (CRF): There is evidence available in the literature both for (23, 25, 56) and against (99) the possibility that the hypothalamic-hypophysial-adrenocortical system may be partially regulated or affected by higher brain centers. Decerebration blocked the "excitatory" effect of ether, Pitressin, or histamine, but not that of epinephrine (88, 91).

Recently, it has been shown that a fraction of an extract of the median eminence or of blood collected from the sella turcica following hypophysectomy contained an ACTH-releasing factor (CRF) (44, 89). This fraction appeared to be free of epinephrine and other substances known to cause ACTH release in intact animals (90).

Anterior pituitary tissue from dog and rat, cultured in roller tubes, lost all ACTH activity by the eighth day, but when explants of hypothalamus or median eminence were added to the cultures, ACTH activity was reinitiated (45). Epinephrine or norepinephrine could not be demonstrated to be present, either in the control cultures, or in the cultures to which portions of the hypothalamus or median eminence had been added.

As a result of surveying the literature, experimental evidence indicates that epinephrine may act via the hypothalamus, adrenocortical vascularization, and/or adrenocortical hormone synthesis to initiate the stress response. Epinephrine probably does not directly stimulate the adrenocortical cells in mammals to release the cortical hormones or the anterior pituitary to release ACTH.

AVIAN ADRENOCORTICAL-MEDULLARY RELATIONSHIPS

Almost all of the information previously presented was derived from studies in which mammals were utilized as experimental animals; much less experimental evidence is available from birds.

The evidence concerning the relationship between hypophysectomy and adrenal function in the chicken is controversial. Hypophysectomy has been reported to cause atrophy of the adrenocortical portion of the gland, resulting in a 20% decrease in the weight of the total gland (64), to cause only slight weight decrease (2), or to have no effect on the weight (66, 78), adrenal ascorbic acid, adrenal cholesterol, or adrenal Δ^4-3-ketocorticosteroids (78).

Repeated injections of ACTH in the intact pigeon and chick caused adrenal hypertrophy (52, 64), which did not disappear after hypophysectomy (1), and a decrease in the adrenal Δ^4-3-keto-corticosteroids (77). Other authors have reported that ACTH did not affect the adrenal weight of the chick (5, 13, 39), nor did it stimulate the adrenal of the quail (33).

Jailer (52) found that chronic administration of epinephrine in the chick resulted in adrenal hypertrophy accompanied by a depletion of the adrenal sudanophilic material. Newcomer (76) has shown that injection of epinephrine in the chick elicited acidophilia, a criterion which had been shown previously to be an acute indicator of ACTH injection or stress in the chicken (75).

Usually, adrenal enlargement in chickens is evoked by severe, chronic stressors only, such as surgical trauma (5), water deprivation (13), or overcrowding (95).

ADRENOMEDULLARY BLOCKING AGENTS

TM-10: Daily administration of choline 2:6-xylyl ether bromide (TM-10) for two weeks depleted the suprarenals of rats of about one-half of their normal content of epinephrine and norepinephrine (16). Restoration of the catecholamines was a slow process requiring about 14 days after withdrawal of the drug. When administered acutely, TM-10 prevented release of norepinephrine from the spleen following stimulation of the splenic nerve (31), but did not influence the release of pressor amines from the electrically stimulated adrenals of the cat (15, 31).

P-286: N,N-diisopropyl-N'-isoamyl-N'-diethylaminoethylurea (P-286) selectively blocked the action of acetylcholine in the adrenal medulla as demonstrated in dogs by Gardier et al. (38). This drug was considered to

have induced a reversible, chemical adrenal medullectomy which had been previously stated to be impossible without affecting all acetylcholine-mediated synapses (62).

Reserpine: Single injections of reserpine have been shown to cause an increased release of epinephrine in the rabbit (67), an almost selective liberation of norepinephrine in the rat (8), and a depletion of norepinephrine, but not epinephrine, from the adrenal medulla of the hamster (9). Acute reserpine treatment of guinea pigs, rats, and humans resulted in hypertrophy of the adrenals, adrenal ascorbic acid depletion, and higher plasma 17-keto and 17-hydroxysteroids (54).

Large, single doses of, or chronic treatment with reserpine resulted in the complete disappearance of epinephrine and norepinephrine in the rat (8, 30), chicken (6), and cat (98).

Callingham and Mann (7) demonstrated that norepinephrine reached a concentration 7 times its initial value in the rat 7 days after catecholamine depletion and then declined to a normal value by 21 days. Reappearance of epinephrine was not apparent until the seventh day and reached a normal value only after 21 days; this indicated that norepinephrine was an immediate precursor to epinephrine. However, Eranko (30) was unable to demonstrate this biphasic response in rats and reported that epinephrine and norepinephrine recovered proportionally after reserpine depletion.

It has become apparent that the adrenal innervation must be intact in the cat if complete depletion of catecholamines from the adrenal medulla is to be brought about by reserpine injections (98). Hillarp (49) reported that reserpine treatment caused a 77% depletion of catecholamines from the intact rat adrenal, but only a 36% decrease in rats with denervated adrenal glands.

CHAPTER III

MATERIALS AND METHODS

TREATMENT OF EXPERIMENTAL BIRDS

Seven to ten-week old, White Leghorn cockerels were used throughout the series of experiments. The chicks were raised in floor pens in a well-ventilated building. They were fed a standard growing ration and watered *ad lib*.

Prior to the beginning of all experiments, the birds were transferred to the laboratory in large, wooden cages and placed in a darkened room for a period of 4 hours which allowed them to remain in an undisturbed state until immediately before use.

Birds were removed singly from the darkened room and subjected to various intervals of physical restraint, which consisted of fastening birds to boards on their backs with rubber bands (76). After the appropriate period of restraint, the birds were removed from the board and separate blood samples for catecholamine and steroid analyses were drawn by cardiac puncture. The birds were sacrificed by decapitation, which was followed by the immediate removal of both adrenal glands. Body weights were recorded after decapitation and removal of the adrenals. The adrenal glands were carefully cleaned of adherent tissue and weighed separately on a Roller-Smith torsion balance to the nearest 0.2 mg. After being individually wrapped in Parafilm along with proper identification, the glands were stored in a deep freeze until analyzed for catecholamine and steroid

content. The non-stressed, control chicks were treated in the same manner with the exception of restraint. The complete process, from the time of drawing the first blood sample to the storage of the adrenal glands in the deep freeze, took less than 5 minutes for each bird.

The treated birds in Experiment II received 10 mg. of 2:6 xylyl ether bromide (TM-10), intraperitoneally (i.p.), per day for 11 days, the last injection being on the day of sacrifice.

Ten mg. of N,N-diisopropyl-N'-isoamyl-N'-diethylaminoethylurea (P-286) was administered i.p. each day for 15 days to the treated birds in Experiment III.

Reserpine was prepared for injection by the Method of Burack (6). The crystals were dissolved in the smallest possible volume of glacial acetic acid. The solution was then diluted with 2 parts water, 1 part ethanol (95%), and 1 part propylene glycol. Partial neutralization of the solution was accomplished by the dropwise addition of concentrated sodium hydroxide until just before the reserpine came out of solution permanently. The birds in Experiment IV were injected intramuscularly with a single dose of 22.6 mg. reserpine 7 days before sacrifice.

EPINEPHRINE AND NOREPINEPHRINE DETERMINATIONS

Plasma epinephrine and norepinephrine concentrations were determined by the ethylenediamine condensation method of Weil-Malherbe and Bone (108) as modified by Mangan and Mason (61) and Taylor (101). Reagents for this determination were prepared in the following manners.

Sodium citrate-sodium thiosulfate solution was made up by dissolving $C_6H_5Na_3O_7.2H_2O$ (2 gm.) and $Na_2S_2O_3$ (3 gm.) in 100 ml. glass-distilled water; the solution was autoclaved just before use. The citrate served as an anticoagulant while the thiosulfate prevented oxidation of the

epinephrine and norepinephrine before chromatographic separation.

Sodium acetate buffer was prepared by passing a 0.2 M solution of sodium acetate over a column of cationic Permutit resin (Permutit Q, sulfonated polystyrene) to remove all traces of heavy metals; the solution was then adjusted to pH 8.4 by the addition of 0.5 N Na_2CO_3. The column used for the preparation of the sodium acetate buffer had a bulb capacity of 120 ml. and a stem approximately 30.5 cm. in length with an inside diameter of 2 cm. At a distance of about 8 cm. from the tip, the stem had a constriction on which was placed a plug of glass wool.

The Permutit column was prepared by placing approximately 30 gm. of Permutit Q in the glass column. The column was washed repeatedly with 2 N HCl, water, and 4% NaCl, the latter being continued until the pH of the filtrate was about 6. Excess NaCl was flushed out with glass-distilled water.

One hundred gm. alumina (Al_2O_3, Fisher Scientific, "for chromatographic analysis, 80-200 mesh") was continuously stirred with 500 ml. of boiling 2 N HCl for 20 minutes, filtered, and washed on a large Buchner funnel with 500 ml. hot 2 N HCl. It was repeatedly washed by decantation with distilled water and dried at 300° C. for 3 hours. The acid wash prevented autoxidation of the catecholamines during the chromatographic separation procedure.

The ethylenediamine (Eastman Kodak Company, Organic) and isobutanol (Merck & Co., "suitable for fluorimetric work") were redistilled immediately prior to use. The ethylenediamine was kept in a dark, well-sealed bottle.

Stock, standard solutions (100 µg./ml.) of L-epinephrine bitartrate and DL-Arterenol were made up in 0.1 N acetic acid; the solutions were kept in the refrigerator since it has been reported that these standards remain stable for at least 6 months if refrigerated (97). Working standards were made immediately prior to use by diluting 1 ml. of the appropriate stock

solution to give a final concentration of 1 μg./ml. The appropriate volumes of the final dilution were placed in the condensation tubes and diluted to 10 ml. with 5 ml. of glass-distilled water and 5 ml. of 0.2 N acetic acid.

Six ml. of blood were drawn from the heart into a 10 ml. syringe which was equipped with a 20 gauge needle and contained 2 ml. of a 2% sodium citrate-3% sodium thiosulfate solution. The blood was then transferred to a chilled, 15 ml., conical, graduated centrifuge tube, the volume of blood plus preservative being noted (V_1). The tubes were placed in an ice bath until four samples had been collected; these were then centrifuged at 1500 rpm for 20 minutes. The plasma was decanted into another chilled, graduated centrifuge tube and the volume noted (V_2). The samples of plasma were quickly placed in a deep freeze where they were stored until analysis.

The samples were thawed in groups of 10 or 12; 0.2 M sodium acetate buffer was added to each sample in amounts equivalent to the plasma volume. The pH was adjusted to 8.4 by the addition of a few drops of 0.5 N sodium carbonate or 0.2 N acetic acid with the aid of a Coleman pH Electrometer, Model 18.

The columns used for the chromatographic isolation of the catecholamines were of the thistle tube type with a stem approximately 16 cm. in length and an inside diameter of 6 mm. A plug of glass wool was placed on a constriction in the stem 1.6 cm. from the tip of the tube. The columns were fitted through one-hole rubber stoppers in 500 ml. filter flasks which were connected to an individually controlled vacuum source. The columns were prepared by pouring a slurry composed of 0.5 to 0.7 gm. of the acid-washed alumina and 2.5 ml. of acetate buffer on the column. The slurry was washed down with 2.5 ml. of acetate buffer. After settling,

mild suction was applied and so regulated that the rate of filtration was 20 to 30 drops per minute.

The plasma-acetate mixture was added to the column followed by 5 ml. of acetate buffer and then 5 ml. of glass-distilled water. These two filtrates were rejected. The adsorbed epinephrine and norepinephrine were eluted into a 15 ml. test tube by passing 5 ml. of 0.2 N acetic acid through the column followed by 5 ml. of glass-distilled water. To the acetic acid eluate which was transferred to a 50 ml., glass-stoppered, centrifuge tube, was added 0.5 ml. of ethylenediamine dihydrochloride solution followed immediately by 0.7 ml. of ethylenediamine. The samples were then maintained at 50° C. for exactly 20 minutes in a constant temperature water bath. At the end of this period, all samples were removed, placed in a cold water bath for 5 minutes, and then saturated with NaCl (about 4 gm.) in order to remove any protein that might have come through the columns. The samples were extracted with 6 ml. of isobutanol on a mechanical shaker for 4 minutes and centrifuged lightly to break up the emulsion. Approximately 2 ml. of the isobutanol extract were transferred to the fluorometer cuvette and its fluorescence was read.

A Farrand Photoelectric Fluorometer, Model A, equipped with a constant voltage transformer was used to measure fluorescence. The fluorometer was equipped with an excitation source of 4050 Å (Corning filters # 3060, 4308, and 5970 placed in that order beginning at the light source) and secondary filter wavelengths of 5150 Å (Corning filters # 3384, 4303, and 5433 positioned in that order from the sample) and 5800 Å (Corning filters # 3480 and 4303 in this order from the sample). Small, matched, non-fluorescent test tubes were employed as cuvettes. The sensitivity of the fluorometer was adjusted (using aperature No. 2) so that a reading of 100 was obtained on the galvanometer scale with 0.25 micrograms of epinephrine as a standard.

The fluorescence of the remaining standards, blanks, and samples was determined at both secondary wavelengths. A standard fluorescence curve for each wavelength was constructed (after subtracting the blank reading) for the standards of epinephrine and norepinephrine. By dividing the slope of the epinephrine curve by that of norepinephrine at each secondary wavelength, the fluorescence ratio of epinephrine to norepinephrine was found.

Using the foregoing information, the following relationship was established: $A + \frac{N}{X} = b$, where A = concentration of epinephrine; N = concentration of norepinephrine; b = apparent epinephrine content of sample measured at 5150 Å; X = fluorescence ratio of epinephrine to norepinephrine at 5150 Å.

Similarly, $A + \frac{N}{Y} = c$, where c = apparent epinephrine content of the sample measured at 5800 Å; Y = ratio of epinephrine to norepinephrine at 5800 Å.

The two equations were solved simultaneously (61).

$$A + \frac{N}{X} = b \qquad (1)$$

$$A + \frac{N}{Y} = c \qquad (2)$$

$$N = \frac{Y \times X}{Y - X} (b - c) = K (b - c) \qquad (3)$$

$$A = b - \frac{N}{X} = c - \frac{N}{Y} \qquad (4)$$

The final catecholamine concentrations were calculated on the basis of micrograms per liter of plasma using the following equation (101).

$$(\text{micrograms of hormone per sample}) (V_1) \times 1{,}000/V_2 (V_1 - P),$$

where P = amount of sodium citrate-thiosulfate used to dilute the blood sample when initially drawn.

In the presence of an alkaline pH and oxygen, epinephrine is oxidized

to adrenochrome, a highly reactive and unstable compound. During the condensation reaction, the free base, ethylenediamine, maintains the alkaline pH of the mixture (pH = 10.4) and the ionized salt, ethylenediamine dihydrochloride, combines with the adrenochrome to trap it in the nascent state, quantitatively producing a highly fluorescent, condensation product (structure unknown). The fluorescent, isobutanol extract of this phase remains stable for at least 24 hours. The sensitivity is such that concentrations as low as 1 μg./liter of plasma can be determined.

According to Persky (81), this method appears to be as reliable, if not the most reliable, as any method for the quantitative estimation of epinephrine and norepinephrine.

Adrenal epinephrine and norepinephrine were determined by the same basic method, the only difference being in the tissue preparation up to the stage of chromatographic separation of the catecholamines.

One adrenal gland at a time, chosen randomly as to right or left, was removed from the deep freeze and homogenized in a Potter-Elvehjem homogenizer containing 2 ml. of cold trichloroacetic acid (TCA). The homogenizer tube was washed twice with 2 ml. of TCA and the washings were combined with the adrenal homogenate. The resulting solution was shaken and centrifuged for 15 minutes at 1500 rpm. The supernatant was decanted, extracted once with 10 ml. of anhydrous ethyl ether, and diluted to 1 liter with distilled water. Four ml. of the diluted extract was combined with 2 ml. of sodium citrate-thiosulfate solution and 6 ml. of acetate buffer; the resulting solution was adjusted to pH 8.4 by the dropwise addition of 0.5 N sodium carbonate. The buffered solution was then passed through the alumina column, eluted, condensed with ethylenediamine dihydrochloride, and the resulting fluorescence determined in the same manner as was that of the plasma samples. If the samples were more fluorescent than the

standard, they were diluted to a readable value with isobutanol, the blank being diluted in the same manner.

Calculation of the absolute hormone content of the adrenals was performed following the same method as was used for calculating the plasma catecholamine content. The final catecholamine concentration of the unknown samples was multiplied by the dilution factor to obtain the absolute amount of epinephrine and norepinephrine per gland. The catecholamine concentration was reported in micrograms per 100 mg. of adrenal weight.

CORTICOSTERONE ANALYSIS

The content of corticosterone in plasma and adrenal glands was measured by the method of Guillemin et al. (43).

At the time of sample collection, 6 ml. of blood were drawn via cardiac puncture into a 10 ml., heparinized syringe equipped with a 20 gauge needle. The samples were centrifuged at 1500 rpm for 20 minutes after which a 2 ml. aliquot of plasma was transferred to another tube and stored in the deep freeze pending final analysis.

The 2 ml. aliquots were removed from the freezer in groups of 10 or 20 and transferred to 50 ml., glass-stoppered centrifuge tubes. The samples were shaken for 15 seconds with 6 ml. of isooctane (2,2,4-trimethylpentane, practical grade) to remove the fats. The isooctane phase was removed by aspiration and discarded. Five ml. of distilled water were added, followed by 15 ml. of chloroform (redistilled from sodium carbonate). The tubes were shaken for 30 seconds and centrifuged for 3 minutes (about 2,000 rpm), the aqueous phase then being removed by aspiration and discarded. One ml. of 0.1 N NaOH was added to the chloroform extract; the tube was shaken for 15 seconds and centrifuged for 3 minutes. Ten ml. of the purified chloroform extract (bottom layer) were transferred to another 50 ml.,

glass-stoppered centrifuge tube containing 2.0 ml. of 30 N H_2SO_4 (prepared by making up 420 ml. of concentrated sulfuric acid to 500 ml. with glass-distilled water). The time was noted at the point of the addition of the chloroform to the acid. The tubes were shaken vigorously for 30 seconds and centrifuged for 3 minutes. About 1 ml. of the acid extract (bottom layer) was transferred into the fluorometer cuvettes. The fluorescence was determined 30 to 40 minutes after the addition of H_2SO_4 with the fluorometer set at 80 for 1.5 μg. corticosterone standard. The fluorometer was fitted with a primary filter yielding a wavelength of 436 λ (Corning filters # 3389 and 5113) and a secondary filter specific for 530-545 λ (Wratten gelatin filters # 16 and 74, Kodak).

The standard was prepared by placing 0.75 ml. of a stock solution containing 2.0 μg. corticosterone (free alcohol) per ml. absolute ethanol (redistilled from 2,4-dinitrophenylhydrazine) in a 50 ml. centrifuge tube and carefully evaporating to dryness. After the addition of 2 ml. of glass-distilled water, the tube was carried through the above procedures along with the plasma samples and a reagent blank containing 2 ml. of glass-distilled water.

Analysis of adrenal corticosterone involved the homogenation of the gland in a Potter-Elvehjem tube containing 4.0 ml. saline (0.85%) plus 1 ml. absolute ethyl alcohol. Following the transfer of the homogenate to a 12 ml., conical, centrifuge tube, 4 ml. of isooctane were added. The tube was then closed with a plastic cap and shaken for 30 seconds. After centrifugation at 2,000 rpm for 3 minutes, the isooctane phase was discarded and 4 ml. of the ethanol-saline extract transferred to a 50 ml., centrifuge tube fitted with a glass stopper. From this point the procedure for plasma was followed. The standard was made up by adding 1 ml. of a stock solution containing 2 μg./ml. of absolute ethanol to a 12 ml.

centrifuge tube containing 4 ml. of saline. The fluorometer was set on 100 with this standard. A reagent blank containing 5 ml. of saline was run along with the standard and adrenal samples.

Two standard curves were constructed following the methods for both plasma and adrenal corticosterone analysis. The fluorescence of the sample, minus the fluorescence of the blank, multiplied by the cotangent of the appropriate standard curve, transposed the fluorescence values into μg. corticosterone per sample. The plasma corticosterone concentration was expressed on the basis of μg./100 ml. of plasma while the adrenal corticosterone content was calculated on the basis of μg./100 mg. of adrenal weight.

In order to determine if treatment effects existed, the data from Experiments II and III were subjected to an analysis of variance followed by Tukey's test for significance.

The following compounds were generously supplied by their respective manufacturers:

P-286, Pitman-Moore Company

TM-10, May and Baker, Ltd.

Reserpine, Ciba Pharmaceutical Products, Inc.

Permutit Q, Permutit Company

Corticosterone, Merk Institute for Therapeutic Research.

CHAPTER IV

RESULTS AND DISCUSSION

Since there is relatively little information available concerning the avian adrenocortical-medullary relationships or the effects of catecholamines on the release of cortical hormones in chickens, the following data will be presented and discussed in the light of what is known about these relations in both mammals and birds. It must be borne in mind that differences in physiologic mechanisms have been pointed out between mammals and birds, but the mass of information available concerning stress in mammals, in contrast to the scarcity of data from birds, is helpful in setting up experiments and drawing possible conclusions from the experimental data. Care was taken to avoid drawing any positive conclusions in this paper unless warranted by the experimental data presented from birds.

EXPERIMENT I

Levels of epinephrine, norepinephrine, and corticosterone in both plasma and adrenal glands, measured following 0, 5, 10, 20, 30, and 60 minutes of restraint are listed in Table I.

It can be seen that there was an immediate and concurrent rise in the concentrations of epinephrine and corticosterone in plasma during restraint (Figure I). A similar type of response pattern was observed in dogs by Walker (103, 104, 105). Newcomer (77) was unable to demonstrate a rise in plasma free, glucuronide, or sulfate bound Δ^4-3-keto-corticosteroids following 6 hours of restraint in chickens; the failure perhaps being the

TABLE I

CONCENTRATIONS OF CATECHOLAMINES AND CORTICOSTERONE IN PLASMA AND ADRENAL GLANDS FOLLOWING VARIOUS INTERVALS OF RESTRAINT

Group	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
Normal Control	7.89±4.96**	27.30±18.83	202.58±18.83	132.73±27.46	2.12±2.42	0.664±0.247
Restraint 5 minutes	10.75±3.48	16.73± 7.34	223.16±53.53	50.89±39.59*	12.48±4.31*	0.782±0.284
Restraint 10 minutes	12.86±5.40	40.07±13.82	177.49±73.22	126.73±86.63	9.95±3.30*	0.796±0.284
Restraint 20 minutes	14.47±7.34	9.79± 1.04*	18.61± 7.29*	22.45± 4.44*	9.95±4.75*	1.178±0.283*
Restraint 30 minutes	18.32±9.39*	16.33± 5.31	32.62±10.24*	31.33± 8.71*	8.89±2.56*	1.049±0.823
Restraint 60 minutes	16.73±9.20*	16.85± 9.28	50.75±37.01*	36.60±16.98*	16.68±4.90*	1.716±0.361*

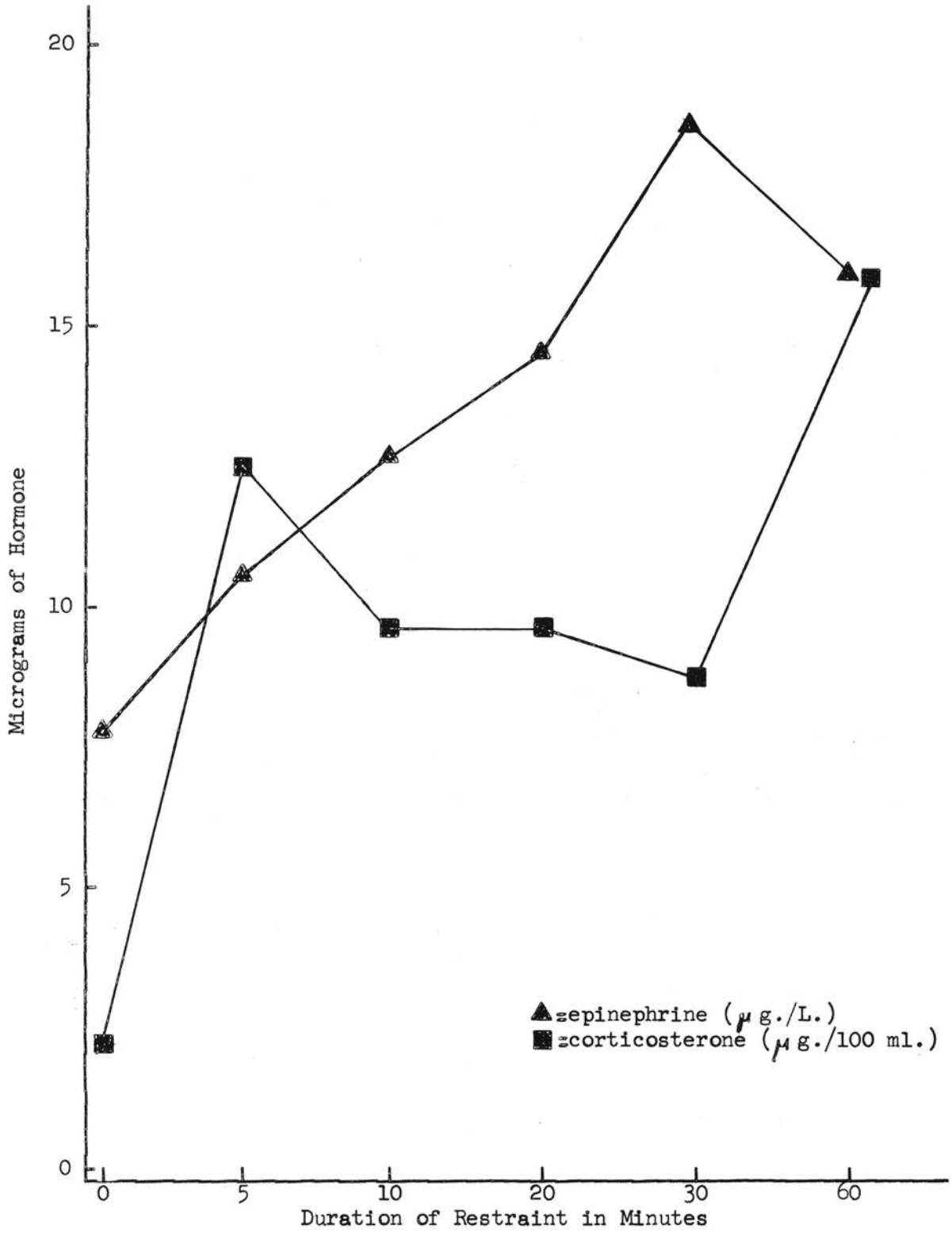
These data were subjected to an analysis of variance which was followed by Tukey's D test for significance.

* indicates a Probability < 0.05.

** indicates standard deviation of the mean.

FIGURE I

CONCENTRATIONS OF EPINEPHRINE AND CORTICOSTERONE IN PLASMA FOLLOWING VARIOUS INTERVALS OF RESTRAINT



result of the less sensitive method that was used for a general class of biologically active steroid hormones.

The concurrent rise in both plasma epinephrine and corticosterone could possibly indicate that catecholamine release has a role in bringing about the general stress response, however, a significant increase ($P<0.05$) in plasma epinephrine came only after 30 minutes of restraint while a significant rise ($P<0.05$) in plasma corticosterone was noted after only 5 minutes of restraint. The fact that epinephrine has a very short life in the peripheral circulation (101) could have been responsible for the longer time period necessary to establish a significant rise in plasma epinephrine.

The response pattern of the plasma corticosterone had an unexpected biphasic nature. After 5 minutes of restraint, the corticosterone concentration rose to a value 6 times as high as the control level, then slowly fell through the 10 and 20 minute intervals and reached the bottom of its decline at the 30 minute interval. From this point it rose to a new high following 60 minutes of immobilization. This same type of response was noted in rats by Knigge et al. (57) who also found that at the time of lowest levels of corticosterone in the plasma, the pituitary contained ACTH in approximately a twice-normal concentration. The drop in steroid release is not the result of exhaustion of corticosterone from the adrenal gland (Table I).

The increased epinephrine concentration in the plasma may have had a direct effect on the release of corticosterone from the cortical cells. The anatomical arrangement of the chicken adrenal is such that the cortical cells, and especially the blood vessels serving the adrenal, are subjected to very high concentrations of the catecholamines before they are diluted in the general circulation. Both epinephrine and norepinephrine increase the amount of blood reaching the cortical cells in the mammalian

adrenal (26, 46, 47), but whether this is true in the avian adrenal is unknown.

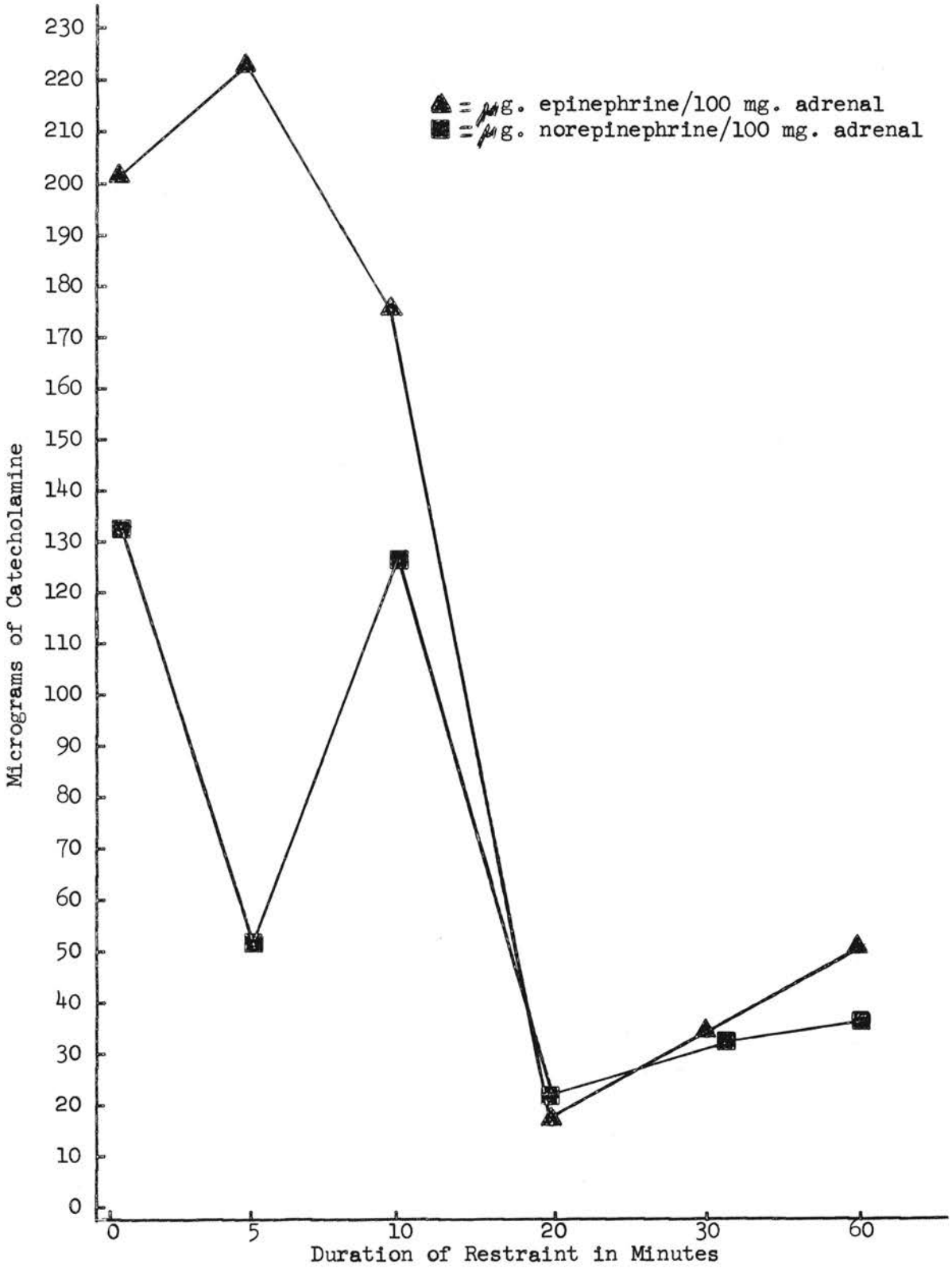
The plasma levels of norepinephrine (Table I) fluctuated a great deal with a significant fall ($P<0.05$) at the 20 minute interval of restraint. The failure of norepinephrine to follow the same pattern of release as did epinephrine would appear to indicate the possibility of individual release mechanisms for the two hormones, a phenomenon that is suspected of being true in mammals (65, 87). However, the concentration of the two catecholamines in the adrenal began to fall after restraint and by 20 minutes, both hormones were following a similar release pattern (Figure II) which indicated that both hormones were released in a similar manner during the stress of immobilization. At the 20, 30, and 60 minute intervals, both epinephrine and norepinephrine levels in the gland were significantly lower ($P<0.05$) than those of the controls.

It is difficult to explain why, if both epinephrine and norepinephrine are being released from the adrenal at a rapid rate, the plasma norepinephrine values did not show a significant increase over the control values as did epinephrine. Whitby (109) recognized that norepinephrine is bound more tightly, in mammals, to blood and tissues than is epinephrine, and therefore, remains in the circulation longer. Further investigation will be necessary before the fate of norepinephrine, in the avian circulation, can be determined. Since norepinephrine disappeared from the adrenal gland, but did not show an increase in the plasma, the possibility exists that during conditions of stress, norepinephrine was converted to epinephrine within the adrenal gland.

Shepherd and West (94) reported that norepinephrine made up 85% of the total adrenal catecholamines in chickens, but the data in this study (with the exception of Experiment IV) are more comparable to those of

FIGURE II

CONCENTRATIONS OF CATECHOLAMINES IN ADRENAL GLANDS
FOLLOWING VARIOUS INTERVALS OF RESTRAINT



Eranko (29) who found that norepinephrine made up only one-third of the total adrenal catecholamines in chickens.

Even though a significantly higher value ($P<0.05$) of corticosterone in the gland was not observed until after 20 minutes of restraint, a gradual and steady increase in the hormone concentration in the plasma was observed at the 5 and 10 minute intervals. It appears unlikely that enough time had elapsed to allow the peripheral utilization of corticosterone, and other cortical hormones, to account for the release of ACTH, resulting in the higher than normal adrenal and plasma corticosterone concentrations so quickly during restraint. The rapidity of the reaction is indicative of the involvement of a direct neural or indirect neurohumoral mechanism or both. If a neurohumoral mechanism is involved, epinephrine would appear to have the characteristics necessary to fulfill the requirements of the neurohumor. It is released in large quantities during stressing conditions, it mobilizes the adrenal cortex and increases the electrical activity of the hypothalamus when injected into test animals, and it is also taken up by the hypothalamus in significant quantities but not by other areas of the brain.

In vitro experiments with mammalian adrenals have demonstrated that epinephrine and norepinephrine can stimulate the rate of synthesis of cortical hormones (14), although earlier work did not demonstrate this phenomenon (92). The data from this study certainly do not contradict the possibility that epinephrine may stimulate the rate of synthesis of cortical hormones in the chick adrenal.

The over-all, general response pattern in normal chickens, following various intervals of physical restraint, has a number of apparent characteristics, as evaluated with the criteria used. There was an immediate rise ($P<0.05$) in plasma corticosterone followed by a drop of short

duration, following which the values then rose to a new peak. The corticosterone concentration in the gland showed a slow, but steady, increase which became significantly higher ($P<0.05$) by 20 minutes.

Epinephrine and norepinephrine disappeared from the gland, both being significantly reduced ($P<0.05$) in concentration by 20 minutes, however, only epinephrine showed a rise in concentration in the plasma. The increased values of epinephrine in the plasma became significant ($P<0.05$) after 30 minutes of restraint, but norepinephrine did not appear to follow a particular pattern of release.

EXPERIMENT II

Numerous reports have been published which state that TM-10 is a satisfactory adrenergic blocking compound in mammals. It did not alter the release of catecholamines from the adrenal medulla of the cat following electrical stimulation when administered acutely (15, 31). However, following daily injections of TM-10 for two weeks, the concentrations of epinephrine and norepinephrine in the adrenal glands of rats were reduced to about one-half their normal values (16). It has been reported that TM-10 interferes with the biosynthesis of epinephrine and norepinephrine (16). Since TM-10 must be chronically administered to mammals to deplete the adrenal gland of its catecholamine content, through a probable interference in synthesis, it was decided to administer TM-10 chronically to chickens and to measure the adrenal and plasma catecholamine and steroid concentrations.

Chronic treatment with TM-10 failed to alter the catecholamine levels either in plasma or in the adrenal glands (Table II). The treatment significantly increased ($P<0.005$) the concentration of corticosterone in the glands, indicating that TM-10 injection may be capable of initiating the

TABLE II

CONCENTRATIONS OF CATECHOLAMINES AND CORTICOSTERONE IN PLASMA AND ADRENAL GLANDS FOLLOWING CHRONIC TREATMENT WITH TM-10

Group	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
Normal Control	8.76±7.47**	32.43± 8.43	112.43±16.62	38.55±24.81	11.40±4.66	0.441±0.062
TM-10	8.27±3.92	30.90±21.50	102.12±68.76	75.44±77.96	12.11±1.23	0.700±0.224***

These data were subjected to Student's "t" test to determine if treatment effects existed.
** indicates standard deviation of the mean. *** indicates a Probability < 0.005.
Each injected bird received 10 mg. TM-10/day for 11 days.

TABLE III

CONCENTRATIONS OF CATECHOLAMINES AND CORTICOSTERONE IN PLASMA AND ADRENAL GLANDS FOLLOWING CHRONIC TREATMENT WITH P-286

Group	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
Normal Control	4.94±8.72***	6.14±3.20	261.36± 78.50	220.19±206.60	3.00±1.09	0.741±0.200
P-286	0.91±0.39	6.83±5.08	363.40±139.80	347.44±212.13	7.58±4.32**	0.538±0.172*

These data were subjected to Student's "t" test to determine if treatment effects existed.
* indicates a Probability < 0.10. ** indicates a Probability < 0.025.
*** indicates standard deviation of the mean.
Each injected bird received 10 mg. P-286/day for 15 days.

stress response. The plasma corticosterone values in the treated birds were high; however, the control values in this case were higher than had been observed in the first experiment.

No explanation can be offered for the failure of this drug to affect the catecholamine values in the adrenal gland or plasma of the chick, unless the dose level was inadequate.

EXPERIMENT III

The report of Gardier et al. (38) that P-286 brought about a reversible, chemical medullectomy in dogs prompted the use of the drug in chickens. The specific blockade of the adrenal medulla that was brought about in dogs was the result of the selective inhibition of the action of acetylcholine in the adrenal medulla (38).

It can be seen in Table III that birds, chronically treated with P-286, demonstrated much lower plasma epinephrine levels, but not reduced norepinephrine levels. The lower plasma epinephrine values in the treated birds were not statistically significant, but a larger number of animals in the two groups might have allowed the values to become significant.

The higher concentration of epinephrine and norepinephrine in the glands of the treated birds, though not significantly different from the control chicks, indicated that P-286 prevented the release of the catecholamines from the medullary cells. In this experiment high corticosterone values were apparent in the plasma while the epinephrine concentration in the plasma was low. This type of response differed from the previous two experiments where high corticosterone levels in the plasma were always accompanied with increased epinephrine values in the plasma.

A significantly lower ($P<0.10$) adrenal corticosterone level existed in the treated cockerels when compared to the controls, another response

that is opposite to that observed in the restrained birds which did not receive P-286. At the present time, two possible explanations for this difference present themselves. If the injection of P-286 were a stress stimulus, as it appeared to be when considering the plasma corticosterone level, the absence of high epinephrine levels in the general circulation indicates that epinephrine has a stimulatory effect on the synthesis of the cortical hormones, since both circulating epinephrine and adrenal corticosterone were lower in the injected birds. This possibility is supported by the evidence reported by Cooper et al. (14) that epinephrine stimulated the rate of conversion of progesterone to 17α, 21-dihydroxy-corticosteroids in adrenocortical homogenates.

On the other hand, information is available that fails to show that epinephrine had any effect on corticosteroid synthesis (92). If this report is correct, the lower adrenal corticosterone values may be the result of the long-term administration of the drug, which may have resulted in a faster depletion than synthesis of corticosterone. Chronic administration of ACTH reduced the concentration of steroid hormones in the adrenal gland of chickens (77). Therefore, if P-286 caused the release of ACTH through its own toxicity, the depletion of corticosterone observed in the adrenal gland may have nothing to do with epinephrine. There is a possibility that both of the ideas mentioned above may be correct.

EXPERIMENT IV

Burack (6) reported that a single dose of reserpine caused depletion of the catecholamines from the adrenal glands of chickens; 7 days after the single dose, the catecholamine levels were at their lowest values.

In this study, cockerels were treated with a single i. m. dose of reserpine, 7 days later the birds were restrained for 30 minutes along

TABLE IV

CONCENTRATIONS OF CATECHOLAMINES AND CORTICOSTERONE IN PLASMA AND ADRENAL GLANDS FOLLOWING RESTRAINT OF CHICKENS CHRONICALLY TREATED WITH RESERPINE

Group	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
I Normal Control	3.88± 4.07*	8.47± 8.89	88.73±42.95	326.03±164.74	4.88±2.64	0.352±0.110
II Restraint 30 minutes	34.07±10.48	27.76±14.92	40.90±36.00	263.81±138.94	9.93±3.76	0.636±0.154
III Reserpine Control	1.29± 1.85	3.79± 3.44	2.56± 2.40	20.96± 21.41	7.40±3.12	0.152±0.053
IV Reserpine, Restraint 30 minutes	0.89± 1.89	14.83±16.93	1.11± 1.08	4.38± 7.05	3.64±1.65	0.413±0.137

These data were subjected to an analysis of variance which was followed by Tukey's D test for significance. Significant differences at a Probability < 0.05.

Plasma Epinephrine: II > I; III; IV. Gland Epinephrine: I > II; III; IV. Gland Norepinephrine: I > III; IV.
Plasma Norepinephrine: II > I; III. II > III; IV. II > III; IV.
Plasma Corticosterone: II > I; III; IV. Gland Corticosterone: II > I; III; IV.
III > IV. IV > III. I III.

* indicates standard deviation of the mean.

Each injected bird received 22.6 mg. reserpine (intramuscularly) in a single dose 7 days before autopsy.

with non-injected controls. Normal and reserpine-treated, non-restrained birds were also carried through the experiment. The results of the measurements are recorded in Table IV.

The epinephrine concentration in the plasma of birds following treatment with reserpine was decreased, but not significantly so. The same condition was observed for the value of norepinephrine in the plasma of birds treated with reserpine. A lower, but not significantly decreased, level was observed.

Reserpine significantly depleted ($P<0.05$) the adrenal glands of both epinephrine and norepinephrine which verifies the report of Burack (6) for chickens and also those reports concerning adrenal catecholamine depletion in rats (8, 30) and cats (98). The norepinephrine level was higher in the adrenal glands of the cockerels treated with reserpine than was the epinephrine value. This fact may be explained by the report of Callingham and Mann (7) who demonstrated that norepinephrine was synthesized more rapidly than was epinephrine following depletion. This information indicated that norepinephrine was a precursor of epinephrine within the adrenal gland (7).

Reserpine treatment caused an increased plasma corticosterone level, but it was not significantly higher than that of the normal control birds. In the gland, the corticosterone value of the reserpine-treated cockerels was significantly lower ($P<0.05$) than that found in the normal controls.

When a comparison was made between the non-injected, restrained birds and the reserpine-treated, restrained birds, many differences appeared. The non-treated, restrained birds demonstrated a significant increase ($P<0.05$) in concentration of epinephrine in the plasma while those birds which were treated with reserpine showed no increase in plasma epinephrine. Restraint caused a significant increase ($P<0.05$) in plasma norepinephrine

in the control birds and an increase in the plasma norepinephrine level in the reserpine-treated birds, but the latter value was not significant. The rise in plasma norepinephrine of the reserpine-treated, restrained birds appeared to be the result of a possible resynthesis of norepinephrine in the adrenal gland. The reserpine-treated control group had a higher norepinephrine concentration than epinephrine in the adrenal gland while the reserpine-treated, restrained group had a much smaller norepinephrine value in the adrenal gland which indicated that the norepinephrine was present to be released from the adrenal glands of the reserpine-treated, restrained birds.

Restraint also caused a significant decrease ($P<0.05$) in the adrenal epinephrine concentration, but there was so little epinephrine left in the adrenal glands of the reserpine-treated birds that a decrease could not be detected in the birds of the reserpine-treated, restrained group. Norepinephrine values in the glands decreased following restraint in the normal birds, but the decrease was not significant. A slight and nonsignificant decrease was also noted in the adrenal norepinephrine level of the reserpine-treated, restrained group.

The corticosterone concentration in plasma of the control, restrained group was significantly increased ($P<0.05$), but was significantly decreased ($P<0.05$) in the reserpine-treated, restrained group.

Restraint resulted in a significantly increased ($P<0.05$) concentration of corticosterone in the adrenal glands of both the non-injected and injected groups. The reserpine-treated, restrained group had a significantly lower ($P<0.05$) adrenal corticosterone content when compared to the non-injected, restrained group.

When viewing the overall picture, it would appear that the high norepinephrine levels in the plasma might have blocked the release of

corticosterone; however, in Experiment I, high values of plasma norepinephrine and corticosterone were found concurrently.

The data indicate that when the release of epinephrine and norepinephrine have been prevented by drug action (P-286 or reserpine) and the birds given no further treatment, the gland corticosterone value fell as did the circulating level of catecholamines. Further evidence for this situation is apparent in Experiment II, for the treated birds (TM-10) had a high adrenal corticosterone level along with a normal or higher catecholamine level in the peripheral circulation. Therefore, it would appear, from the information available, that in the absence of normal levels of epinephrine and/or norepinephrine in the plasma, the corticosterone content of the adrenal gland was reduced.

The data from this study indicate that the release of catecholamines from the adrenal gland is not necessary prerequisite for at least some stimulation of the adrenocortical tissue to occur during stress; however, the reserpine-treated, restrained group of Experiment IV had the lowest plasma corticosterone value indicating that, even though synthesis was occuring, epinephrine, norepinephrine, or both, may be necessary for the release of corticosterone to occur normally under conditions of stress. In other words, it is probable that epinephrine and/or norepinephrine work in a synergistic, or facilitory, manner with ACTH in stimulating either synthesis or release of corticosterone, or both.

The pattern of release of epinephrine, norepinephrine, and corticosterone, in Experiments I and IV, indicated a facilitory reaction between the catecholamines and ACTH in bringing about the release of corticosterone, since the rise in both hormones (epinephrine and corticosterone) came at approximately the same time (Figure I). If large increases in epinephrine and/or norepinephrine had been noted prior to an increased plasma

corticosterone level, one could justify the necessity of the catecholamines in bringing about the subsequent release of corticosterone. Or, if the rise in corticosterone had appeared long before a rise in the catecholamines, the necessity of epinephrine and norepinephrine could have been discounted. Also the failure of the adrenomedullary blocking agents to inhibit completely the stress response following restraint, indicated that the previously discussed synergistic or facilitory possibilities would appropriately apply to these data.

CHAPTER V

SUMMARY AND CONCLUSIONS

Seven to ten-week old cockerels were subjected to physical restraint of various intervals of time (0, 5, 10, 20, 30, and 60 minutes) which were followed by the determination of epinephrine, norepinephrine, and corticosterone concentrations in both plasma and adrenal glands.

Physical restraint resulted in an immediate and significant rise in both epinephrine and corticosterone values in plasma, a significant decrease in the epinephrine and norepinephrine content of the adrenal gland, and a significant increase in the level of corticosterone in the adrenal gland. The concentration of norepinephrine in plasma was highly variable, with a significant decrease being noted after 20 minutes of restraint, which was followed by a rise through the 30 and 60 minute intervals of restraint.

TM-10 treatment for 11 days had no effect on the concentration of epinephrine or norepinephrine in plasma or adrenal glands. A significant increase was observed in the amount of corticosterone in the adrenal glands of the treated birds.

Daily administration of P-286 for 15 days caused the amount of epinephrine in the plasma, but not that of norepinephrine, to be reduced. An increase in the level of both hormones was noted in the adrenal glands of the treated birds, indicating that P-286 blocked the release of catecholamines from the adrenal glands. A significantly lower level of corticosterone in the adrenal glands of treated birds was demonstrated; the

concentration of this hormone in the plasma was higher than that observed in the control birds.

A great reduction of epinephrine and norepinephrine from plasma and adrenal glands was brought about by a single dose of reserpine. Reserpine treatment induced a significantly higher corticosterone level in the plasma and a significantly lower value of corticosterone in the adrenal glands.

No difference was observed in the amount of epinephrine in the plasma or adrenal glands of the reserpine-treated birds following 30 minutes of restraint; however, there was a slight increase in the level of norepinephrine in plasma with a small decline in the concentration of norepinephrine in the adrenal glands. Restraint failed to increase the amount of corticosterone in the plasma of reserpine-treated birds, even though there was a significant increase in the concentration of corticosterone in the adrenal glands of the same birds. However, this latter increase was not as great as was seen in the normal, restrained birds.

The experimental evidence from this study demonstrates that the release of epinephrine and corticosterone in birds, after acute restraint, occurs concomitantly. It appears likely that epinephrine has an effect in bringing about adrenocortical mobilization in birds during stress, since it is present in the peripheral plasma in a quantity great enough to cause mobilization of the adrenal cortex. The absence of a rise in the concentration of corticosterone in plasma following restraint of reserpine-treated birds is also indicative of a role for epinephrine in the stress response in birds.

If it is true that epinephrine has a stimulatory effect on the pituitary-adrenal axis, it is likely that it works in a facilitory manner with a nervous mechanism in bringing about an activation of the adrenal cortex.

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A P P E N D I X

EXPERIMENT I

Normal Controls

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	4.75	13.75	105.77	105.77	0.00	0.663
2			156.78	202.47	5.00	0.774
3	5.15	9.57	193.28	196.76	4.75	0.825
4	19.63	9.16	360.64	168.08	0.00	0.504
5	2.82	26.38			1.60	0.726
6	5.87	10.91	416.67	24.04	1.60	0.512
7	9.42	8.69	243.25	77.39	6.85	0.519
8	6.19	4.76	321.82	96.15	6.35	1.398
9	4.85	7.37	275.22	112.55	0.55	
10	17.18	12.31	236.70	60.33	0.00	0.665
11	8.39	28.33	122.43	286.79	2.10	1.000
12	1.55	56.67	175.64	156.74	3.98	0.475
13	5.20	38.62	179.89	132.42	5.30	0.720
14	6.08	23.36	89.63	143.23	0.00	0.508
15	4.09	22.50	115.45	91.22	1.05	0.559
16	11.95	46.21			0.00	0.537
17	8.25	56.14	115.00	10.00	1.05	0.794
18	5.52	55.71	141.05	21.89	0.00	0.413
19	6.79	33.71	134.30	315.82		0.758
20	16.19	54.52	262.93	187.50	0.00	0.264
Mean	7.89	27.30	202.58	132.73	2.12	0.664
S. D.	4.96	18.83	94.70	27.46	2.42	0.247

EXPERIMENT I

Restraint, 5 Minutes

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	15.67	23.02	255.34	65.09	9.50	0.873
2	11.43	15.24	231.25	43.75	8.45	0.767
3	6.91	6.91	129.02	11.73	8.45	1.437
4	8.36	16.73	253.57	50.00	11.65	0.833
5	9.43	25.12	175.64	15.97	15.85	
6	7.20	26.32	200.53	20.05	10.60	0.794
7	12.80	20.16	293.78	120.75	14.80	0.445
8	11.78	18.63	286.52	108.37	13.75	0.605
9	16.47	6.59	237.10	64.49	22.20	0.539
10	7.40	8.54	168.88	8.66	9.50	0.749
Mean	10.75	16.73	223.16	50.89	12.48	0.782
S. D.	3.48	7.34	53.53	39.59	4.31	.284

EXPERIMENT I

Restraint, 10 Minutes

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1			142.67	85.18	7.40	1.094
2	13.93	42.62	188.49	224.45	14.80	0.730
3	5.33	15.11	133.93	38.27	6.35	1.404
4	10.44	32.89	269.94	140.99	11.65	0.621
5	19.21	46.32	144.23	68.42	9.50	
6	18.18	60.85	341.03	283.97	8.45	0.452
7	9.66	50.11	176.75	200.17	12.70	0.769
8	20.24	48.81	116.21	8.50	6.90	0.705
9	6.91	37.78	114.83	101.71	14.80	0.662
10	11.82	26.15	146.80	115.66	6.90	0.724
Mean	12.86	40.07	177.49	126.73	9.95	0.796
S. D.	5.40	13.82	73.22	86.63	3.30	.284

EXPERIMENT I

Restraint, 20 Minutes

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	9.94	10.18	14.70	19.50	10.60	0.939
2	13.07	8.10	31.72	20.99	6.35	1.262
3	25.50	6.32	20.45	20.00	11.65	1.321
4	4.20	5.68	27.00	16.55	8.45	0.946
5	15.60	9.31	14.16	19.09	7.40	1.065
6	5.68	16.54	14.95	23.77	5.30	1.458
7	13.60	10.13	12.65	24.29	21.15	1.264
8	26.93	14.93	25.00	24.44	10.60	1.178
9	14.92	9.75	17.22	23.13	11.65	1.668
10	15.28	6.94	8.30	32.71	6.35	0.682
Mean	14.47	9.79	18.61	22.45	9.95	1.178
S. D.	7.34	1.04	7.29	4.44	4.57	.283

EXPERIMENT I

Restraint, 30 Minutes

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	21.96	6.27	53.57	29.76	11.65	1.346
2	14.62	14.36	18.71	35.93	6.35	1.660
3	12.89	18.52	23.12	46.23	8.45	0.990
4	19.61	15.69	33.61	28.89	6.35	2.051
5	12.38	12.62	19.67	20.49	6.35	2.281
6	39.49	15.13	33.30	44.76	6.35	0.299
7	9.33	17.33	34.48	29.63	11.65	0.357
8	28.27	27.33	38.56	26.79	10.60	0.827
9	11.73	17.87	28.71	20.93	8.45	0.287
10	12.87	18.20	42.42	29.92	12.70	0.388
Mean	18.32	16.33	32.62	31.33	8.89	1.049
S. D.	9.39	5.31	10.74	8.71	2.56	.823

EXPERIMENT I

Restraint, 60 Minutes

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	14.06	10.91	26.39	52.29	19.05	1.277
2	20.80	25.93	17.96	28.04	12.70	1.780
3	3.33	36.92	14.24	37.97		1.555
4	10.00	12.80	78.41	69.32	8.45	1.526
5	13.09	16.73	5.06	26.82	14.80	2.493
6	23.21	14.32	80.22	5.13	21.15	1.746
7	15.43	12.03	83.19	44.89	16.90	1.927
8	29.60	22.40	117.19	30.21	23.25	1.798
9	7.03	12.12	42.39	33.76	12.65	1.839
10	30.77	4.36	42.40	37.55	21.15	1.220
Mean	16.73	16.85	50.75	36.60	16.68	1.716
S. D.	9.20	9.28	37.01	16.98	4.90	.361

EXPERIMENT II

Normal Controls

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	6.27	27.73	113.12	27.97		
2	5.60	28.27	52.08	68.23	12.69	0.470
3	6.67	31.80	49.93	21.89	19.04	0.515
4	3.47	38.47	116.84	14.84	6.35	0.391
5	5.33	25.60	108.81	40.99	15.86	0.507
6	17.33	50.93	49.31	29.84	7.40	0.329
7	26.13	34.67	169.79	32.20	14.81	0.448
8	0.80	27.47	110.34	41.12	11.63	0.463
9	6.93	37.73	148.81	14.88	9.52	0.380
10	9.07	21.60	205.29	93.52	5.29	0.462
Mean	8.76	32.43	112.43	38.55	11.40	0.441
S. D.	7.47	8.43	16.62	24.81	4.66	.062

EXPERIMENT II

TM-10 Treated

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	4.27	72.53	40.71	21.42	12.69	0.749
2	12.27	26.93	169.90	68.77	12.69	0.477
3	15.73	62.67	80.25	39.06	11.63	0.737
4	7.73	23.47	187.31	202.15	13.75	0.398
5	10.13	30.47	148.99	218.65	12.16	0.593
6	11.20	14.13	95.34	40.25	10.58	1.060
7	5.60	17.60	37.55	35.64	10.58	0.662
8	4.27	14.13	33.23	17.37	13.75	1.000
9	4.80	6.13	125.84	35.65	12.69	0.428
10	6.67	32.80			10.58	0.891
Mean	8.27	30.09	102.12	75.44	12.11	0.700
S. D.	3.92	21.50	68.76	77.96	1.23	0.224

EXPERIMENT III

Normal Controls

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	2.22	6.17	363.50	347.40	1.06	0.866
2	1.26	4.28	184.29	18.69	4.23	0.546
3	0.73	9.21	240.65	79.44	2.64	0.798
4	22.72	9.88	279.15	224.80	3.17	1.049
5	1.70	1.21	332.28	85.90	3.70	0.625
6	1.00	6.10	168.27	564.90	3.17	0.564
Mean	4.94	6.14	261.36	220.19	3.00	0.741
S. D.	8.72	3.20	78.50	206.60	1.09	0.200

EXPERIMENT III

P-286 Treated

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	2.62	10.00	207.94	412.80	6.87	0.433
2	0.00	7.95	426.40	84.70	3.70	0.307
3	0.00	3.15	441.84	455.06	12.16	0.721
4	0.00	5.00	568.57	630.32	2.64	0.756
5	2.34	6.78	262.65	107.00	13.22	0.508
6	0.48	8.10	273.03	394.74	6.87	0.500
Mean	0.91	6.83	363.40	347.44	7.58	0.538
S. D.	0.39	5.08	139.80	212.13	4.32	0.172

EXPERIMENT IV

Normal Controls

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	7.73	3.73	66.96	99.70	6.35	0.548
2	2.40	5.87	32.69	23.08	7.40	0.425
3	2.40	2.13	66.91	385.82	6.35	0.283
4	8.53	2.13	174.49	374.37	4.76	0.356
5	0.00	0.00	79.67	510.99	4.23	0.393
6	0.00	12.27	48.46	468.20	4.23	0.363
7	10.67	20.53	120.85	286.87	8.46	0.233
8	3.20	4.53	97.83	433.70	2.12	0.180
9	0.00	25.07	110.68	351.56	0.00	0.385
Mean	3.88	8.47	88.73	326.03	4.88	0.352
S. D.	4.07	8.89	42.95	164.74	2.64	0.110

EXPERIMENT IV

Restraint, 30 Minutes

Animal Number	Plasma Epinephrine µg./L.	Plasma Norepinephrine µg./L.	Gland Epinephrine µg./100 mg.	Gland Norepinephrine µg./100 mg.	Plasma Corticosterone µg./100 ml.	Gland Corticosterone µg./100 mg.
1	33.00	27.99	2.13	450.09	10.05	0.740
2	18.93	12.80	61.50	425.53	10.05	0.469
3	36.80	45.07	72.30	390.78	6.87	0.406
4	38.67	36.80	107.34	180.06	5.82	0.636
5	27.20	22.13	60.61	505.73	14.28	0.838
6	30.13	25.33	18.58	118.24	7.93	0.597
7	49.07	53.33	26.50	57.67	16.39	0.854
8	49.07	7.73	17.80	214.29	5.82	0.548
9	23.73	18.67	3.36	31.88	12.16	0.639
Mean	34.07	27.76	40.90	263.81	9.93	0.636
S. D.	10.48	14.92	36.00	138.94	3.76	0.154

EXPERIMENT IV

Reserpine-Treated Controls

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	0.00	0.00	7.72	67.99	12.69	0.129
2	1.33	4.00	0.84	9.23	7.93	0.115
3	0.00	1.07	4.46	36.27	4.23	0.131
4	4.80	7.20	1.89	28.54	7.93	0.076
5	0.00	0.00	2.50	8.54	7.40	0.154
6	1.25	5.35	0.00	24.49	4.23	0.161
7	4.00	1.87	1.06	1.77	4.23	0.189
8	0.27	10.13	3.67	10.26	6.35	0.150
9	0.00	4.53	0.91	1.52	11.63	0.267
Mean	1.29	3.79	2.56	20.96	7.40	0.152
S. D.	1.85	3.44	2.40	21.41	3.12	0.053

EXPERIMENT IV

Reserpine-Treated; Restraint 30 Minutes

Animal Number	Plasma Epinephrine μg./L.	Plasma Norepinephrine μg./L.	Gland Epinephrine μg./100 mg.	Gland Norepinephrine μg./100 mg.	Plasma Corticosterone μg./100 ml.	Gland Corticosterone μg./100 mg.
1	0.00	0.00	3.90	22.73	3.17	0.176
2	2.67	15.27	1.03	1.72	2.12	0.471
3	0.00	12.27	1.14	1.90	5.29	0.314
4	0.00	13.86	1.19	2.00	4.76	0.386
5	0.00	1.33	1.36	2.27	4.76	0.402
6	0.00	4.00	0.00	0.00	4.23	0.431
7	5.33	30.40	0.76	1.26	4.23	0.439
8	0.00	52.33	1.04	1.74	0.00	0.694
9	0.00	4.00	0.61	5.79	4.23	0.401
Mean	0.89	14.83	1.11	4.38	3.64	0.413
S. D.	1.89	16.93	1.08	7.05	1.65	0.137

VITA

Jack Dennis Connally

Candidate for the Degree of

Master of Science

Thesis: CATECHOLAMINES AND CORTICOSTERONE IN ADRENAL GLANDS AND PLASMA FOLLOWING RESTRAINT IN CHICKENS

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