A FUNCTIONAL INTERRELATIONSHIP OF THE CORTICAL AND MEDULLARY TISSUES OF THE AVIAN ADRENAL

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NOMENCLATURE FOR APPENDIX B

Acidophil Count	Number of acidophils per 0.09 mm^3 blood
Bk	Corticosterone
E	Epinephrine
n	Number of animals per group
NE	Norepinephrine
PNMT	Phenylethanolamine-N-methyl transferase
±S.E.	±Standard error of the mean
Units	μmoles of metanephrine-^{14}C formed per hour per 100 mg adrenal tissue

CHAPTER I

INTRODUCTION

Most of the information concerning the role of the adrenal gland in response to stressors has come from research which involved mammals as the experimental animals. In contrast, there are very few data available on the response of the avian adrenal to stressors.

In mammals and birds, the adrenal is composed of two, distinct types of tissues: cortical (interrenal) tissue and medullary (chromaffin) tissue. These two tissues vary in both their embryological origins and the chemical nature of their hormones as well as many other factors.

In the past there had been a general tendency for endocrinologists to restrict their study of the adrenal to the cortical tissue, whereas pharmacologists and neurophysiologists concentrated their efforts toward study of the adrenal to the medullary tissue. It has been only recently that some investigators have suggested that because of the close anatomical relationship of the mammalian adrenal cortex and medulla, there may exist a functional interaction of these two tissues. In contrast to the situation in mammals, the cortical and medullary tissues of the avian adrenal are intermingled with one another; thus, there is an even closer anatomical relationship and presumably a greater possibility of a functional interrelationship of these two tissues in the bird.

The objectives of this study were 1) to evaluate the activity of the avian adrenal in response to stressors and 2) to obtain evidence that

there is a functional interrelationship of the cortical and medullary tissues of the avian adrenal.

The nonspecific stressor which was used throughout this study was a three-hour period of immobilization. To assess comprehensively the adrenal's activity under stress, the major hormones (corticosterone, epinephrine, and norepinephrine) of both the cortical and medullary cells were quantitatively measured in plasma and adrenal tissue. Phenylethanolamine-N-methyl transferase (PNMT) which catalyzes the conversion of norepinephrine to epinephrine was also assayed in the avian adrenal.

CHAPTER II

REVIEW OF LITERATURE

Introduction

In 1936, Selye demonstrated that the rat responds in a stereotypical manner to a wide variety of factors such as exercise, heat, cold, trauma, and intoxications with sublethal doses of drugs such as adrenalin, atropine, and morphine. Each of these stimuli cause specific effects upon the organism characteristic of that particular stimulus. The above-mentioned agents also have a common feature in that they all cause the organism to be in a state of general "stress". Selye (1951) stated that the stereotypical response to these damaging agents, which is super-imposed upon all the specific responses, represents the manifestations of nonspecific stress.

Selye (1936) reported that the outstanding manifestations of the stress response in rats were: adrenocortical enlargement with increased number of cellular lipoid granules, thymicolymphatic involution, and gastrointestinal ulcers. Selye noted that whereas many of the organs of the body showed involutional or degenerative changes, the adrenal cortex appeared to flourish on stressors.

There has been a considerable amount of research performed on the role of the mammalian adrenal gland in the stress response, but very little information is available on the avian adrenal's response to stressors. The purpose of this literature review is to discuss some of

the important aspects of avian adrenal physiology and adjustments which the avian adrenal makes in response to stressors. The review has been generally limited to studies in the avian class with only brief comparisons with results found in non-avian animals.

Anatomy and Histology of the Avian Adrenal

Avian adrenals are paired, oval-shaped organs which lie anterior and medial to the cephalic lobe of the kidneys and are located immediately anterior to the bifurcation of the posterior vena cava.

As in mammals, there are two major types of tissue found in the avian adrenal each with a different embryological origin. The interrenal (cortical) cells arise from the periotoneal epithelium which is mesodermal in origin, and the chromaffin (medullary) cells arise from the sympathetic nerve trunks which are ectodermal in origin (Hay, 1914).

In contrast to the situation in mammals in which the adrenal is divided into a well-defined medulla and cortex, the medullary and cortical cells of the avian adrenal are intermingled with one another (Hartman and Brownell, 1949). The adrenals of birds consist of clusters of chromaffin cells situated between cords of cortical tissue. The chromaffin islets (clusters) are irregular in shape and size and are sometimes represented by a single isolated cell inserted between interrenal cells (Cuello, 1970). There are many blood sinusoids which are in contact with both the cortical and medullary tissues.

In studies involving White Leghorn chickens, Oakberg (1951) reported that the proportion of the total adrenal made up of interrenal tissue is slightly larger in females (52%) than it is in males (50%). There is also a wide variation in the relative proportions of these two

tissues among the avian species. The interrenal tissue of pigeons comprised 65% of the total adrenal (Miller and Riddle, 1939), whereas, the interrenal tissue of pelicans comprised 85-90% of the adrenal (Knouff and Hartman, 1951).

Interrenal Tissue

Contrary to the situation in mammals, the interrenal tissue of birds is not zonated into three distinct zones. An exception is in the brown pelican where there are three zones corresponding in position and general appearance to the zones found in the mammalian cortex (Knouff and Hartman, 1951).

The most obvious cytoplasmic feature of the avian and mammalian interrenal cell is the presence of lipid globules which stain for cholesterol (Hartman and Brownell, 1949). These lipid droplets are closely associated with the endoplasmic reticulum (Symington, 1962). It has also been noted that there is an inverse relationship between the number of mitochondria and the size of the lipid vacuoles in the various cortical cells (Hartman and Brownell, 1949; Symington, 1962). In mammals the zona reticularis is rich in mitochondia but has little lipid content; the reverse is true for the zona fasciculata. With the application of a nonspecific stressor or ACTH, there is a very marked decrease in the lipid content of the adrenal. Symington (1962) stated that the zona reticularis is the area of steriod synthesis for normal daily adjustments, whereas the zona fasciculata serves as a storage place for the steroid precursors. Under the condition of stress, the precursors are quickly utilized in the synthesis of the glucocorticoids.

Chromaffin Tissue

The adrenal medullary cells are modified postganglionic cells which remain in close contact with the preganglionic fibers of the sympathetic nervous system (Dawson, 1953). The sympathetic nerve fibers form plexuses which entangle the chromaffin cells and terminate within the medullary portion of the adrenal (Hartman and Brownell, 1949). Except for some vasomotor fibers to blood vessels in the adrenal cortex, the cortical cells themselves receive no neural innervation.

Eranko (1957) is credited with first reporting the presence of two types of cells in avian chromaffin tissue: epinephrine- and norepinephrine-containing cells. By using density gradient centrifugation of medullary extracts, Shumann (1957) isolated a chromaffin granular fraction which contained mainly epinephrine, whereas another fraction contained mainly norepinephrine.

Additional evidence supporting the view that there are two cell types is based on the capacity of different stimuli to release epinephrine or norepinephrine selectively (Rubin and Miele, 1968). The stimulation of certain areas of the hypothalamus resulted in enhanced secretion of epinephrine, whereas stimulation of other areas in the hypothalamus resulted in increased secretion of norepinephrine (Folkow and von Euler, 1954).

Using the formalin or iodate reaction to distinguish noradrenalin- from adrenalin-storing cells, Eranko (1957) reported that one third of the total catecholamine content of the hen adrenal was noradrenaline (norepinephrine). In contrast to the situation in chickens, Cuello (1970) found that most of the chromaffin cells in the pigeon adrenal

stained for noradrenalin.

Criteria of Stress

One of the major problems in studying stress in birds has been the lack of adequate criteria for evaluating the effects of stressors on birds. Many of the criteria frequently used in mammalian research have proved unsatisfactory in birds. The following are a few of the criteria which have been examined in birds with varying amounts of success.

Adrenal Weight

Adrenal hypertrophy in rats was one of the original manifestations of stress which was noted by Selye in 1936. In contrast to the situation in rats, adrenal hypertrophy was reported not to occur following the application of various noxious stressors in birds (Bates et al., 1940). Other authors have reported that there was an increased adrenal weight following chronic administration of corticotropin (ACTH) extract or epinephrine (Jailer and Boas, 1950).

Ascorbic Acid Depletion

Slover (1956) reported a 16.5% decrease in adrenal ascorbic acid content twelve hours after ACTH administration in chickens, whereas other investigators reported that ACTH or epinephrine did not lead to decreased ascorbic acid in the adrenals of chickens or quail (Jailer and Boas, 1950; Zarrow and Baldini, 1952).

More recent research has implicated the bursa of Fabricius in the adrenal ascorbic acid depletion (AAAD) response. Perek and Eilat (1960) demonstrated that AAAD did occur with ACTH administration in bursectomized

chickens. They claimed that the presence of the bursa of Fabricius inhibits the AAAD response. In contrast, Freeman et al. (1966) found that the absence of the bursa did not result in AAAD when the chickens were stressed with ACTH. Freeman (1969) suggested that the loss of the bursa inhibits the AAAD response.

Cholesterol Depletion

Adrenal cholesterol depletion has been successfully used as an indicator of stress or increased ACTH secretion in rats (Fortier, 1962). The functional significance of adrenal cholesterol depletion in rats is based upon the fact that cholesterol serves as a precursor of adrenal corticosteroids (Sandor, 1969).

There has been less success in using adrenal cholesterol depletion as a criterion of stress or ACTH secretion in birds. Elton et al. (1959) found that neither ACTH nor severe cold results in a decrease in the adrenal concentration of cholesterol in the chicken. It has also been found that hypophysectomy was ineffective in producing a change in the adrenal cholesterol content of 3-5-month old chickens (Newcomer, 1959a). Slover (1956) reported that the administration of ACTH to chicks resulted in a 19% decrease in the adrenal level of cholesterol.

Acidophilia

Acidophilia, which is an increase in blood heterophils and eosinophils, has proved to be very satisfactory as an indicator of stress or ACTH secretion in chickens (Newcomer, 1957, 1958). Newcomer (1958) reported that a significant acidophilia followed administration of ACTH or a nonspecific stressor which was in the form of immobilization. Six

hours of restraint resulted in an eight-fold increase in the number of acidophils. Other nonspecific stressors such as acute anoxia, starvation, handling, centrifugation, injections of epinephrine or formaldehyde, and cold (4°C) combined with wetness all led to acidophilia in chickens (Newcomer, 1958; Wolford and Ringer, 1962; Burton and Guion, 1968). Injections of adrenal cortical extracts or glucocorticoids also resulted in acidophilia (Newcomer, 1958; Siegel, 1968).

Acidophilia has proved to be statistically useful in determining if an individual bird is in a state of stress. Thus, instead of having to utilize a large number of birds to evaluate whether a particular environmental situation is stressful, one bird can be used.

Hypophysectomy markedly inhibited, but did not block, acidophilia following immobilization; this suggested to Newcomer (1958) that a nonpituitary factor was probably involved in the stressor-induced acidophilia. No investigator has yet elucidated the physiological factors responsible for acidophilia in birds, but the glucocorticoids have been strongly implicated in the response mechanism (Wolford and Ringer, 1962; Burton and Guion, 1968).

Quantitative Measurement of Adrenal Hormones

One of the best indicators of a gland's activity is the plasma concentration of hormones secreted by the gland. It has only been recently that good quantitative analyses for the adrenal hormones have been developed.

Cortical Hormones. The first useful chemical assay of the glucocorticoids was developed by Porter and Silber (1950) and Nelson and

Samuels (1952). They based their assay on the ability of the 17,21-dihydroxy-20-ketone grouping to react with phenylhydrazine in acid solution to form colored compounds.

The method developed by Sweat (1954) was the first sensitive assay for cortisol and corticosterone quantification. This method depended upon the reaction of the steroids with sulfuric acid to form fluorescent compounds and was specific for the 11-hydroxy group found on these steroids.

Brief or short-cut methods, which eliminate chromatographic separation, have proved unsatisfactory in the bird due to the presence of many interfering chromagens in avian plasma (Frankel, 1970). Specific fluorometric assays which involve thin layer chromatography are now available for the quantitative measurement of the glucocorticoids (Frankel et al., 1967; Ganjam et al., 1970).

Methods other than fluorometry such as protein binding (Murphy et al., 1963) and gas phase chromatography (Baily, 1968) have also been used with varying amounts of success.

Catecholamines. The ability to quantitatively measure catecholamines is older than that for the corticoids, but there is much less satisfaction with the available methods. The vast number of publications appearing each year on new methods for the estimation of the catecholamines indicates that a suitable method has not been found yet, particularly when it comes to measuring the small amounts of catecholamines in the plasma.

Almost all of the new methods are merely modifications of procedures reported by Natelson et al. (1949), Shaw (1938), von Euler and

Lishajko (1961), and Weil-Malherbe and Bone (1952). Most of these assays involve the selective absorption of the catecholamines onto aluminum oxide followed by the oxidation of the eluate to a trihydroxyindole derivative or the condensation of the eluate with ethylenediamine.

The ethylenediamine method was reported to be satisfactory for measuring relative levels rather than absolute levels of norepinephrine and epinephrine, but is limited to systems in which interfering substances are low or absent (Vikotora, 1968).

One major problem with the trihydroxyindole method has been the instability of the oxidation products particularly in alkaline solution; thus, reducing agents such as ascorbic acid have been added to protect the lutins.

Haggendal (1962) replaced ascorbic acid with 2,3-dimercaptopropanol and obtained low stable blanks. Weil-Malherbe and Bigelow (1968) further modified Haggendal's method after recognizing that norepinephrine and epinephrine could be differentiated by conducting the oxidations at two different pHs.

Cortical Hormones

Levels of Corticosterone

Although small amounts of other adrenal steroids such as aldosterone, hydrocorticosterone, hydrocortisone, and cortisol have been reported in some avian species, the principle hormone of the avian interrenal gland is corticosterone (Frankel, 1970). It is probably for this reason that corticosterone is the only steroid which has been satisfactorily measured and identified in avian plasma.

Phillips and Chester Jones (1957) were the first to report the presence of corticosterone in an avian species. Using a catheter to remove adrenal venous blood of a capon, they reported that the concentrations of three corticosteroids (in μg/100 ml adrenal venous blood) were as follows: corticosterone, 312; cortisol, 3; and cortisone, 2. This high concentration of corticosterone and the existence of 17-hydroxycorticoids in the adrenal venous blood of chickens have never been substantiated (Frankel, 1970).

Nagra et al. (1960) made the first direct determination of corticosterone in avian adrenal venous blood. They found that there were approximately 40 μg of corticosterone per 100 ml venous plasma in pheasants, turkeys, and three different breeds of chickens. They also reported that concentrations in the peripheral plasma were approximately 10 μg/100 ml plasma. They were unable to detect cortisol in either the peripheral or adrenal venous plasma in any of the tested species. In later studies, Nagra and co-workers (1963a, b) reported that concentrations of corticosterone were less than half of their earlier findings. The lowest concentration of corticosterone reported in the adrenal venous blood of cockerels was 7.3 μg/100 ml plasma (Frankel et al., 1967c).

Substantial differences in peripheral plasma corticosterone levels have been found in birds from similar orders; Macchi et al. (1967) found concentrations of 19.35 μg/100 ml plasma for the domestic duck and 5.20 μg/100 ml plasma for the herring gull.

It should be pointed out that levels of corticosterone reported for plasma may be quite different from the true values for whole blood. There has been some evidence that corticosteroids are associated with red blood cells. Frankel (1970) reported that the corticosterone content

was 25% higher when whole blood was extracted versus extracting only plasma. In mammalian blood a significant amount of the corticosteroids exists in a conjugated form, but the levels of corticosteroid conjugates in avian blood, tissue and urine are unknown at this time (Frankel, 1970).

Synthesis of Corticosterone

The pathways for steroidogenesis in the avian adrenal have not been completely elucidated but some studies have been made in this area. The major pathway for the steroidogenesis of corticosterone in the avian adrenal gland apparently is very similar to that in the mammalian adrenal (Frankel, 1970). Sandor et al. (1965) showed a significant production of cortical hormones from acetate-1-^{14}C in both duck and goose adrenals; however, much difficulty has been encountered in establishing cholesterol as the obligatory precursor for corticosteroids (Frankel, 1970). The major pathway for the synthesis of corticosterone from progesterone appears to be as follows: progesterone to 11-desoxycorticosterone (DOC) to corticosterone (Sandor and Lanthier, 1963; Vinson and Whitehouse, 1969). Whitehouse and Vinson (1967) reported a significant production of progesterone from labeled prenenolone with duck adrenal slices. Sandor (1969) has postulated an alternative pathway of corticosterone synthesis via 11-hydroxyprogesterone, but he had only limited success in showing this as a possibility.

Elevation of Corticosterone

Circadian and Circennian Rhythms. There does appear to be a diurnal rhythm in adrenal function in birds as is already well established

in mammals. In birds, it has been shown that plasma corticosterone levels increased in the early morning hours (five to nine a.m.) (Frankel, 1970). There have also been some reports of a circennian rhythm of corticosterone secretion in birds (Resko et al., 1964). It was found that plasma corticosterone levels of chickens were at their maximum in October, November and December, whereas they were minimal in March (Frankel, 1970). In contrast, Macchi et al. (1967) found that the plasma corticosterone concentration of ducks was the highest in the spring.

Effects of Corticotropin (ACTH) or Stressors. As is true in mammals, elevated levels of ACTH result in increased plasma concentration of corticosterone in the bird (Frankel, 1970). Early investigators found that large doses of ACTH led to a decreased corticosterone concentration in the adrenal venous blood (Phillips and Chester Jones, 1957), but these results have never been substantiated by other workers (Urist and Deutsch, 1960; Nagra et al., 1960, 1963a; Frankel et al., 1967c; Macchi et al., 1967). These latter investigators all agree that plasma corticosterone was significantly increased with the administration of ACTH.

Using in vitro studies, deRoos (1961) demonstrated that the addition of ACTH to the incubation medium markedly increased corticosterone production. Greenman et al. (1967) injected ACTH into chickens, waited 15 minutes, and then removed the adrenals for incubation studies. They found that there was 200% more corticosterone produced by the adrenal from ACTH-treated birds as compared to those from the saline injected birds.

Thus far, no purified avian ACTH has been made available for ex-

perimentation, so all of the above-mentioned studies have been done using mammalian corticotropin. It has been found that extract from chicken or duck adenohypophyseal tissue did result in a significant increase in corticosterone production (deRoos and deRoos, 1964; Macchi et al., 1967).

Corticosterone concentration in adrenal venous blood of chickens was found to be markedly increased with surgically inflicted "stress" (Nagra et al., 1963a; Frankel et al., 1967c). In contrast, Macchi et al. (1967) reported that the same treatment in gulls did not lead to increased concentration of corticosterone. Water deprivation or extreme cold resulted in increased levels of corticosterone in peripheral blood of turkeys (Brown, 1961); in the adrenal venous blood of cockerels, but not pheasants (Nagra et al., 1963a). Nagra et al. (1963a) also reported that the serial withdrawal of blood per se from chickens led to a marked increase in their plasma corticosterone.

In measuring the general class of Δ^4-3-keto-corticosteroids, Newcomer (1959) found that the administration of ACTH or the induction of stress by immobilization resulted in a significant decrease of these steroids in both the plasma and adrenals. He suggested that the failure to detect the expectant increase in plasma corticoids might have been due to the insensitivity of the method employed.

Hypophyseal Regulation of Cortical Secretion. In mammals, the elevated adrenal corticoid levels which occur following the application of nonspecific stressors is generally believed to be mediated via the central nervous system and its control of corticotrophin release. The role played by the pituitary-adrenal axis in response to stressors has

been reviewed on several occasions (Selye, 1951; Mangili et al., 1962; and Fortier, 1962). There are also good reviews available on the regulation of the adenohypophysis and the role stressors might play in this regulation (Mangili et al., 1966; McCann, 1968).

In birds, the regulation of adrenal function by the hypophysis is not as well understood as the situation in mammals. Frankel (1970) has written a comprehensive review on the hormonal control of the avian interrenal gland.

One fact which has been substantiated many times is that the avian adrenal is still functional in the hypophysectomized bird (Frankel, 1970). Hypophysectomy did result in decreased plasma corticosterone concentrations in the cockerel, pigeon, quail, and duck, but in all cases, there were still significant levels of corticosterone in the plasma when comparing the results to that of hypophysectomized rats. The plasma level of corticosterone in the cockerel fell to one third of that for the intact bird 36 hours after surgery and remained at this concentration up to 42 days later (Frankel et al., 1967b).

Frankel (1970) suggested that the adrenals of hypophysectomized birds do not exhibit autonomy, but instead are regulated by substance(s) which can be inhibited as with negative feedback inhibition and stimulated by stressors. In order to explain the continued functioning of the adrenal in the hypophysectomized bird, one theory which has been postulated, but never substantiated, has been an extra-hypophyseal source of the ACTH such as the pineal gland (Van Tienhoven, 1969).

Medullary Hormones

Levels of Epinephrine and Norepinephrine

As in the mammal, the principle hormones of the adrenal medulla (chromaffin tissue) are epinephrine and norepinephrine. In mammals, almost all of the circulating epinephrine comes from the adrenal medulla, whereas norepinephrine comes from the adrenal medulla and sympathetic nerve endings (Wurtman, 1965).

Based on histological observations of the avian adrenal tissue and on quantitative assays of epinephrine and norepinephrine in plasma and adrenal tissue, the reported ratios of these two catecholamines have a wide range of values. Most avian physiologists agree that unlike most mammals, the avian plasma and adrenals contain more epinephrine than norepinephrine (Eranko, 1957; Callingham, 1966; Lin and Sturkie, 1968; Sturkie et al., 1970; and Sturkie, 1970). On the contrary some investigators have reported that norepinephrine represents 50-86% of the total adrenal catecholamines in the avian adrenal (Shepherd and West, 1951; Ghosh, 1962; and Callingham, 1965). Cuello (1970) found in a recent histological study of the penguin adrenal that most of the chromaffin cells stained for norepinephrine.

It has been only recently that some quantitative studies of the catecholamines in blood and adrenal tissue of birds have appeared in the literature. Sturkie et al. (1970) measured the levels of epinephrine and norepinephrine in the blood and heart of ducks, pigeons, turkeys, and chickens. Except for the duck, the ratio of epinephrine to norepinephrine in the plasma was considerably greater than one. Values for epinephrine and norepinephrine in the plasma of cockerels

were 8.68 μg/liter and 0.843 μg/liter respectively. In another paper, Sturkie (1970) reported a much smaller value for the epinephrine to norepinephrine ratio in plasma. The epinephrine concentration was 6.076 μg/liter and norepinephrine was 1.564 μg/liter with a resulting ratio of approximately four.

The ratio of epinephrine to norepinephrine in the avian adrenal was found to be approximately 2.5 for twelve-week old chickens kept at room temperature (Lin and Sturkie, 1968). Epinephrine and norepinephrine were reported to be 3734 μg/g of adrenal tissue and 1525 μg/g of adrenal tissue respectively in the chicken adrenal. These investigators also found that the percent of catecholamines made up by norepinephrine decreased somewhat with age.

Epinephrine and Norepinephrine Synthesis

Norepinephrine. The steps involved in the synthesis of norepinephrine and epinephrine have been eluciadated in the mammal (Wurtman, 1965). The biosynthesis is initiated by the active uptake of circulating l-tyrosine by chromaffin cells. The hydroxylation of tyrosine to l-dihydroxyphenylalanine (DOPA) is thought to be the rate-limiting step in the synthesis of dopamine and norepinephrine (Levitt et al., 1965; Weiner and Rabadjija, 1968). The next step involves the decarboxylation of DOPA to form dopamine and is catalyzed by DOPA decarboxylase. Tyrosine hydroxylase is confined to the mitochondrial fraction of the chromaffin cell (Levitt et al., 1965), whereas DOPA decarboxylase is apparently not bound to any subcellular particle (Lovenberg et al., 1962). The final step in the synthesis of norepinephrine is the β-oxidation of dopamine which is catalyzed by dopamine-β-oxidase (Fellman, 1959).

This final step occurs in the chromaffin granule.

Epinephrine. The conversion of norepinephrine to epinephrine involves the transfer of a methyl group to norepinephrine and is catalyzed by phenylethanolamine-N-methyl transferase (PNMT) (Kirshner and Goodall, 1957). In mammals, PNMT is highly localized in the adrenal medulla, whereas the other enzymes involved in the synthesis of the catecholamines are found to a significant extent in various tissues especially the sympathetic nerve endings (Wurtman, 1965). The methylation of norepinephrine in the chromaffin cell occurs in the cytoplasm and not in the storage granules.

There appears to be a negative feedback inhibition in operation for the synthesis of both norepinephrine and epinephrine in mammals. Tyrosine hydroxylase activity has been shown to be inhibited by norepinephrine *in vivo* (Thoenen et al., 1969) and *in vitro* (Udenfriend et al., 1965); PNMT activity was inhibited by epinephrine *in vitro* (Connett and Kirshner, 1970).

Elevation of Plasma Epinephrine and Norepinephrine by Stressors

As in mammals, the plasma catecholamine levels of birds were significantly increased with nonspecific stressors (Connally, 1961; Lin and Sturkie, 1968; Sturkie, 1970; Sturkie et al., 1970). Sturkie et al. (1970) stated that the wide variation of catecholamine levels, which has been reported, was probably due to various conditions of handling, excitement, and blood sampling, all of which caused a release of adrenal and neuronal catecholamines. They found that trained male birds which

were adapted to their surroundings and handling had less catecholamines in their blood than those untrained birds at the time of autopsy.

Subjecting birds to cold temperature (0°C) resulted in a significant increase in plasma catecholamine levels (Sturkie, 1970; Lin and Sturkie, 1968). There was also a significant increase in the adrenal levels of these catecholamines with exposure to cold for 20 weeks.

By using immobilization to initiate stress in rats, Kvetnansky and co-workers have done considerable research concerning the secretion and synthesis of the catecholamines by the adrenal medulla (1970a, 1970b, 1970c, 1971a, and 1971b). Kvetnansky et al. (1970b) found that the urinary excretion of both epinephrine and norepinephrine increased significantly after one or more periods of immobilization. The adrenal epinephrine concentration decreased significantly with immobilization, but there was no change in the adrenal norepinephrine levels.

In an unpublished work by Connally (1961), adrenal and plasma catecholamine levels were also measured in the chicken following various intervals of immobilization. Connally (1961) found that after only five minutes of restraint, there was a notable increase in plasma epinephrine, but not norepinephrine.

Cortical-Medullary Interrelationship

Introduction

In mammals, the adrenal medulla and cortex have a close anatomical relationship with an intra-adrenal portal circulation being present between these two tissues. The mammalian adrenal gland is richly supplied with blood by many arteries which enter the gland at different

points (Hartman and Brownell, 1949). These arteries form a plexus in the gland's capsule and give rise to a sinusoidal network of cortical arteries which surround the cords of cortical cells. There is a double vascularization to the mammalian adrenal medulla (Hartman and Brownell, 1949). The adrenal medulla not only has its own direct arterial blood supply, it also receives blood that has drained the adrenal cortical cells. Thus, the portal blood which comes directly to the medulla from the cortex contains high concentrations of the cortical hormones.

Shepherd and West (1951) found that in examining adrenals from various mammals, there was a direct correlation between the size of the adrenal cortex and the percent epinephrine present in the adrenal medulla. Coupland (1953) reported that in species, in which the chromaffin cells were not surrounded by cortical cells, the chromaffin cells contained only norepinephrine. From these observations, these investigators suggested that the juxtaposed adrenal cortex in mammals was influencing the methylation of norepinephrine to form epinephrine.

Lempinen (1964, 1968) found that if he administered glucocorticoids to neonatal rats, he could prolong the existence of extra-medullary chromaffin tissue which normally atrophied soon after birth.

Effect of Glucocorticoids on PNMT Activity

The first work published on the adrenocortical control of PNMT activity in rats indicated that hypophysectomy resulted in a marked decrease in adrenal weight and PNMT activity within several days (Wurtman and Axelrod, 1965). The level of epinephrine and the percent of total catecholamines represented by epinephrine also decreased after hypophysectomy (Hokfelt, 1951; Wurtman and Axelrod, 1965; Wurtman et al., 1968).

The PNMT activity and epinephrine levels could be restored by supplementing ACTH or glucocorticoids (Wurtman, 1966; Pohorecky and Wurtman, 1971).

Whereas small doses of ACTH were effective in elevating PNMT activity back to normal, it was necessary to administer large amounts of the glucocorticoids to restore the enzyme activity (Wurtman, 1966). When considering the adrenal anatomy and the high concentrations of cortical hormones normally available to the adrenal medulla, the required doses of corticoids were reasonable.

Fuller and Hunt (1967) found the resting PNMT activity in the rat to be maximal and that further stimulation of adrenocortical secretion did not result in an increase in activity. Only after periods of reduced cortical activity, as seen after hypophysectomy, could they show an increase activity of PNMT with glucocorticoids or ACTH administration.

Long-term elevation of circulating ACTH was shown to increase PNMT activity in the adrenals of intact rats. Animals bearing transplantable ACTH-secreting tumors (Vernikos-Danellis et al., 1968) or possessing unilateral adrenalectomy (Ciaranello et al., 1969) exhibited much higher PNMT activity than did the normal controls. Furthermore, rats subjected to chronic stress by repeated immobilizations had an elevation of PNMT activity in the adrenal gland (Kvetnansky et al., 1970c; 1971b).

Reis et al. (1971) found that intermittent electrical stimulation of certain hypothalamic areas which produced fighting behavior in cats, resulted in an increased adrenal PNMT activity. No change in enzyme activity occurred when the hypothalamic stimulation failed to elicit the fighting behavior; thus, the elevation in PNMT activity appeared to be specific for certain areas of the hypothalamus in cats.

It has only been recently that the presence of PNMT activity in the bird adrenal has been reported (Wasserman and Bernard, 1971). Using chick embryos, Wasserman and Bernard (1971) found that there was a good positive correlation between the appearance of a well-developed adrenal cortex and increased levels of epinephrine and PNMT activity. Wasserman and Bernard (1971) demonstrated the elevation of PNMT activity in the chick adrenal by giving large doses of exogenous corticosterone.

Hormone Specificity

Hypophysectomized rats were treated with partially purified preparations of the six adenohypophyseal hormones to test the specificity of PNMT elevation by ACTH (Wurtman, 1966). The administration of ACTH resulted in a 160% increase in PNMT activity, whereas doses of follicle-stimulating hormone, luteinizing hormone, prolactin, thyroid-stimulating hormone and growth hormone had no effect on PNMT activity.

To examine the ability of various steroids to elevate PNMT activity, large doses of compounds representing the four classes of steroidal hormones (estrogens, androgens, mineral corticoids, and glucocorticoids) were administered to hypophysectomized rats (Wurtman, 1966). Dexamethasone and aldosterone resulted in increases of PNMT activity by 195% and 50% respectively, whereas estradiol and testosterone had no detectable effect.

The most potent natural steroid to cause PNMT elevation was hydrocortisone which is hydroxylated at the 11-, 17- and 21-positions (Pohorecky and Wurtman, 1968). The increase in activity accompanying the hydroxylation of various progesterone derivatives at the 11-, 17- or 21-positions was found to be the same for all three positions.

Insulin has been reported to cause increased activity of PNMT in rat adrenals (Kitabchi et al., 1967). It was suggested that insulin's effect was not directly upon the enzyme, but was a result of the decrease of end-product inhibition with the release of epinephrine (Pohorecky and Wurtman, 1971).

Possible Mechanisms for PNMT Elevation

Coupland and MacDougall (1966) reported that addition of hydrocortisone, but no desoxycorticosterone, to tissue cultures of extra-adrenal chromaffin cells resulted in the appearance of epinephrine. These cells normally contain only norepinephrine.

Wurtman and Axelrod (1966) added hydrocortisone and dexamethasone to adrenal homogenates from intact and hypophysectomized rats and found that there was no effect on PNMT activity. They concluded that the mechanism of PNMT elevation with glucocorticoids was not by enzyme activation.

In the same study puromycin and actinomycin D were administered to groups of hypophysectomized rats to test the possibility that the increased activity of PNMT with glucocorticoids was by enzymatic induction (enzyme synthesis). It was found that neither puromycin nor actinomycin D changed the basal activity of PNMT, but both drugs blocked the elevation of activity normally seen with dexamethasone-treatment. From these data, Wurtman (1966) suggested that glucocorticoids elevate PNMT activity by increasing the rate of synthesis of this enzyme.

CHAPTER III

MATERIALS AND METHODS

Materials

Reagents

The dichloromethane, benzene, toluene, and methanol which were used were of "nanograde" quality (Mallinckrodt Chemical Works). The dichloromethane was distilled in an all-glass system for further purification. The organic solvents, iso-octane and chloroform were "spectrograde" in quality (Mallinckrodt Chemical Works). Ethanol was 100% analytical grade (U.S. Industrial Chemical Co.) and was further purified by distillation over glass. Water was twice distilled using an all-glass system, ether-washed, and redistilled. Concentrated hydrochloric, sulfuric and acetic acids were analytical grade (Baker Chemical Co.).

Glassware

Glassware was rinsed with tap water after use, soaked in detergent (Sparkleen, Fisher Chemical Co.), rinsed, and placed in chromic acid overnight. The glassware was then rinsed seven or eight times with tap water, resoaked in detergent, rinsed again with tap water, and placed in dilute hydrochloric acid overnight. After being rinsed seven or eight times each with tap water, distilled water, and glass distilled water, the glassware was finally rinsed two or three times with nanograde methanol

and air dried. The microcuvettes used in the fluorometric determination were soaked in concentrated nitric acid overnight, rinsed with tap water, distilled water, and methanol, and then air dried.

Aluminum Oxide

The aluminum oxide for catecholamine absorption was prepared in the hood according to the procedure of Anton and Sayre (1962). One hundred grams of aluminum oxide (Al_2O_3, Fisher Scientific, 80-200 mesh) were stirred continously with 500 ml of 2N HCl in a covered beaker at 90-100°C for 45 minutes. The aluminum oxide was then allowed to settle for 1.5 minutes, and the supernatant along with the finer particules of Al_2O_3 was discarded. The precipitate was washed two times with 250 ml of 2N HCl at 70°C for 10 minutes, discarding the supernatant and finer particles each time. The Al_2O_3 was given a final acid wash with 500 ml 2N HCl at 50°C for 10 minutes. The precipitate was then repeatedly washed (approximately 20 times) with 200 ml portions of distilled water until a pH 3.4 was obtained. Lastly, the aluminum oxide was heated at 120°C for one hour, 200°C for two hours, and stored in a dessicator. The purpose of the acid wash was to prevent any autoxidation of the catecholamines during their chromatographic separation.

Preparation of Thin Layer Chromatography Plates

Silica gel (Silicar TLC-7GF, Mallinckrodt Chemical Works) was prepared by washing three times with dilute acetic acid over a sintered glass filter, rinsing three times in boiling triple distilled water, and finally washing two times with boiling methanol. The silica gel was then dried 24 hours at 110°C and kept in a dessicator until use. A

slurry containing 24 g silica gel and 65 ml water was spread 0.25 mm thick onto thin layer chromatography plates. The plates were dried and activated by placing them in an oven at 110°C for 24 hours.

Standard Solutions

Corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione), obtained from Merck Sharp and Dohme Research Lab, was recrystallized to its constant melting point (180-181°C), dissolved in ethanol at a concentration of 1 mg/ml, and refrigerated at 5°C until use.

L-epinephrine and L-norepinephrine bitartrates (Sigma Chemical Co.) were dissolved in 0.1 N HCl at a concentration of 100 μg/ml (corrected for bitartrate) and kept refrigerated. New stock solutions were made up every 30 days, and working standards which contained concentrations of 1 μg/ml for each catecholamine were made just prior to use.

Radioisotopes

Corticosterone-1,2-^{3}H, DL-epinephrine-7-^{3}H, and DL-norepinephrine-7-^{14}C, obtained from New England Nuclear Corp., were purified by thin layer chromatography. Their radiochemical purities were checked every 60 days with a radiochromatogram scanner (Packard, Model 7201), and their identities made by spotting authentic standards in adjacent lanes of the thin layer plates. The radiochemical purities of the catecholamines and corticosterone labeled standards were estimated to be 96-98% by measuring the areas under the radiochromatogram tracing. Corticosterone-1,2-^{3}H was stored in ethanol at 5°C. The catecholamines were dissolved in 0.1 N HCl and also kept refrigerated.

S-adenosyl-L-methionine-methyl-^{14}C obtained from New England Nuclear Corp. was checked prior to each use for its radiochemical purity by paper chromatography on Packard radiochromatogram scanner. The purity was found to be consistently greater than 92% as estimated by measuring the area underneath the chromatogram tracing. S-adenosyl-L-methionine was stored in H_2SO_4 (pH 2-3) and kept frozen until use.

Methods

General Procedures

The birds used throughout the series of experiments were eight- to nine-week old, White Leghorn cockerels. The birds were raised in well-ventilated metal cages. When necessary, they were moved from small pens to larger pens to accommodate their growing sizes. They were watered and fed a standard growing ration (see Appendix A) ad libitum.

The night prior to the day of the experiment, the birds were placed in wooden floor pens in a darkened room in order to allow the birds to be undisturbed until immediately before use. The birds were randomly chosen from these pens and placed in the various groups.

The three-hour restraint procedure consisted of placing the birds on their backs and fastening their wings to boards with rubber bands. The legs of the birds were also tied together to prevent them from inflicting injury upon themselves or the birds fastened adjacent to them.

At the end of this period of restraint, 30-35 ml of blood were collected from each bird by cardiac puncture, and placed in chilled, heparinized centrifuge tubes. The birds were sacrificed by decapitation, and approximately 0.2 ml of blood was collected from the neck wound of each

bird for the acidophil count.

The body weights were recorded, and the adrenals immediately removed and placed in ice-cold isotonic KCl. The adrenals and blood samples were kept at 5°C at all times.

The non-restrained, control birds were treated in the same manner with the exception of the immobilization. The entire process from the time of the cardiac puncture to the removal the adrenal glands required less than five minutes.

The adrenal glands were carefully cleaned of adherent tissue and their individual weights were measured on a Roller-Smith torsion balance to the nearest milligram. One adrenal gland was randomly chosen from each chicken, wrapped in parafilm, and stored in the deep-freeze until corticosterone analysis was made. The other adrenal was placed in three ml of ice-cold isotonic KCl for assay of phenylethanolamine-N-methyl transferase (PNMT).

The 30-35 ml blood samples were centrifuged at 1500 rpm for 15 minutes. The plasma was quickly drawn off with a syringe and placed in clean chilled tubes. For each plasma sample, a 2-3 ml aliquot was saved for corticosterone analysis and the remainder kept for the catecholamine determinations. These tubes were kept at -20°C until the analyses for the catecholamines and corticosterone were performed. These analyses were done within three months from the time the plasma was collected.

Acidophil Counts

The blood collected after decapitation was shaken vigorously and an appropriate amount of blood was quickly drawn into a red blood cell pipette. The blood was diluted 200 times with Wiseman's (1931) staining

solution. The pipette was allowed to stand for at least 24 hours and then shaken on a mechanical shaker for one to two hours. After the pipette had been thoroughly shaken, a Levy-Hausser hemocytometer was charged with a spot of the diluted blood. The number of acidophils per 0.09 mm^3 was obtained by two different investigators and an average was made of the two counts. Neither investigator knew the source of the blood; their counts never varied from one another more than four acidophils on any of the samples.

PNMT Assay

The assay for the enzyme, phenylethanolamine-N-methyl transferase (PNMT), was that of Axelrod (1962) with some modifications for the species involved. The assay was performed on the day of autopsy.

One randomly chosen adrenal gland was homogenized in three ml of ice-cold isotonic KCl with a Potter-Elvehjem homogenizer. The whole homogenate was transferred to a 4 ml Beckman cellulose nitrate tube, capped, and centrifuged for 30 minutes at 40,000 rpm on a Beckman Ultracentrifuge (Model L-2) at 0°C.

A 0.2 ml aliquot of the resulting supernatant was removed for the enzyme assay, and the remainder of the whole adrenal homogenate was frozen and stored for the tissue catecholamine determinations. A 50 μl aliquot of the supernatant was transferred to a 15 ml centrifuge tube which contained 10 μl (0.0001 M) of S-adenosylmethionine-methyl-^{14}C and 0.5 ml (0.2 M) phosphate buffer (pH 7.4). Finally, 37.5 μl (1 μg/μl) of the substrate, DL-normetanephrine (Sigma Chemical Co.) were added and the tubes were incubated for one hour at 42°C. Duplicate incubation tubes were made of each sample. A "blank" which contained all of the

above material with the exception of the substrate was included in each incubation. At the end of one hour, the incubation reaction was stopped by adding 0.5 ml of 0.5 M borate buffer, pH 10.

The reaction mixture was vortexed for one minute with six ml of toulene:isoamyl alcohol mixture (3:2;v:v) in order to extract the reaction product, metanephrine-^{14}C. The extraction tubes were centrifuged at 1500 rpm for 10 minutes to insure separation of the layers. Four ml of the toulene:isoamyl alcohol layer were added to a scintillation vial containing one ml of ethanol. Bray's solution was used for the liquid scintillation counting as described under the section on scintillation counting. The blank, in which no substrate had been added, showed very little extractable radioactivity. The blank value was never greater than five percent of any of the values for the samples. The blank value was subtracted from all sample values when calculating the enzyme activity.

The extractable product showed a single radioactive peak with a R_f value equal to that of authentic metanephrine when chromatographed in solvent system of n-butanol:acetic acid:water (4:1:1;v:v:v).

The cpm obtained from the liquid scintillation counting were converted to mμmoles of product formed per hour per mg tissue using the following formula.

$$\text{metanephrine-}^{14}\text{C (m}\mu\text{moles/hour/mg)} = \frac{C}{E} \times \frac{1}{N} \times \frac{1}{SA} \times \frac{VO}{AO} \times \frac{VS}{AS} \times \frac{1}{W}$$

C = cpm (corrected)

E = counting efficiency of ^{14}C

SA = specific activity of S-adenosylmethionine-methyl-^{14}C (mμcuries/mμmole)

N = number of dpm/mμcurie (2.22×10^3)

VO = volume of organic extract (6 ml)

AO = aliquot of organic extract taken for counting (4 ml)

VS = volume of adrenal homogenate (3 ml)

AS = aliquot of adrenal homogenate taken for incubation (0.05 ml)

W = weight of adrenal (mg)

Corticosterone Determination

The method used in this study for the measurement of corticosterone in plasma and tissue was the fluorometric method of Ganjam et al. (1970) with some modifications. To check percent recovery through the method, a corticosterone-1,2-^{3}H standard containing 1500 dpm was added to 80 ml extraction tubes and evaporated to dryness under nitrogen at 30°C.

Plasma Corticosterone. Plasma samples were thawed and 2-3 ml of each sample were added to extraction tubes containing the labeled corticosterone. The tubes were mildly vortexed and allowed to set for 20 minutes at 5°C in order to insure equilibration of the labeled and unlabeled corticosterone. Each sample was washed with 2.5 volumes of iso-octane (2,2,4-trimethyl pentane) and centrifuged 10 minutes at 1500 rpm. The iso-octane was removed with a Pasteur pipette. Iso-octane washing removed lipids less polar than C-21 glucocorticoids.

The plasma was extracted with 40 ml of ice-cold dichloromethane and was then discarded. Four ml of 0.1 N NaOH were used to wash the dichloromethane extract. The alkali was shaken with the organic extract, quickly removed by aspiration, and discarded. The NaOH was not allowed to contact the dichloromethane extract more than 20-30 seconds. Immediately following the NaOH wash, the organic extract was washed three

times with 10 ml portions of water; the water being aspirated and discarded each time. The saponification step was used to remove estrogens as well as other phenolic and acidic steroids.

The resulting dichloromethane extract was evaporated to dryness under a stream of nitrogen at 30°C. The tubes were then washed with three, two, and one ml of methanol:chloroform (1:1;v:v) to concentrate the residue to the tip of the tube.

Samples were spotted on thin layer chromatography (TLC) plates which had been divided into nine lanes of 2 cm each. Six of the lanes were used for unknown samples, the two end lanes for known standards, and one lane for a silica gel blank.

Samples were spotted with capillary tubes in each of the lanes using methanol:chloroform (1:1) as the solvent. The TLC plates were developed in a system of chloroform:methanol:water (90:10:0.5;v:v:v) and then placed briefly under an ultraviolet light to locate areas corresponding to the chromatographed standard. Using this solvent system, corticosterone had a R_f value of 0.60.

The areas of each of the six unknown lanes corresponding to the standard corticosterone were removed by scraping off the silica gel and placing them in 15 ml test tubes. The samples were extracted three times using a benzene-water partition system. The benzene extract was pooled, placed into tubes, and evaporated to dryness under nitrogen.

One ml of methanol was added to the residue and 0.2 ml was taken for scintillation counting. The scintillation vials were evaported to dryness under nitrogen and 10 ml of toulene scintillation fluid was added. The samples were counted according to the procedure discussed in the section on single label scintillation counting. Tubes with the re-

maining 0.8 ml were evaporated to dryness along with standard tubes containing 0, 0.01, 0.02, 0.04, 0.10, and 0.20 μg/ml of corticosterone. One ml of H_2SO_4:C_2H_5OH (65:35;v:v) was added to the residue in each of the tubes.

Fluorescence was measured on an Aminco Bowman Spectrophotoflurometer 1.25 hours after the addition of the alcohol-acid mixture. The following settings were used to read the resulting fluorescence: 1) excitation wave length of 472 mμ through a 3 mm slit; 2) emission wave length of 524 mμ through a 3 mm slit; 3) meter multiplier 0.03; 4) sensitivity 35-50; and 5) turret setting 2.

The micrograms of corticosterone per milliliter of plasma were calculated using the following formula.

$$\text{Corticosterone } (\mu g/ml) = \frac{C}{Sd} \times \frac{S}{A} \times \frac{1}{V} \times \frac{100}{R}$$

where:

C = concentration of standard (μg/ml)

Sd = reading of standard (fluorescence units)

S = reading of sample (fluorescence units)

A = aliquot of sample (ml)

V = volume of plasma extracted (ml)

R = % recovery

The percent recovery of corticosterone and the catecholamines were calculated with the following formula.

$$\% \text{ Recovery} = \frac{\text{net sample cpm}}{\text{net standard cpm}} \times \frac{100}{0.2}$$

where:

0.2 = sample aliquot taken for scintillation counting.

Adrenal Corticosterone. Each adrenal was homogenized in two ml of 100% ethanol to precipitate protein. The homogenate was then centrifuged at 1500 rpm for 10 minutes, and the resulting supernatant was added to an 80 ml extraction tube which contained labeled corticosterone. Two ml of water were then added to each of the extraction tubes. The tubes were gently vortexed and allowed to stand for 20 minutes in order to permit equilibration of the labeled and unlabeled corticosterone. Two and a half volumes of iso-octane were added and the corticosterone was extracted and separated in the same fashion as described under plasma corticosterone. Aliquots for scintillation counting were taken and the fluorescence was measured using the same methods as for the plasma.

The formula used to calculate the amount of corticosterone in adrenal tissue is:

$$\text{Corticosterone } (\mu g/mg) = \frac{C}{Sd} \times \frac{S}{A} \times \frac{1}{W} \times \frac{100}{R}$$

where:

C = concentration of standard (μg/ml)

Sd = reading of standard (fluorescence units)

S = reading of sample (fluorescence units)

A = sample aliquot (ml)

W = weight of adrenal tissue (mg)

R = % Recovery

Epinephrine and Norepinephrine Determinations

The method used for the determination of epinephrine and norepinephrine in plasma and adrenal tissue was that of Weil-Malherbe and Bigelow (1968) with some modifications. To moniter losses of the catecholamines

through the method, 50 μl of a standard containing DL-norepinephrine-7-^{14}C and DL-epinephrine-7-^{3}H (2000 dpm each) were added to 80 ml extraction tubes in which absorption of catecholamines would be made.

Samples containing 10-15 ml of plasma were thawed, placed in the extraction tubes with the radioactive standards, and allowed to equilibrate for 20 minutes. Aluminum oxide (0.3-0.4 g) was shaken with the plasma for three minutes for absorption of the catecholamines. The tubes were then centrifuged for 10 minutes at 1500 rpm, and the aqueous phase removed by aspiration and discarded. The aluminum oxide was washed three times with 10 ml portions of ice-cold water, centrifuged, and the washes discarded each time.

The catecholamines were eluted from the aluminum oxide by mixing two 2.5 ml portions of 0.1 N acetic acid with the aluminum oxide for 2 minutes each. From the pooled eluate, one ml was taken for the epinephrine determination, two ml were taken for the norepinephrine determination, and one ml was taken for the calculation of percent recovery. The measurement of radioactivity for the calculation of percent recovery was done according to the double labeling procedure outlined in the section on scintillation counting. The percent recoveries for epinephrine and norepinephrine never varied from one another more than 3% for the same sample.

Plasma Epinephrine. Epinephrine standards containing 0, 0.001, 0.002, 0.004, 0.010, and 0.020 μg were made up to a total volume of one ml with 0.1 N acetic acid. Tubes containing one ml of each of the sample eluates, along with the epinephrine standards, were mixed with 0.3 ml of 0.1 M formic acid, 0.05 ml of 0.01 M cupric acetate, and 0.05

ml of 0.25% ferricyanide. After five minutes, 0.3 ml of 1 N NaOH-mercaptoethanol reagent was added and then mixed. The NaOH-mercaptoethanol reagent was made by mixing equal volumes of 1% β-mercaptoethanol in 20% Na_2SO_3 (1:1;v:v) and 1 N NaOH (0.5 N for the norepinephrine determination) immediately prior to use. The mercaptoethanol provided low and stable blanks. Following a four minute wait, 0.3 ml of 1 N acetic acid was added to each tube and mixed thoroughly. The tubes were centrifuged for five minutes at 600 rpm and the supernatant transferred to fluorometric curvettes.

Fluorescence was measured on an Aminco Bowman spectrophotofluorometer using the following instrument settings: 1) excitation wavelength of 415 mμ through a 3 mm slit; 2) emmission wavelength of 500 mμ through a 3 mm slit; 3) meter multiplier 0.001; 4) sensitivity 30-50; and 5) turret setting 2.

The calculation for the determination of epinephrine in plasma was as follows.

$$\text{Epinephrine } (\mu g/ml) = \frac{C}{Sd} \times \frac{S}{A} \times \frac{1}{V} \times \frac{100}{R}$$

where:

C = concentration of standard (μg/ml)

Sd = reading of standard (fluorescence units)

S = reading of sample (fluorescence units)

A = sample aliquot of eluate taken for reading (ml)

V = volume of plasma extracted (ml)

R = % Recovery

A norepinephrine internal standard was included in all of the epinephrine determinations. The internal standard was made by adding .02

μg norepinephrine to one of the epinephrine standards before the fluorescence reading was made. This precaution was done to check for any possible interference by norepinephrine at the pH and wavelength used for the epinephrine determination. It was found that the norepinephrine internal standard consistently did not give off any additional fluorescence and thus no correction was made.

Plasma Norepinephrine. Tubes containing 0, 0.002, 0.004, 0.01, and 0.02 μg of standard norepinephrine were prepared and made up to 2 ml with 0.1 N acetic acid. The 2 ml eluates along with the standards were individually adjusted to pH 5.9 ± 0.1 with 0.5 N and 0.1 N NaOH using a Coleman pH meter. The volume of each was made up to 3 ml and 0.1 ml of 0.25% ferricyanide was added. After 5 minutes, 0.5 ml of 0.5 N NaOH-mercaptoethanol reagent was added to each and left for 4 minutes. After adding and mixing 0.3 ml of 1 N acetic acid, the tubes were centrifuged 5 minutes at 600 rpm.

The supernatant was placed in a fluorometer cuvette and read on an Aminco Bowman spectrophotofluorometer using the following settings: 1) excitation wavelength of 395 mμ through a 3 mm slit; 2) emmission wavelength of 475 mμ through a 3 mm slit; 3) meter multiplier 0.001; 4) sensitivity 30-50; and 5) turret setting 2.

It was found that no correction had to be made for any interference by epinephrine internal standard (0.02 μg) at the pH and wavelength used for norepinephrine.

The formula used for norepinephrine determination in plasma was that used for the estimation of epinephrine, with the exception that the aliquot size for norepinephrine was twice that used for epinephrine.

Adrenal Epinephrine and Norepinephrine. Adrenal epinephrine and norepinephrine were measured using the same methods as those for plasma. The only difference was in the preparation of the tissue up to the time of absorption on the aluminum oxide.

The whole adrenal homogenate from the enzyme assay was treated with five ml of 0.4 N perchloric acid, centrifuged and the supernatant removed. Perchloric acid was used as the protein precipitant because it has been reported that perchloric acid resulted in a better percent recovery and smaller blank value than the more common precipitants such as trichloroacetic acid (Anton and Sayre, 1962).

The supernatant was transferred to extraction tubes containing the radioactive labeled standards. The absorption of the tissue catecholamines on Al_2O_3 followed by their elution and fluorometric determinations were performed in the same manner as the steps used for the plasma catecholamines. The same fluorometric settings were used with the exception that the meter multiplier was set at 0.03 and 0.01 for epinephrine and norepinephrine respectively. The epinephrine and norepinephrine standards used for the tissue catecholamine determinations contained zero, one, two, four, and six μg.

The amount of epinephrine and norepinephrine found in the adrenal tissue was determined using the following formula.

$$\begin{matrix}\text{Epinephrine } (\mu g/mg) \\ \text{or} \\ \text{Norepinephrine } (\mu g/mg)\end{matrix} = \frac{C}{Sd} \times \frac{S}{A} \times \frac{VH}{AH} \times \frac{1}{W} \times \frac{100}{R}$$

where:

C = concentration of standard (μg/ml)

Sd = reading of standard (fluorescence units)

S = reading of sample (fluorescence units)

A = sample aliquot of eluate taken for reading (ml)

VH = volume of adrenal homogenate (ml)

AH = volume of adrenal homogenate taken for analysis (ml)

W = weight of adrenal (mg)

R = % Recovery

Scintillation Counting

All samples were either counted on a two channel Packard Tri-Carb Scintillation Spectrometer (Series 314E) or a three channel Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3003) equipped with a ^{137}Cs automatic external standard device.

Scintillation Liquids. Bray's (1960) solution, which was used for determination of percent recovery of catecholamines, was made up of the following: 1) 60 g napthalene; 2) 4 g 2,5-diphenyloxalate (PPO); 3) 0.2 g 1,4-bis-2-(5 phenyloxasolyl)-benzene (POPOP); 4) 100 ml methanol; 5) 10 ml ethylene glycol; 6) enough p-dioxane to give a total of one liter. The scintillation mixture, used for the determination of the percent recovery of corticosterone and the enzyme activity, was one liter of toulene containing 0.0505 g POPOP and 5.05 g PPO. The scintillation mixtures were stored in dark bottles in the cold and used within 30 days of their preparation.

Single Label Counting. The determination of percent recovery for corticosterone was done on the two channel Packard spectrometer which had efficiences of 79% and 31% for ^{14}C and ^{3}H respectively. Ratio of background cpm to sample cpm fell within the range of 0.008 to 0.03. Quenching was minimal and uniform among samples; thus, no correction was

made for it in the calculations. Each sample was counted three periods of 10 minutes each.

A Packard three-channel spectrometer which operated at 71% efficiency for ^{14}C was used to measure the activity of metanephrine-^{14}C which was formed in the enzyme assay. Each sample was counted ten periods of 10 minutes each. The level of quenching was low, but varied from sample to sample; therefore, a quench correction was made utilizing the automatic external standardization method with ^{137}Cs as a radiation source.

Double Label Counting. Double labeled samples in this study contained DL-epinephrine-7-^{3}H and DL-norephinephrine-7-^{14}C dissolved in Bray's solution. A Packard three channel spectrometer which operated at efficiences of 26% for ^{3}H and 60% for ^{14}C with double label settings was used. Each sample was counted three periods of 20 minutes each. The level of quenching was corrected by use of the automatic external standardization procedure.

Statistical Analysis of Data

Data were subjected to analysis of variance (Snedecor and Cochran, 1967). When analysis of variance revealed a significant variation due to treatment, the differences were detected by Duncan's New Multiple Range Test (Steel and Torrie, 1960).

CHAPTER IV

RESULTS

Experiment I

The objectives of the first experiment were 1) to determine if immobilization is effective in provoking a variety of responses previously associated with stress and ACTH release and 2) to measure these responses quantitatively in the chicken.

The responses which were measured were 1) acidophilia, 2) corticosterone levels in plasma and adrenal tissue, 3) epinephrine and norepinephrine concentrations in plasma and adrenal tissue, and 4) phenylethanolamine-N-methyl transferase (PNMT) activity in adrenal tissue.

There were three groups of birds included in Experiment I as follows:

1. The normal, control group which was given neither ACTH nor immobilized for three hours.
2. The ACTH group which was given one IU of ACTH per kg of body weight (ACTHAR, Armour Pharmaceutical Co.) one hour prior to autopsy.
3. The restrained group which was immobilized for three hours prior to autopsy.

All birds were handled the same number of times, but only the restrained group was immobilized for the stated period. All the birds

were injected the same number of times, but only the ACTH-treated animals were given ACTH, whereas the others were given the ACTH vehicle, water.

Acidophilia

Table I presents the mean values for the number of acidophils/0.09 mm^3 of blood for Experiment I.

A pronounced acidophilia occurred in both the restrained birds and the ACTH-treated birds as compared to the controls ($P<0.01$ in each comparison). The nonstressed control group had an average acidophil count of only 13.9/0.09 mm^3 of blood, whereas the numbers of acidophils for the groups given either ACTH or restrained were 26.3 and 36.2 respectively. The value obtained for the restrained group proved to be significantly larger ($P<0.01$) than that for the ACTH group.

Corticosterone

The results from the quantitative analyses of corticosterone in plasma and adrenal tissue are presented in Table II. Corticosterone was chosen for analysis because it is the predominate adrenal corticosteroid in birds (Frankel, 1970).

The purity and structure of the corticosterone isolated from approximately 200 ml of avian plasma was established by comparing its mass spectrum with that of authentic corticosterone. Figure 1 shows the mass spectrum of authentic corticosterone and Figure 2 shows the mass spectrum of the compound isolated from chicken plasma.

Plasma Corticosterone. Table II shows that the plasma corticosterone levels significantly increased ($P<0.01$) after the administration

TABLE I

THE NUMBER[1] OF ACIDOPHILS FOLLOWING ACTH TREATMENT OR IMMOBILIZATION

Group	Treatment	Acidophil Count	±S.E.[2]
I	Control	13.9	0.9
II	ACTH	26.3 (.01)[3]*	2.1
III	Restraint	36.2 (.01)*+	2.7

[1]Number of acidophils per 0.09 mm^3 blood.

[2]Standard error of the mean.

[3]Number in parentheses indicates value of P.

*Compared to control group (I).

+Compared to ACTH group (II).

TABLE II

ADRENAL AND PLASMA CONCENTRATIONS OF CORTICOSTERONE FOLLOWING ACTH TREATMENT OR IMMOBILIZATION

Group	Treatment	Adrenal Corticosterone μg/gm	Plasma Corticosterone μg/100 ml
I	Control	3.99 ±.62[1]	2.06 ±.43
II	ACTH	4.52 ±.60	4.37 ±.58 (.01)[2]*
III	Restraint	4.78 ±.62	5.04 ±.60 (.01)*

[1]Standard error of the mean.

[2]Number in parentheses indicates P value.

*Compared to control group (I).

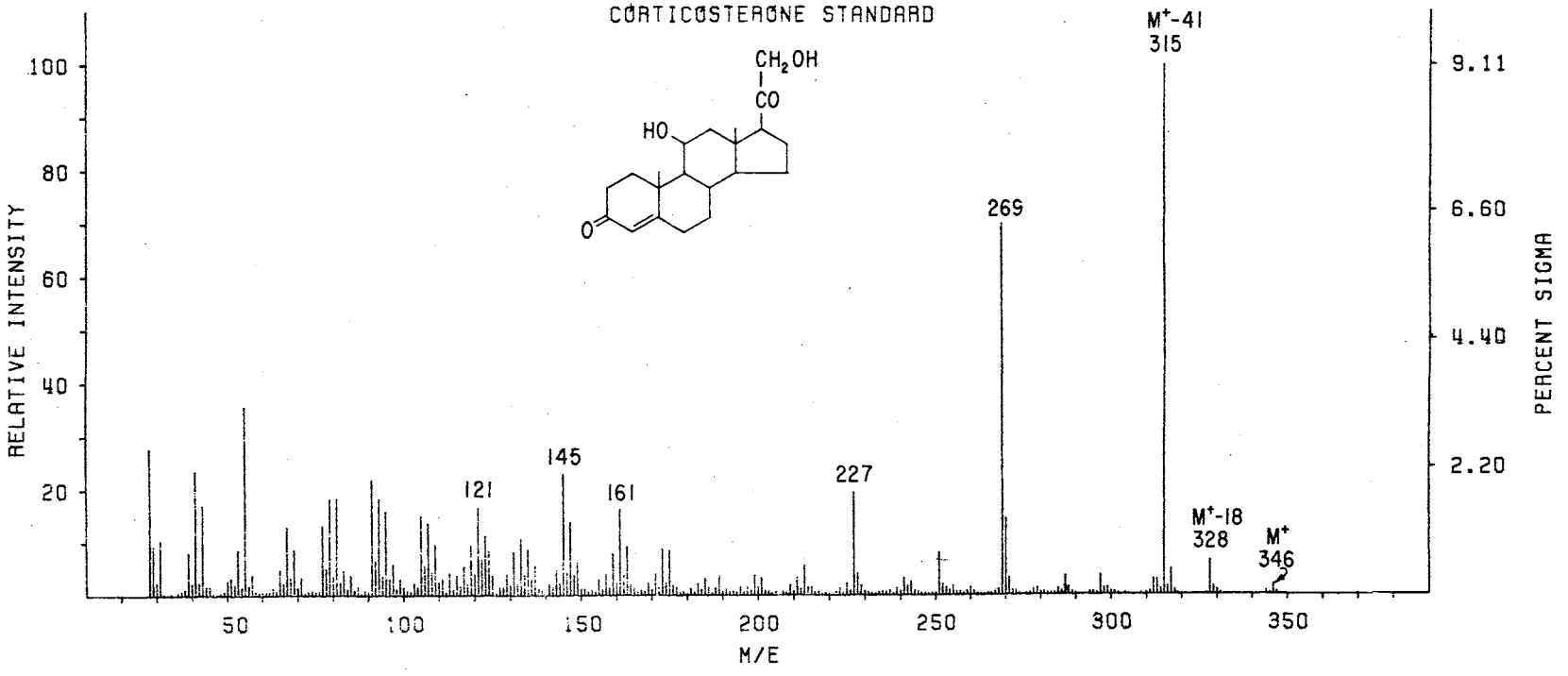

Figure 1. Mass Spectrum of Standard Corticosterone.

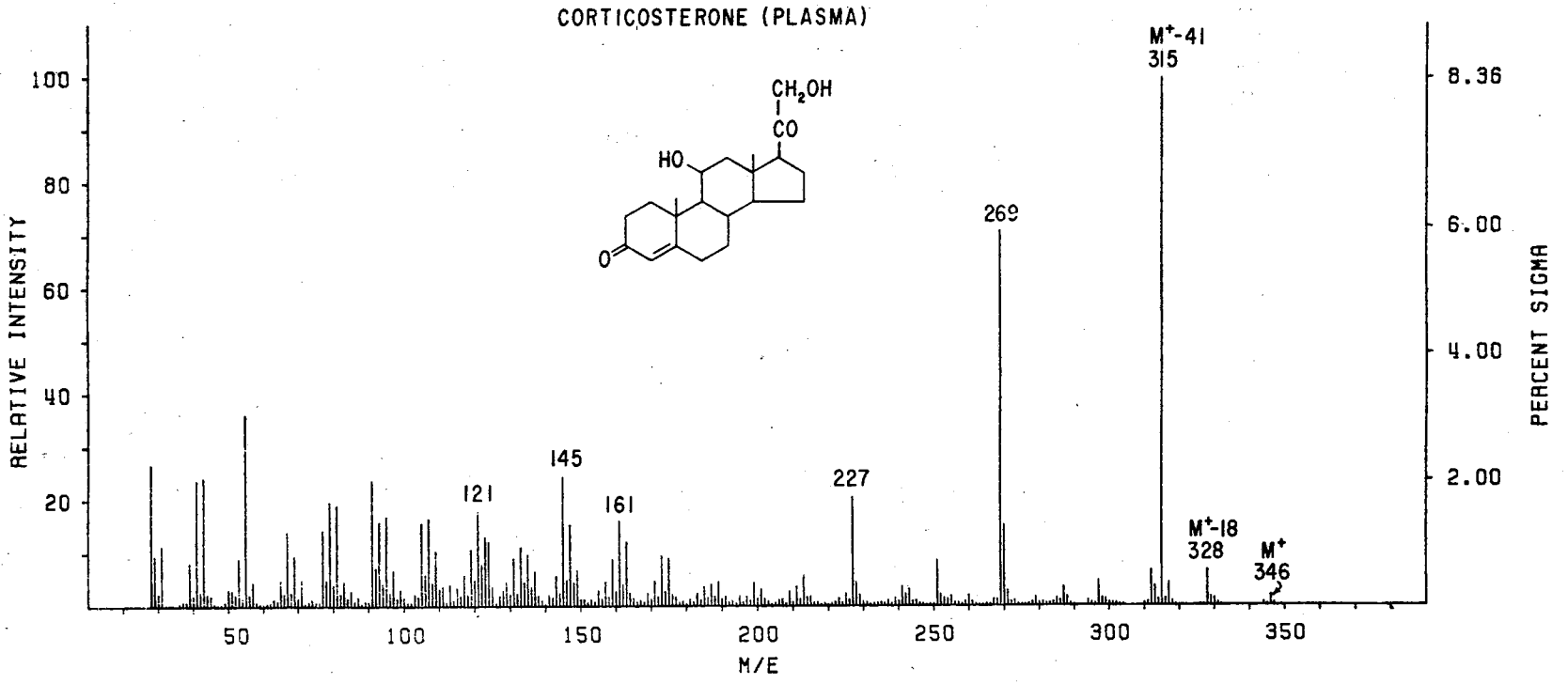

Figure 2. Mass Spectrum of Corticosterone Isolated from the Peripheral Plasma of White Leghorn Cockerels.

of ACTH or restraint as compared to those of the control animals. The mean value of plasma corticosterone for the control group was 2.06 μg/100 ml plasma. With the application of immobilization or ACTH, the plasma levels rose to 5.04 μg/100 ml and 4.37 μg/100 ml respectively.

Apparently the dosage of ACTH administered and the period of restraint used were equally effective in provoking the increase in plasma corticosterone concentration since there was no statistical difference ($P>0.4$) between these two experimental groups.

Adrenal Corticosterone. Table II also presents the mean values for the adrenal levels of corticosterone in the three groups.

There was no detectable difference ($P>0.2$) in the adrenal concentrations of corticosterone between any of the three groups. The values for the control group, the ACTH group and the restrained group were 3.99, 4.52, and 4.78 μg/gram of adrenal tissue respectively.

Epinephrine and Norepinephrine

Table III presents the levels of epinephrine and norepinephrine in both plasma and adrenals of the three groups in the first experiment.

Plasma Epinephrine and Norepinephrine. It can be seen that the levels of these two catecholamines increased significantly when given ACTH or immobilized for three hours. The control value for epinephrine in the plasma was 3.38 μg/liter, whereas the mean epinephrine values for the ACTH group and the restrained group were 16.91 and 17.92 μg/liter respectively. Similar results were obtained for the plasma concentration of norepinephrine. The plasma level of norepinephrine increased from a mean value of 1.23 μg/liter for the control group to values of 5.96

TABLE III

ADRENAL AND PLASMA CONCENTRATIONS OF EPINEPHRINE (E) AND NOREPINEPHRINE (NE) FOLLOWING ACTH TREATMENT OR IMMOBILIZATION

Group and Treatment	Adrenal Concentration			Plasma Concentration		
	E μg/100 mg	NE μg/100 mg	E/NE	E μg/liter	NE μg/liter	E/NE
I Control	174.22 ±11.22[1]	82.44 ±7.28	2.18 ±.10	3.38 ±.68	1.23 ±.25	2.65 ±.27
II ACTH	141.74 ±4.80 (.05)[2]*	60.90 ±4.45 (.05)*	2.38 ±.12	16.91 ±2.03 (.01)*	5.96 ±.40 (.01)*	2.62 ±.10
III Restraint	134.36 ±8.07 (.05)*	65.01 ±5.12 (.05)*	2.15 ±.16	17.92 ±1.15 (.01)*	6.21 ±.49 (.01)*	2.94 ±.13

[1]Standard error of the mean.

[2]Number in parentheses indicates the P value.

*Compared to control group (I).

μg/liter and 6.21 μg/liter for the ACTH group and the restrained group respectively.

Table III also presents the ratios of these two catecholamines in plasma. Plasma norepinephrine was obviously in smaller concentrations than epinephrine in the chicken. The ratio of epinephrine to norepinephrine in the control group had a mean value of 2.65. The administration of ACTH or restraint did not statistically alter this ratio. ($P>0.2$).

Adrenal Epinephrine and Norepinephrine. Accompanying the increases in plasma levels of epinephrine and norepinephrine, there were decreases in the adrenal levels of these two catecholamines. The application of restraint or ACTH resulted in adrenal levels of 70-80% of the normal, control values for epinephrine and norepinephrine. As was found for the plasma, the ratio of epinephrine to norepinephrine in the adrenal did not significantly change ($P>0.2$) in the two experimental groups when compared to the control ratio. The adrenal ratio of epinephrine to norepinephrine was 2.18 in the control group.

PNMT Activity

The effects of ACTH administration or immobilization on the adrenal PNMT activity are presented in Table IV. The activity of this enzyme (Units) represents the number of mμmoles of ^{14}C-metanephrine formed per hour of incubation per 100 mg of avian adrenal tissue.

The control group had a mean value of 1.495 Units. With the administration of ACTH, the activity significantly rose ($P<0.01$) to a value of 2.441 Units. Three hours of restraint was also effective in significantly increasing ($P<0.01$) the PNMT activity as compared to that

TABLE IV

ADRENAL PNMT ACTIVITY FOLLOWING ACTH TREATMENT OR IMMOBILIZATION

Group	Treatment	PNMT Units[1]	±S.E.[2]
I	Control	1.495	±.186
II	ACTH	2.441 (.01)[3]*	±.174
III	Restraint	2.175 (.01)*	±.137

[1]Units of activity represent mμmoles of product formed/hour/100 mg adrenal.

[2]Standard error of the mean.

[3]Number in parentheses indicates P value.

*Compared to control group (I).

of the controls. There was no detectable difference between the activities of PNMT in the ACTH group and the restrained group.

Experiment II

With the elevations of plasma corticosterone and catecholamines (epinephrine and norepinephrine), it was obvious from Experiment I that immobilization was effective in activating both the adrenal medullary and cortical cells of the avian adrenal. By using immobilization as a stressing agent, the objective of Experiment II was to see if a physiological interrelationship of these two adrenal tissues could be shown under the condition of stress. It was desired to see what change or changes would occur in the stress response if the production of corticosterone was blocked. Metopirone (2-methyl-1,2 bis (3'-pyridyl)-1-propanone), obtained from CIBA Pharmaceutical Co., was the agent used to inhibit the synthesis of corticosterone.

There were four groups included in Experiment II as follows:

1. Group I was the normal control group.
2. Group II was the restrained group; animals in this group were immobilized three hours prior to autopsy.
3. Group III was the Metopirone control group; animals in this group were injected IP with 20 mg Metopirone per kg body weight four hours prior to autopsy.
4. Group IV was the experimental Metopirone group; animals in this group were given 20 mg Metopirone per kg body weight one hour prior to the three-hour period of immobilization.

The controls and restrained controls were injected with only the Metopirone vehicle which was propylene glycol. All animals were handled

in the same fashion.

Acidophilia

Table V presents the results from the acidophil count for Experiment II. The control value was 13/0.09 mm^3 blood and this was statistically equal ($P>0.4$) to that of the Metopirone control group which had a mean value of 17/0.09 mm^3 blood. Obviously the drug alone has no direct effect upon the number of acidophils.

As was found in Experiment I, three hours of immobilization resulted in a marked increase in the number of acidophils per 0.09 mm^3 blood as compared to the mean number in the controls ($P<0.01$). The mean value obtained for the acidophil count in the restrained controls was 34.2/0.09 mm^3 blood.

It can be seen from the results for Group IV (Metopirone plus restraint) that the administration of Metopirone did not block the acidophilia occurring with immobilization. There was no detectable difference ($P>0.5$) between the counts for the restrained control group and the restrained group given Metopirone.

Plasma Corticosterone

The results from the analyses of corticosterone in the plasma are shown in Table VI. Metopirone was apparently quite effective in blocking the production of corticosterone as indicated by the low levels of this steroid in the drug-treated groups. There were some animals in the two Metopirone-treated groups which had undetectable amounts of corticosterone in the plasma (see Appendix B). The plasma corticosterone concentration of the Metopirone control group was only 36% of that for the normal

TABLE V

THE NUMBER[1] OF ACIDOPHILS FOLLOWING METOPIRONE TREATMENT AND/OR IMMOBILIZATION

Group	Treatment	Acidophil Count	±S.E.[2]
I	Control	13.0	±1.2
II	Restraint	34.2 (.01)[3]*	±2.4
III	Metopirone	17.0	±3.1
IV	Metopirone + Restraint	36.7 (.01)*	±4.7

[1]Number of acidophils per 0.09 mm^3 blood.

[2]Standard error of the mean.

[3]Number in parentheses indicates the value of P.

*Compared to normal control group (I) or Metopirone control group (III).

TABLE VI

PLASMA CONCENTRATIONS OF CORTICOSTERONE FOLLOWING METOPIRONE TREATMENT AND/OR IMMOBILIZATION

Group	Treatment	Corticosterone μg/100 ml	±S.E.[1]
I	Control	2.40	±.32
II	Restraint	5.24 (.01)[2]*	±.61
III	Metopirone	0.86 (.01)*+	±.32
IV	Metopirone + Restraint	0.96 (.01)*+	±.31

[1]Standard error of the mean.

[2]Number in parentheses indicates P value.

*Compared to normal control group (I).

+Compared to restrained control group (II).

controls.

Whereas immobilization resulted in increased levels of plasma corticosterone, immobilization following Metopirone-treatment did not. In fact, the plasma concentration of corticosterone for the group given Metopirone and restrained was significantly smaller ($P<0.01$) than the mean value for the normal controls. In addition, there was no detectable difference ($P>0.5$) between the corticosterone mean value (0.86 μg/100 ml plasma) for the Metopirone control group and the corticosterone value, (0.96 μg/100 ml plasma) for the restrained group given Metopirone.

It was noted in the chromatographic separation of corticosterone that a very distinct spot, with a R_f value less than that for corticosterone, appeared on each of the lanes which had samples from the Metopirone-treated birds, but not on any of the other lanes. On further investigation, it was found that the unknown spots had R_f values corresponding to those of authenic 11-desoxycorticosterone in several solvent systems.

Epinephrine and Norepinephrine

Table VII presents the data from the quantitative analyses of norepinephrine and epinephrine in both the plasma and adrenal tissue for Experiment II.

As was found in Experiment I, immobilization resulted in elevated plasma levels of epinephrine and norepinephrine with an accompanying decrease in their adrenal levels. When compared to the normal controls, the plasma concentrations of epinephrine and norepinephrine for the restrained controls increased 300% and 283% respectively, while their adrenal levels decreased 49% and 43% respectively. Metopirone alone

TABLE VII

ADRENAL AND PLASMA CONCENTRATIONS OF EPINEPHRINE (E) AND NOREPINEPHRINE (NE) FOLLOWING METOPIRONE TREATMENT AND/OR IMMOBILIZATION

Group and Treatment	Adrenal Concentration			Plasma Concentration		
	E μg/100 mg	NE μg/100 mg	E/NE	E μg/liter	NE μg/liter	E/NE
I Control	215.64 ±23.73[1]	93.42 ±11.40	2.34 ±.11	3.65 ±.82	1.20 ±.25	3.06 ±.29
II Restraint	111.21 ±17.28 (.05)[2]*	53.00 ±7.91 (.05)*	2.13 ±.21	14.59 ±1.66 (.01)*	4.60 ±.54 (.01)*	3.24 ±.33
III Metopirone	180.90 ±22.84	94.47 ±15.46	1.99 ±.12	3.73 ±.70	1.33 ±.29	2.99 ±.24
IV Metopirone + Restraint	67.43 ±11.30 (.05)*+	48.50 ±3.74 (.01)*	1.38 ±.17 (.05)*+	8.17 ±1.32 (.01)*+	4.90 ±.40 (.01)*	1.63 ±.16 (.01)*+

[1]Standard error of the mean.

[2]Number in parentheses indicates the P value.

*Compared to normal control group (I) or Metopirone control group (III).

+Compared to restrained control group (II).

apparently did not affect the plasma or adrenal levels of these catecholamines as evidenced by the statistical insignificance ($P>0.2$) of the differences between the values for the normal controls and the Metopirone controls.

In contrast, Metopirone administration in conjunction with restraint did have some noticeable effects on catecholamine levels in the plasma and the adrenal. In the group given Metopirone and restrained, there was an increase in both plasma epinephrine and norepinephrine following immobilization. Whereas the plasma norepinephrine levels for the restrained controls and for the restrained given Metopirone were statistically equal ($P>0.5$), the plasma epinephrine concentrations of these two groups were significantly different ($P<0.01$). Plasma epinephrine in the restrained group given Metopirone had a mean value of 8.17 μg/liter plasma which has significantly higher ($P<0.01$) than the normal controls' value of 3.65 μg/liter, but which was also signficantly smaller than the restrained controls' value of 14.59 μg/liter. The differential effect that Metopirone-treatment had upon the plasma concentrations of epinephrine and norepinephrine is further reflected in the plasma ratio of these two catecholamines. It can be found in Table VII that the ratios of plasma epinephrine:norepinephrine for Groups I, II, and III had a range of 2.99-3.24, whereas the plasma ratio declined to 1.63 for the restrained group given Metopirone. Thus, Metopirone administration resulted in a higher proportion of norepinephrine being secreted into the plasma under the condition of stress.

The adrenal levels of epinephrine and norepinephrine showed similar responses to Metopirone-treatment. The decrease in concentration of adrenal epinephrine occurring with immobilization was even greater in the

Metopirone-treated group. Whereas the adrenal level of epinephrine fell to 111.21 μg/100 mg adrenal tissue in the restrained controls, it diminished to 67.43 μg/100 mg adrenal tissue in the restrained group given Metopirone. The adrenal norepinephrine concentration of 53.0 μg/100 mg adrenal tissue for the restrained controls was not noticeably different ($P>0.5$) than that of 48.5 μg/100 mg for the immobilized group given Metopirone. Again, the dissimilarity of the two catecholamine's responses to restraint with Metopirone pretreatment is reflected in their adrenal ratios. The ratios of adrenal epinephrine:norepinephrine for Groups I, II, and III had a range of 1.99-2.34, but the ratio decreased to 1.38 in the immobilized animals pretreated with Metopirone. It appears that the group given Metopirone has less depletion of norepinephrine than epinephrine with immobilization. It was also found that there was an observable difference between the ratios of the adrenal catecholamines for the normal control group and the Metopirone control group, but this difference did not prove to be statistically significant at the 0.05 level.

PNMT Activity

Table VIII summarizes the data obtained in Experiment II for the assay of PNMT activity in the adrenals.

As was noted in Experiment I, three hours of immobilization resulted in a significant increase in PNMT activity as compared to that of the normal controls ($P<0.05$). The restrained controls had a mean value of 3.010 Units of PNMT activity versus 1.666 Units in the normal controls.

In contrast, immobilization preceded by Metopirone-treatment did not lead to an increase in PNMT activity. The values for the PNMT

TABLE VIII

ADRENAL PNMT ACTIVITY FOLLOWING METOPIRONE TREATMENT AND/OR IMMOBILIZATION

Group	Treatment	PNMT Units[1]	±S.E.[2]
I	Control	1.666	±.186
II	Restraint	3.010 (.05)[3]*	±.382
III	Metopirone	1.734	±.250
IV	Metopirone + Restraint	1.563 (.01)+	±.237

[1]Units of activity represent mμmoles product formed/hour/100 mg adrenal.

[2]Standard error of the mean.

[3]Number in parentheses indicates P value.

*Compared to normal control group (I) or Metopirone control group (III).

+Compared to restrained control group (II).

activities in the normal control group, the Metopirone control group, and the restrained plus Metopirone group were statistically equal ($P>0.5$)

Experiment III

The purpose of Experiment III was to determine if the elevated PNMT activity occurring with immobilization was by enzyme activation or by enzyme induction (synthesis). Puromycin, an inhibitor of protein synthesis, was employed in this experiment in conjunction with immobilization. Puromycin (Nutritional Biochemical Corp.) was given to the cockerels IP at a dosage of 35 mg per kg of body weight.

There were five groups included in Experiment III as follows:

1. Group I was the normal control group.
2. Group II was the restrained control group; animals in this group were immobilized for three hours prior to autopsy.
3. Group III was the puromycin control group; animals in this group were given puromycin six hours prior to autopsy.
4. Group IV was the puromycin experimental group; animals in this group were given puromycin three hours prior to the three-hour restraint period.
5. Group V was the corticosterone plus puromycin experimental group; animals in this group were given puromycin three hours prior to immobilization and 4 mg/kg body weight of corticosterone one-half hour prior to immobilization.

All animals were handled in the same fashion.

Acidophilia

Table IX summarizes the data obtained from the acidophil counts in Experiment III. As in the two previous experiments, the acidophil count rose markedly ($P<0.01$) with immobilization. The normal controls had a mean value of 14.4/0.09 mm^3 blood, whereas after three hours of restraint the count increased to 39.9/0.09 mm^3 blood.

Pretreatment with puromycin did not have any effect on the acidophil count in the puromycin controls; the mean value of the puromycin controls was no different than that of the normal controls ($P>0.1$).

Puromycin apparently did inhibit the acidophilia associated with immobilization. It was found that the mean value of 15.5/0.09 mm^3 blood for the group given puromycin prior to immobilization was not significantly different ($P>0.2$) from either that of the normal controls or that of the puromycin controls.

Group V, which was given puromycin and corticosterone prior to restraint, also did not show any acidophilia with immobilization. The mean value for Group V was 14.0/0.09 mm^3 blood.

Plasma Corticosterone

The data obtained from the measurement of plasma corticosterone in Experiment III are presented in Table X.

Immobilization alone led to a 169% increase in the concentration of plasma corticosterone as compared to that of the normal controls. On the contrary, the increased plasma corticosterone accompanying restraint was abolished with the pretreatment of puromycin. The mean value for the restrained group given puromycin was even smaller than the level

TABLE IX

THE NUMBER[1] OF ACIDOPHILS FOLLOWING PUROMYCIN TREATMENT AND/OR IMMOBILIZATION

Group	Treatment	Acidophil Count	±S.E.[2]
I	Control	14.4	±1.2
II	Restraint	39.9 (.01)[3]*	±2.7
III	Puromycin	17.5	±1.4
IV	Puromycin + Restraint	15.5 (.01)+	±1.5
V	Puromycin + Corticosterone + Restraint	14.0 (.01)+	±1.8

[1]Number of acidophils per 0.09 mm^3 blood.

[2]Standard error of the mean.

[3]Number in parentheses indicates P value.

*Compared to normal control group (I) or puromycin control group (III).

+Compared to restrained control group (II).

TABLE X

PLASMA CONCENTRATIONS OF CORTICOSTERONE FOLLOWING PUROMYCIN TREATMENT AND/OR IMMOBILIZATION

Group	Treatment	Corticosterone μg/100 ml	±S.E.[1]
I	Control	2.45	±.39
II	Restraint	6.59 (.01)[2]*	±.40
III	Puromycin	2.11	±.21
IV	Puromycin + Restraint	1.96 (.01)+	±.35
V	Puromycin + Corticosterone + Restraint	20.20 (.01)*+	±1.79

[1]Standard error of the mean.

[2]Number in parentheses indicates P value.

*Compared to normal control group (I) or puromycin control group (III).

+Compared to restrained control group (II).

found in the normal control group, but this difference did not prove to be statistically significant ($P>0.2$).

Group V which was given corticosterone prior to immobilization had a very large amount of corticosterone remaining in the peripheral blood at the time of autopsy. The mean value of plasma corticosterone for this group was 20.20 μg/100 ml plasma.

Epinephrine and Norepinephrine

Values obtained for epinephrine and norepinephrine levels in plasma and tissue are summarized in Table XI.

Immobilization led to a marked increase ($P<0.01$) in the plasma levels of both epinephrine and norepinephrine with an accompanying decrease ($P<0.01$) in their adrenal levels as compared to the normal controls' levels. Puromycin did not significantly alter ($P>0.1$) the concentrations of either epinephrine or norepinephrine in the plasma or adrenals as compared to those levels in the normal control group.

When immobilization was preceded by puromycin (Group IV), the epinephrine and norepinephrine levels in plasma also rose significantly ($P<0.05$) as compared to those of the normal controls, but not to such an extent as was found in the restrained controls. The mean value of plasma epinephrine of 10.72 μg/liter for Group IV was significantly larger ($P<0.05$) than the values for the normal controls and the puromycin controls, but it was also significantly smaller ($P<0.05$) than the value for the restrained controls. Similarily, the mean value obtained for the plasma concentration of norepinephrine in Group IV was both significantly smaller ($P<0.05$) than the restrained controls' and larger ($P<0.05$) than the normal or puromycin controls.

TABLE XI

ADRENAL AND PLASMA CONCENTRATIONS OF EPINEPHRINE (E) AND NOREPINEPHRINE (NE) FOLLOWING PUROMYCIN TREATMENT AND/OR IMMOBILIZATION

Group and Treatment	Adrenal Concentration E μg/100 mg	Adrenal Concentration NE μg/100 mg	Adrenal Concentration E/NE	Plasma Concentration E μg/liter	Plasma Concentration NE μg/liter	Plasma Concentration E/NE
I Control	220.04 ±18.66[1]	101.57 ±10.09	2.19 ±.07	3.56 ±.46	1.32 ±.16	2.75 ±.18
II Restraint	122.02 ±9.92 (.01)[2]*	55.96 ±4.38 (.01)*	2.18 ±.03	15.21 ±1.18 (.01)*	5.48 ±.54 (.01)*	2.86 ±.13
III Puromycin	224.21 ±20.14	102.06 ±10.01	2.21 ±.10	4.13 ±.20	1.65 ±.13	2.55 ±.13
IV Puromycin + Restraint	96.61 ±11.27 (.01)*	42.01 ±4.68 (.01)*	2.32 ±.09	10.72 ±.77 (.05)*+	4.06 ±.36 (.05)*+	2.68 ±.17
V Puromycin + Corticosterone	94.94 ±8.61 (.01)*	46.15 ±6.10 (.01)*	2.14 ±.12	8.10 ±1.31 (.01)*+	3.02 ±.78 (.01)*+	2.79 ±.22

[1] ± standard error of the mean.

[2] Number in parentheses indicates the P value.

*Compared to the control group (I) or the puromycin control group (III).

+Compared to restrained control group (II).

Immobilization alone resulted in adrenal epinephrine and norepinephrine values of 122.02 μg/100 mg and 55.96 μg/100 mg respectively. Immobilization preceded by puromycin-treatment resulted in adrenal epinephrine and norepinephrine concentrations of 96.61 μg/100 mg and 42.01 μg/100 mg respectively. The differences between the values for the restrained group given puromycin and the restrained controls did not prove to be significant at the 0.05 level.

Large doses of corticosterone (Group V) did not overcome the effects that puromycin had upon the plasma and adrenal levels of epinephrine and norepinephrine. The values of the plasma and adrenal catecholamines for Group IV were not detectably different ($P>0.2$) from those of Group V.

PNMT Activity

Table XII presents the results of the PNMT assay in Experiment III. It can be seen by comparing the PNMT activity of the normal control group with that of the puromycin control group that puromycin alone did not alter ($P>0.4$) the enzyme activity.

As in the two previous experiments, there was a significant increase in PNMT activity following three hours of restraint when compared to the normal controls ($P<0.05$). The mean values for the PNMT activities of the restrained controls and the normal controls were 3.019 Units and 1.707 Units respectively.

This increase in activity occurring with restraint was abolished when immobilization was preceded with puromycin administration. The restrained group given puromycin had a mean value of 1.861 Units for the enzyme activity which was not significantly different ($P>0.5$) from the values for the normal or puromycin control groups.

TABLE XII

ADRENAL PNMT ACTIVITY FOLLOWING PUROMYCIN TREATMENT AND/OR IMMOBILIZATION

Group	Treatment	PNMT Units[1]	±S.E.[2]
I	Control	1.707	±.201
II	Restraint	3.019 (.05)[3]*	±.244
III	Puromycin	1.986	±.288
IV	Puromycin + Restraint	1.861 (.01)+	±.227
V	Puromycin + Corticosterone + Restraint	1.672 (.01)+	±.169

[1]Units of activity represent mμmoles product formed/hour/100 mg adrenal.

[2]Standard error of the mean.

[3]Number in parentheses indicates the P value.

*Compared to normal control group (I) or puromycin control group (III).

+Compared to restrained control group (II).

Large levels of circulating corticosterone apparently were unable to overcome the effect of puromycin on PNMT activity. Group V which was given puromycin and corticosterone prior to immobilization had a mean value of 1.672 Units for its PNMT activity which was statistically equal ($P>0.5$) to that of Group IV.

CHAPTER V

DISCUSSION

Introduction

By using a well-established criterion of stress in birds (acidophilia), it was found that three hours of immobilization was effective as a stressing agent. Furthermore, it was found from Experiment I that the changes occurring with immobilization paralleled those occurring with high levels of circulating ACTH. The major hormones of the avian adrenal cortical and medullary cells were quantitatively measured to assess comprehensively the response of the avian adrenal to stressors.

Evidence which has been collected from the mammalian class has suggested that the adrenal cortex influences the synthesis of epinephrine in the adrenal medulla. The possibility of a functional interrelationship of the cortical and medullary tissues of the avian adrenal has also been investigated in the present study.

Acidophilia

The present study found that a marked acidophilia occurred after immobilization or ACTH injections (Table I). These findings confirm the data reported by earlier investigators that various nonspecific stressors result in acidophilia in birds (Newcomer, 1957; 1958; Wolford and Ringer, 1962; Burton and Smith, 1965; Burton et al., 1967; Besch et al., 1967).

Newcomer (1957) reported mean values for the acidophil counts following immobilization to be much higher than those obtained in the present study. From 16-day old birds he obtained a mean value of 129.23 acidophils/0.09 mm^3 blood six hours after the initiation of restraint. The reasons for the difference in magnitude between Newcomer's results and those of the present study are unknown, but the following factors may account for some of the difference: 1) difference in the ages of the two groups of birds; 2) difference in the length of time after the treatment (immobilization) began; and 3) difference in the conditions of restraint.

In the present investigation, the chickens were fastened to boards, as were those in Newcomer's study, but they also had their feet bound together so as to prevent injury to themselves or the bird adjacent to them. The feet binding may have provided for less "stressful" conditions.

Siegel (1968) reported that with the administration of ACTH to four-week old cockerels, the heterophil (acidophil) count increased throughout the treatment period; thus, the longer the treatment period, the higher the acidophil count.

Because of the acidophilia which resulted from injections of ACTH or glucocorticoids, the corticosteroids have been strongly implicated as the cause for acidophilia in birds (Wolford and Ringer, 1962; Burton and Guion, 1968). As of yet, no investigator has actually elucidated the physiologic mechanism for acidophilia in birds. From the data presented in Tables V and VI, it appears to this author that acidophilia is not a direct effect of increased plasma corticosterone. Metopirone was effective in lowering the plasma corticosterone in both the unrestrained

and restrained birds, but was unable to prevent acidophilia from occurring with immobilization.

Newcomer (1958) found that hypophysectomy in birds did not abolish the acidophilia accompanying immobilization, but hypophysectomy did reduce the magnitude of acidophilia.

It has been found that the administration of an inhibitor of norepinephrine synthesis (α-meta-methyl-tyrosine) prior to immobilization prevented the acidophilia which normally occurred with immobilization (Unpublished findings; see Appendix C). The administration of α-meta-methyl-tyrosine prior to immobilization also resulted in significantly smaller ($P<0.05$) concentrations of plasma epinephrine and norepinephrine when compared to those of the restrained controls. From these data along with those collected from the Metopirone experiment, it is the opinion of this author that epinephrine and/or norepinephrine may be responsible for the avian acidophilic response. The mechanism by which these catecholamines influence acidophilia is not known at this time.

From Experiment III, it was seen that puromycin was effective in blocking the acidophilia associated with restraint (Table IX). It is not known at this time whether the inhibition was a result of puromycin acting directly upon hemopoietic tissue or whether it was acting indirectly via some unknown substance. It has been shown that another protein inhibitor, chloramphenicol, was effective in blocking proliferating cells such as is found in hemopoietic tissue. Two days of therapy with chloroamphenicol led to a marked reduction in the number of circulating granulocytes (Bush and Lane, 1967). Page (1964) demonstrated that the administration of puromycin prior to the initiation of an inflammation was effective in preventing the lymphocytic response to inflammation.

Corticosterone

Adrenal Corticosterone

From Experiment I (Table II) it was found that the control group had an adrenal concentration of corticosterone of 3.99 μg/g adrenal tissue. This value is within the range of 1-6 μg/g which was reported by Connally (1961) for the control value in chickens. The control birds described by Connally were of the same age and sex, and were handled in the same general manner as those in the present study.

In the present study, it was found that immobilization or ACTH-treatment did not result in a detectable difference ($P>0.2$) in adrenal corticosterone concentrations. Apparently the adrenal level of corticosterone does not serve as a good criterion of stress. It was for this reason that the analysis of adrenal corticosterone was abandoned in Experiments II and III.

It can also be seen in the present study (Table II) that the absolute level of corticosterone in the bird adrenal is small as compared to the circulating level of this steroid. It was found in this study that on the basis of 100 mg for the average weight of a pair of avian adrenals, there is only 0.399 μg of corticosterone in the adrenal tissue of the normal control bird. In reference to human adrenal glands, Symington (1962) stated that in contrast to other endocrine glands, the adrenal cortex does not appear to store its hormones to any appreciable degree. Corticosterone is released into the plasma shortly after its synthesis in the adrenal cortex (Symington, 1962).

Since immobilization in the present study resulted in an increase in the concentration of plasma corticosterone without altering the con-

centration of adrenal corticosterone, it is obvious that immobilization did result in an increased synthesis of this steroid in the bird.

Plasma Corticosterone

The data collected in this study (Table II) confirm earlier findings that the administration of ACTH or the application of a nonspecific stressor results in elevated plasma corticosterone in birds (Urist and Deutsch, 1960; Nagra et al., 1963a; Frankel et al., 1967c; Macchi et al., 1967).

In mammals, nonspecific stressors such as immobilization are thought to cause increased plasma corticosterone by stimulating the release of ACTH from the hypophysis. In contrast, the hypophysis in birds is apparently not an absolute requirement for functioning of the interrenal gland (Frankel, 1970). It has been shown that the hypophysectomized bird responds to stress with acidophilia and elevated levels of plasma corticosterone (Newcomer, 1958; Frankel, 1970). Therefore, it cannot be determined at this time whether the increased levels of corticosterone seen with immobilization in the chicken is entirely a result of increased levels of circulating ACTH or whether other factors are involved.

The data presented in Table VI substantiate findings in the literature that Metopirone is effective in suppressing the output of 11-β-hydroxycorticoids such as corticosterone (Liddle et al., 1958; Nagra et al., 1963; 1965; Bhattacharyya et al., 1967). Nagra and co-workers (1963) noted a 48% decrease in plasma corticosterone one hour after Metopirone injection in the pheasant. In vitro studies have also shown that the production of 11-β-hydroxy steroids was significantly decreased when Metopirone was present in the incubation media (Liddle et at., 1958).

In mammals the anterior pituitary responds to the lowered levels of the 11-β-hydroxy steroids by secreting more ACTH (Cushman et al., 1963). In studies with man and dog, Cushman et al. (1963) noted that plasma ACTH increased significantly with Metopirone-treatment. It was these increased levels of circulating ACTH which probably accounted for the histological changes which were noted in the pigeon adrenal (Bhattacharyya et al., 1967). Bhattacharyya et al. (1967) found that Metopirone administration resulted in extra-vascularization and multiplication of the adrenal cortical cells of the pigeon.

Metopirone appears to be specific for the blockage of the 11-β-hydroxylation step in steroidogenesis (Liddle et al., 1957). In the present study Metopirone-treatment resulted in a noticeable increase of 11-desoxycorticosterone. This statement is based on the observation that spots with R_f values corresponding to authentic 11-desoxycorticosterone appeared on only those lanes of the thin layer chromatography plates which had samples from Metopirone-treated birds. No quantitative measurement of 11-desoxycorticosterone was made in any of the three experiments in the present study. It would have been interesting to see if immobilization had resulted in an increased plasma concentration of 11-desoxycorticosterone when the production of corticosterone was blocked by Metopirone.

It was found in Experiment III (Table X) that pretreatment with puromycin blocked the elevation of plasma corticosterone normally seen with immobilization. Puromycin probably prevented the increase in plasma corticosterone by inhibiting some enzymatic step in the synthesis of this corticosteroid. Kowal (1970) found that puromycin was effective in blocking adrenal steroidogenesis between cholesterol and pregnenolone.

PNMT Activity

Elevation by Immobilization

Throughout this study, it was shown that a single three-hour period of immobilization was effective in elevating the PNMT activity of the chicken adrenal. The PNMT activities measured in the restrained control groups of the various experiments (Tables IV, VIII, and XII) were significantly greater ($P<0.05$) than those activities found in the normal controls. This study on the bird is the first demonstration that the acute application of a stressor (3 hours of immobilization) can result in a rapid increase in the activity of PNMT.

In contrast, many investigators have concluded that an increase in PNMT activity cannot be demonstrated in rats except under conditions of reduced adrenal cortical activity such as occurs with hypophysectomy (Fuller and Hunt, 1967; Leach and Lipscomb, 1969).

Kvetnansky and Mikulaj (1970b) demonstrated that repeated daily periods of immobilization of rats did result in a significant increase in PNMT activity. They were unable to detect an increase in PNMT activity immediately following a single period of immobilization, but they did detect a small rise in activity a few hours after its termination.

Axelrod et al. (1970) have studied the effects of psychosocial interaction on PNMT activity over a long period of time. They found that the rats which had a high level of social interaction (caged with a large number of rats of both sexes) had a marked increase in PNMT activity as compared to the PNMT activities of the controls and of the socially deprived animals.

The increased PNMT activity in rats appears to be important in response to long-term "stress" conditions (Fuller and Hunt, 1967; Ciaranello et al., 1969; Kvetnansky et al., 1970c), whereas the present study indicates that increased PNMT activity in chickens may be important in the acute adrenal stress response. The rapid increase in PNMT activity, occurring with the acute application of stressors in chickens, may provide a means of sustaining an increased output of epinephrine in times of "stress".

The present study also confirms recent findings that PNMT activity could be elevated in the intact bird (Wasserman and Bernard, 1971). Wasserman and Bernard found that injections of corticosterone or dexamethasone into intact birds resulted in a significant increase in PNMT activity in the chicken adrenals. Furthermore the present study indicates that an increase in PNMT activity can be demonstrated in the intact bird by elevating endogenos levels of plasma corticosterone by applying a stressor (immobilization).

Influence by Corticosterone

In contrast to most published studies on the influence of corticoids (corticosterone) on PNMT activity, the present evaluation of this influence was not done by injecting exogenous steroids into the experimental animal. Instead, the endogenous levels of circulating corticosterone were elevated by subjecting the animal to immobilization. By increasing the endogenous levels of corticosterone, this study demonstrates that the increase in PNMT activity seen with increased plasma corticosterone is physiological and not pharmacological.

Another weakness of many of the previous studies is the lack of data on the plasma levels of the corticosteroids. Thus, it cannot be substantiated whether the various stressors or ACTH administration did in fact increase the levels of circulating glucocorticoids. The present study does quantitatively measure plasma corticosterone in each bird included in the study.

It was found that there was a direct correlation between the level of plasma corticosterone and the activity of PNMT in the adrenal. Three hours of immobilization resulted in a significant increase ($P<0.05$) of both plasma corticosterone and PNMT activity. When corticosterone production was blocked by Metopirone, immobilization did not lead to an increase in PNMT activity (Tables VI and VIII).

In birds, it appears that the elevation of PNMT activity by corticosteroids depends upon the presence of an hydroxyl group in the 11-position. It was stated in the section under corticosterone that Metopirone-treatment resulted in an elevation of plasma 11-desoxycorticosterone. The structure of 11-desoxycorticosterone is the same as corticosterone with the exception of the hydroxyl group in the 11-position. In the present study, the increased concentration of plasma 11-desoxycorticosterone appeared to be ineffective in elevating the activity of PNMT. In contrast, Pohorecky and Wurtman (1968) reported that in the rat, the PNMT activity could be elevated equally by hydroxylating various progesterone derivatives at the 11-, 17-, or 21-positions.

Assuming that a negative feedback mechanism is present in birds, the data collected in Experiment II (Table VIII) indicate that the elevated PNMT activity seen with immobilization is not a direct result of an increase in ACTH secretion. There is previous evidence that increased

plasma corticosterone has a negative feedback on ACTH secretion in birds (Frankel, 1970). If there is a negative feedback mechanism in operation, it seems probable that the ACTH levels in the Metopirone-treated birds would be increased. The PNMT activities of the Metopirone control group and the Metopirone plus restraint group were not detectably different ($P>0.5$) from that of the normal control group.

On the contrary, the administration of dexamethasone, a synthetic glucocorticoid, should lead to decreased levels of circulating ACTH if a negative feedback mechanism is operable. It has been found that the administration of dexamethasone to intact chickens resulted in a significant increase in PNMT activity (Unpublished findings - see Appendix C).

The data from Experiment II also suggest that there exists a basal level of enzyme activity which is unaffected by a decrease in circulating levels of corticosterone. Although the levels of plasma corticosterone were significantly diminished ($P<0.01$) in the Metopirone-treated groups, the PNMT activities of these groups were statistically equal ($P>0.5$) to the enzyme activity of the normal control group. Wasserman and Bernard (1971) found that the daily administration of Metopirone or aminoglutethimide (inhibitors of steroid synthesis) for 8-10 days had no effect upon the PNMT activity in chickens.

Since the adrenal gland of birds is capable of functioning to some extent without the hypophysis, it would be of interest in future studies to see if hypophysectomy would result in PNMT activity and if supplementing hypophysectomized birds with ACTH would increase PNMT activity as it does in mammals (Pohorecky and Wurtman, 1971).

Mechanism of PNMT Elevation

It was found in Experiment III that the rise in PNMT activity occurring with immobilization could be prevented by the pretreatment with puromycin (Table XII). There was no detectable difference ($P>0.4$) between the PNMT activities of the puromycin-treated groups and that of the normal control group. Obviously puromycin did not alter existing levels of PNMT activity, but it did block an increase in activity which normally occurred with restraint. Wurtman and Axelrod (1966) found that the administration of puromycin or actinomycin D had no effect upon the basal activity of PNMT in rats, but these drugs were effective in blocking the rise of PNMT activity seen with dexamethasone-treatment.

Since puromycin had been shown to block steroidogenesis (Kowal, 1970), one of the groups (Group V) in Experiment III was given large doses of exogenous corticosterone to insure high circulating levels of this steroid. At the time of autopsy, the concentration of plasma corticosterone in Group V was 20.20 μg/100 ml of plasma, but this high of a level of circulating corticosterone was ineffective in overcoming the inhibition of PNMT activity caused by puromycin-treatment.

The data collected in Experiment III suggest that the mechanism for PNMT elevation is not enzyme activation by corticosterone. Instead, the results indicate that the increased PNMT activity seen with immobilization is a result of *de novo* enzyme synthesis (enzyme induction).

The author fully realizes that this study does not unequivocally prove enzyme induction as the mechanism responsible for the elevation of PNMT activity. To prove enzyme induction, future investigators should try to show actual changes in the concentration of PNMT.

Epinephrine and Norepinephrine

The data collected in the present study confirm earlier investigations which showed that the plasma concentration of epinephrine is greater than norepinephrine in chickens (Lin and Sturkie, 1968; Sturkie, 1970a; Sturkie et al., 1970). The ratio of epinephrine to norepinephrine in plasma of the present study had a range of 2.65-3.06 for the normal control birds.

As discussed earlier, the amount of published literature on the concentrations of catecholamines in birds is small and the data which are available are varied. In the same year, Sturkie (Sturkie et al., 1970; Sturkie, 1970) had two publications on the plasma catecholamine levels in chickens. In eight-week old chickens, he found an epinephrine (E) to norepinephrine (NE) ratio of approximately 4, whereas in 17-month old chickens, he found the E/NE to be approximately 10. No experiments have been performed to see if the variations seen in Sturkie's two papers are due to age differences or some other factors.

The ratio of E/NE concentration in the chicken adrenal obtained in the present study is comparable to that found by Lin and Sturkie (1968). In the present investigation, the adrenal E/NE of the normal control groups had a range of 2.18-2.34 (Tables III, VII, and XI), whereas Lin and Sturkie obtained a ratio of 2.46.

It was found that three hours of immobilization did not significantly alter the E/NE ratios in either the adrenal or the plasma (Tables III, VII, and XI). The application of immobilization resulted in a marked increase ($P<0.05$) in the concentrations of these two catecholamines in the plasma with accompanying decreases in their

adrenal levels. Since the ratios did remain the same, it appears that immobilization is equally effective in evoking the release of epinephrine and norepinephrine from avian adrenal chromaffin cells.

When immobilization was preceded by Metopirone-treatment, there was not an equal release of epinephrine and norepinephrine (Table VII). In the restrained group given Metopirone the plasma epinephrine concentration did significantly increase ($P<0.01$) as compared to that of the normal control but the level was not as great ($P<0.01$) as was found in the restrained controls. Immobilization following Metopirone-treatment also resulted in an even greater depletion ($P<0.05$) of epinephrine from the adrenal than was seen in the restrained control group. It is the opinion of this author that the differences in adrenal and plasma epinephrine concentrations of the restrained control group and the restrained group given Metopirone are due to a difference in the rate of synthesis of this catecholamine. This opinion is based upon the observation that an increase in PNMT activity normally occurring with immobilization was blocked by Metopirone administration. The higher PNMT activity (Table VIII) of the restrained control group as compared to that of the restrained group given Metopirone indicates that the rate of epinephrine synthesis is probably greater than that in the restrained group given Metopirone. Thus the drug-treated group cannot sustain the increased output of epinephrine in response to immobilization to as great an extent as the nondrug-treated group.

In contrast to a difference in epinephrine concentrations, there was no difference ($P>0.5$) in the norepinephrine concentrations in the plasma or adrenals between the restrained control group and the restrained group given Metopirone (Table VII).

The differential effect of Metopirone on the concentrations of epinephrine and norepinephrine in the plasma and adrenal tissue is further emphasized in the ratios of these catecholamines. The ratios indicate that there is a proportionally larger ($P<0.05$) amount of norepinephrine in the restrained group given Metopirone than is found in the other groups.

The data show that in the absence of corticosterone, the levels of epinephrine are affected to a much greater extent than norepinephrine. It appears from the results of the catecholamine determinations that a decrease in corticosterone results in a decrease in the production of epinephrine. On the contrary, diminished levels of corticosterone apparently have very little, if any, effect on norepinephrine synthesis.

In evaluating corticosterone influence on catecholamine synthesis, the present study is the first to demonstrate the differential effect that decreased levels of corticosterone have on catecholamine levels in the plasma and adrenal of any species. The data collected in this study indicate that the site of this influence is the conversion of norepinephrine to epinephrine which is catalyzed by PNMT.

From the puromycin experiment (Table XI), it was found that immobilization following puromycin-treatment led to increased plasma concentrations of both epinephrine and norepinephrine ($P<0.01$) as compared to those of the normal controls and the puromycin controls. The plasma levels were also significantly smaller ($P<0.05$) than those for the restrained controls. Furthermore, the adrenals of the puromycin-treated group appeared to be depleted of catecholamines to a greater extent with immobilization than those of the restrained controls, but this difference did not prove to be statistically significant ($P>0.1$).

These data suggest that puromycin did not inhibit the releasing mechanism of catecholamines from the adrenal, but that it did interfere with the synthesis of epinephrine and norepinephrine. The ratios of E/NE in the plasma and adrenals were the same with or without drug-treatment; thus, puromycin was equally effective in blocking the production of epinephrine and norepinephrine. The administration of large doses of corticosterone did not overcome the effect of puromycin on the catecholamine concentrations.

At this time, it is not known what influence the glucocorticoids may have upon other enzymes involved in catecholamine synthesis. Wurtman and Axelrod (1966) found that glucocorticoids did not stimulate an increase in activity of tyrosine hydroxylase in rats. Mueller et al. (1970) reported that hypophysectomy or adrenal denervation resulted in decreased activity of tyrosine hydroxylase. The administration of ACTH, but not glucocorticoids, prevented the decrease from occurring in hypophysectomized rats. Kvetnansky et al. (1970) found that in hypophysectomized rats, the adrenal tyrosine hydroxylase activity increased with stress, but the PNMT activity did not. It has also been shown that adrenal denervation prevented the elevation of tyrosine hydroxylase activity, but not PNMT activity, from occurring with immobilization (Kvetnansky et al., 1970a).

Based on the observations presented in the previous paragraph, this author suggests that acetylcholine, which is released from the sympathetic preganglionic nerve endings, might be the factor responsible for the elevation of tyrosine hydroxylase activity seen with stress. The observed effects of ACTH on tyrosine hydroxylase activity may be mediated through an increase in sympathetic nerve activity.

Less information is available concerning dopamine-β-hydroxylase, but it has been shown that the activity of this enzyme also decreases with hypophysectomy (Weinshilboum and Axelrod, 1970). Repeated injections of ACTH, but not dexamethasone, elevated this activity after hypophysectomy.

It is conceivable that the regulation of catecholamine synthesis might have several loci and that there may be several different factors responsible for this regulation.

From the data collected in the present study on the avian adrenal, it is concluded that corticosterone does cause an increase in PNMT activity in times of "stress". It is suggested that the minute-to-minute regulation of PNMT activity may be by some mechanism such as end-product inhibition, but that in times of emergency (or adaptation to stressors), the regulatory mechanism is by way of corticosterone (glucocorticoids). Elevated levels of corticosterone thus provide a means of replenishing the adrenal with epinephrine under the condition of stress. Furthermore, it appears that this regulation of PNMT activity by corticosterone is by enzyme induction.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The present study was composed of three experiments in which eight- to nine-week old White Leghorn cockerels were utilized. The immobilization procedure used throughout this study consisted of placing the chickens on their backs and tying them to boards for a period of three hours.

At the end of this period of restraint, 30-35 ml of blood were collected by way of a cardiac puncture. The birds were killed and the adrenals removed.

Subsequently, each blood sample was quantitatively analyzed for corticosterone, epinephrine, and norepinephrine. A blood acidophil count was also determined for each bird. Epinephrine, norepinephrine, and corticosterone were quantitatively measured in the adrenals of each bird. The adrenals were also quantitatively assayed for phenylethanolamine-N-methyl transferase (PNMT) activity.

It was found in Experiment I that the administration of ACTH or immobilization resulted in a significant increase in plasma norepinephrine and epinephrine concentrations with an accompanying decrease in their adrenal levels. ACTH-treatment or immobilization was also effective in significantly increasing plasma corticosterone concentration, adrenal PNMT activity, and blood acidophil count. On the contrary, neither ACTH nor restraint resulted in a detectable change in the concentration of

adrenal corticosterone.

With the administration of Metopirone, it was found in Experiment II that the plasma corticosterone was significantly decreased in both the nonrestrained group and the restrained group. Metopirone-treatment prior to immobilization was also effective in preventing the increase in PNMT activity and acidophilia which normally occurred with immobilization. Immobilization following pretreatment with Metopirone resulted in a plasma epinephrine concentration which was significantly greater than that for the normal control group, but was significantly smaller than that for the restrained control group. The adrenals of the Metopirone group appeared to be depleted of epinephrine to a greater extent than those of the restrained controls after immobilization. On the contrary, the plasma and adrenal concentrations of norepinephrine for the restrained control group and the restrained group given Metopirone were statistically equal.

In Experiment III, it was found that puromycin given prior to immobilization was effective in blocking many of the responses associated with restraint. The acidophil count, PNMT activity, and plasma corticosterone of the restrained group given puromycin (Group IV) were statistically equal to the respective values in the normal control group. The plasma epinephrine and norepinephrine concentrations were significantly elevated in Group IV, but the levels were also significantly smaller than the concentrations in the restrained control group. Other than to elevate the levels of plasma corticosterone, large doses of corticosterone did not alter the effects resulting from puromycin-treatment.

The objectives of this study were 1) to investigate the stress response of the avian adrenal and 2) to investigate the possibility of a functional interrelationship of the cortical and medullary tissues of the avian adrenal.

It is concluded that the avian adrenal responds to a nonspecific stressor (immobilization) or ACTH administration by secreting large amounts of its major hormones (corticosterone, epinephrine, and norepinephrine)into the circulation. The data in this study also indicate that a single application of ACTH or immobilization increases the activity of at least one of the enzymes involved in the synthesis of epinephrine, namely PNMT.

The increased activity of PNMT which occurs with immobilization seems to be a result of increased plasma corticosterone levels. Whereas corticosterone does appear to have an influence on the synthesis of epinephrine, it apparently has little, if any, effect on the synthesis of norepinephrine. Furthermore, the observed elevation of adrenal PNMT activity by corticosterone seems to be by enzyme induction (enzyme synthesis) and not by enzyme activation.

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APPENDIX A

TABLE XIII

COMPOSITION OF RATION

Ingredients	Pounds
Corn, ground yellow	235.7
Milo, ground yellow	417.2
Oats, ground	65.2
Soybean meal (50%)	111.9
Corn Gluten meal (60%)	20.0
Fish meal (60%)	18.9
Alfalfa meal (17%)	22.0
Meat and bone scrap (50%)	24.1
Yeast culture	14.2
Distillers solubles	14.2
Whey, dried	14.2
dl-Methionine	0.6
Dicalcium phosphate	34.3
VMC-60	2.5
Salt	5.0
TOTAL	1000.0

APPENDIX B

TABLE XIV

EXPERIMENT I - NORMAL, CONTROL BIRDS

Animal No.	Acidophil Count	E μg/100 mg	NE μg/100 mg	E/NE Tissue	E μg/liter	NE μg/liter	E/NE Plasma	Bk μg/gm	Bk μg/100 ml	PNMT Units
1	15	165.48	85.43	1.94	4.97	1.64	3.03	3.36	0.80	1.384
2	12	161.20	91.77	1.76	3.74	0.28	3.61	3.08	1.07	1.170
3	18	252.60	126.00	2.00	1.96	0.49	3.97	1.93	1.16	2.080
4	15	169.24	81.47	2.08	8.20	2.32	3.53	3.21	1.13	1.025
5	16	192.96	82.45	2.34	1.74	0.81	2.15	3.91	1.16	2.821
6	13	169.24	67.70	2.50	2.91	1.00	2.91	3.10	2.67	1.262
7	8	142.68	57.05	2.50	2.58	1.55	1.66	5.43	4.96	1.085
8	13	118.68	43.96	2.70	2.83	1.73	1.64	3.93	1.39	1.231
9	16	195.48	102.88	1.90	0.36	0.18	2.00	3.05	3.57	1.883
10	13	174.68	85.63	2.04	4.53	2.31	1.96	8.99	2.66	1.010
Mean	13.9	174.22	82.44	2.18	3.38	1.23	2.65	3.99	2.06	1.495
±S.E.	0.9	11.22	7.28	0.10	0.68	0.25	0.27	0.62	0.43	0.186

TABLE XV

EXPERIMENT I - RESTRAINED BIRDS

Animal No.	Acidophil Count	E μg/100 mg	NE μg/100 mg	E/NE Tissue	E μg/liter	NE μg/liter	E/NE Plasma	Bk μg/gm	Bk μg/100 ml	PNMT Units
1	26	99.04	54.36	1.82	18.25	6.89	2.65	8.45	3.80	1.791
2	28	113.40	31.88	3.56	19.74	8.12	2.43	4.09	5.31	1.555
3	25	125.40	54.08	2.32	20.39	7.03	2.90	4.78	5.68	2.634
4	33	135.68	67.84	2.00	13.08	3.60	3.63	4.80	5.74	2.041
5	47	138.36	70.02	1.98	23.01	7.30	3.15	6.39	2.76	2.620
6	36	187.56	87.44	2.15	20.50	5.82	3.52	2.25	4.08	2.170
7	50	152.28	80.24	1.90	16.59	5.76	2.88	4.41	3.06	1.526
8	35	107.16	55.76	1.92	12.55	4.67	2.69	3.68	4.26	2.615
9	42	317.04	75.60	1.81	14.17	4.71	3.01	2.29	9.03	2.468
10	40	147.68	72.90	2.03	20.91	8.23	2.54	6.67	6.73	2.326
Mean	36.2	134.36	65.01	2.15	17.92	6.21	2.94	4.78	5.04	2.175
±S.E.	2.7	8.07	5.12	0.16	1.15	0.49	0.13	0.61	0.60	0.137

TABLE XVI

EXPERIMENT I - ACTH-TREATED BIRDS

Animal No.	Acidophil Count	E µg/100 mg	NE µg/100 mg	E/NE Tissue	E µg/liter	NE µg/liter	E/NE Plasma	Bk µg/gm	Bk µg/100 ml	PNMT Units
1	18	115.68	41.76	2.77	26.49	5.96	2.50	3.96	6.31	2.225
2	26	150.00	64.65	2.32	12.81	4.27	3.00	5.41	4.23	2.206
3	33	160.92	85.08	1.89	22.55	9.47	2.38	4.63	5.34	3.396
4	30	159.44	69.32	2.30	8.69	4.16	2.09	5.64	6.42	2.810
5	21	317.52	66.36	2.07	9.43	3.83	2.46	2.97	3.93	1.891
6	19	145.76	58.30	2.50	14.89	5.21	2.86	3.77	4.02	2.153
7	34	---	---	---	16.09	5.55	2.90	8.36	3.31	---
8	36	130.60	56.78	2.30	19.79	7.52	2.63	2.48	5.21	2.373
9	21	142.44	63.31	2.25	21.48	7.70	2.79	5.94	6.69	1.892
10	25	133.32	42.64	3.13	---	---	---	2.05	4.96	3.026
Mean	26.3	141.74	60.90	2.39	16.91	5.96	2.62	4.52	4.37	2.441
±S.E.	2.1	4.80	4.45	0.12	2.03	0.40	0.10	0.60	0.58	0.174

TABLE XVII

EXPERIMENT II - NORMAL, CONTROL BIRDS

Animal No.	Acidophil Count	E µg/100 mg	NE µg/100 mg	E/NE Tissue	E µg/liter	NE µg/liter	E/NE Plasma	Bk µg/100 ml	PNMT Units
1	13	300.98	129.67	2.32	4.09	1.49	2.74	3.79	1.565
2	16	209.15	88.23	2.37	4.36	1.93	2.26	2.08	2.448
3	9	126.12	53.67	2.35	6.73	1.65	4.08	2.26	1.933
4	15	188.87	87.31	2.16	2.04	0.82	2.49	1.42	1.209
5	15	225.85	80.39	2.81	3.75	1.02	3.68	2.19	1.516
6	10	242.95	121.23	2.00	0.93	0.30	3.10	2.68	1.326
Mean	13.0	215.64	93.42	2.34	3.65	1.20	3.06	2.40	1.666
±S.E.	1.2	23.73	11.40	0.11	0.82	0.25	0.29	0.32	0.186

TABLE XVIII

EXPERIMENT II - RESTRAINED BIRDS

Animal No.	Acidophil Count	E µg/100 mg	NE µg/100 mg	E/NE Tissue	E µg/liter	NE µg/liter	E/NE Tissue	Bk µg/100 ml	PNMT Units
1	40	93.83	50.56	1.86	14.61	5.24	2.79	6.04	4.679
2	41	142.46	54.60	2.61	18.68	6.81	2.74	7.58	3.169
3	36	79.95	42.12	1.90	9.74	4.02	2.42	5.65	3.236
4	32	180.05	90.19	2.00	9.53	3.00	3.18	4.79	2.204
5	29	103.42	35.36	2.90	17.43	4.76	3.66	3.86	2.171
6	27	67.55	44.88	1.51	17.56	3.77	4.66	3.54	2.602
Mean	34.2	111.21	53.00	2.13	14.59	4.60	3.24	5.24	3.010
±S.E.	2.4	17.28	7.91	0.21	1.66	0.54	0.33	0.61	0.382

TABLE XIX

EXPERIMENT II - METOPIRONE BIRDS

Animal No.	Acidophil Count	E µg/100 mg	NE µg/100 mg	E/NE Tissue	E µg/liter	NE µg/liter	E/NE Plasma	Bk µg/100 ml	PNMT Units
1	20	155.02	66.49	2.33	4.67	2.12	2.20	1.70	1.382
2	--	237.99	139.87	1.70	2.96	1.23	2.41	0	2.748
3	26	167.48	93.95	1.78	5.06	1.48	3.42	0.96	1.147
4	13	262.02	140.61	1.86	2.03	0.56	3.62	0.69	1.777
5	8	137.33	73.05	1.88	1.77	0.51	3.47	1.84	1.244
6	18	125.59	52.83	2.38	5.88	2.08	2.83	0	2.105
Mean	17.0	180.90	94.47	1.99	3.73	1.33	2.99	0.86	1.734
±S.E.	3.1	22.84	15.46	0.12	0.70	0.29	0.24	0.33	0.250

TABLE XX

EXPERIMENT II - EXPERIMENTAL METOPIRONE BIRDS

Animal No.	Acidophil Count	E μg/100 mg	NE μg/100 mg	E/NE Tissue	E μg/liter	NE μg/liter	E/NE Plasma	Bk μg/100 ml	PNMT Units
1	29	46.04	35.21	1.31	9.45	5.53	1.71	1.39	1.882
2	45	37.70	56.86	0.66	13.33	6.29	2.12	0.68	0.694
3	28	97.43	55.26	1.76	8.05	4.14	1.94	0	2.277
4	24	67.87	45.36	1.50	8.66	5.36	1.62	1.41	1.869
5	54	104.03	56.83	1.83	4.18	3.65	1.15	2.01	1.574
6	40	51.53	41.51	1.24	5.38	4.43	1.21	0.29	1.080
Mean	36.7	67.43	48.50	1.38	8.17	4.90	1.63	0.96	1.563
±S.E.	4.7	11.30	3.74	0.17	1.32	0.40	0.16	0.31	0.237

TABLE XXI

EXPERIMENT III - NORMAL, CONTROL BIRDS

Animal No.	Acidophil Count	E μg/100 mg	NE μg/100 mg	E/NE Tissue	E μg/liter	NE μg/liter	E/NE Plasma	Bk μg/100 ml	PNMT Units
1	16	245.97	131.05	1.88	3.62	1.65	2.19	2.15	2.163
2	10	193.05	88.83	2.17	5.15	1.72	2.99	1.75	2.230
3	13	171.23	73.30	2.34	5.21	1.79	2.91	1.32	1.792
4	13	315.17	141.06	2.23	2.05	0.57	3.61	2.45	2.215
5	20	200.50	95.55	2.10	3.10	1.23	2.52	3.07	1.451
6	14	183.97	73.16	2.51	3.18	1.20	2.65	4.01	0.971
7	15	230.41	108.01	2.13	2.58	1.08	2.40	--	1.126
Mean	14.4	220.04	101.57	2.19	3.56	1.32	2.75	2.45	1.707
±S.E.	1.2	18.66	10.09	0.07	0.46	0.16	0.18	0.39	0.201

TABLE XXII

EXPERIMENT III - RESTRAINED BIRDS

Animal No.	Acidophil Count	E μg/100 mg	NE μg/100 mg	E/NE Tissue	E μg/liter	NE μg/liter	E/NE Plasma	Bk μg/100 ml	PNMT Units
1	48	151.82	67.28	2.26	15.62	6.43	2.43	5.85	2.636
2	27	124.01	59.81	2.07	20.15	7.81	2.58	8.72	3.378
3	36	104.16	48.43	2.15	10.12	3.22	3.41	6.01	2.204
4	33	142.86	62.93	2.27	13.71	4.79	2.86	5.71	3.891
5	42	82.85	37.82	2.19	16.90	5.75	2.94	6.47	2.842
6	32	145.98	68.28	2.14	16.08	5.10	3.15	7.16	3.707
7	40	102.46	47.15	2.17	13.89	5.24	2.65	6.23	2.475
Mean	36.9	122.02	55.96	2.18	15.21	5.48	2.86	6.59	3.019
±S.E.	2.7	9.92	4.38	0.07	1.18	0.54	0.13	0.40	0.244

TABLE XXIII

EXPERIMENT III - PUROMYCIN BIRDS

Animal No.	Acidophil Count	E µg/100 mg	NE µg/100 mg	E/NE Tissue	E µg/liter	NE µg/liter	E/NE Plasma	Bk µg/100 ml	PNMT Units
1	18	314.56	145.40	2.16	4.01	1.63	2.46	1.85	1.748
2	13	207.10	101.98	2.03	3.86	1.85	2.09	1.98	2.068
3	15	173.17	83.16	2.08	5.10	2.16	2.36	2.63	1.468
4	22	234.77	90.09	2.61	3.72	1.24	3.00	1.41	3.019
5	21	192.61	79.29	2.43	4.15	1.49	2.79	2.78	2.458
6	16	223.06	112.43	1.98	3.95	1.51	2.62	2.03	1.050
Mean	17.5	224.21	102.06	2.21	4.13	1.65	2.55	2.11	1.968
±S.E.	1.4	20.14	10.01	0.10	0.20	0.13	0.13	0.21	0.288

TABLE XXIV

EXPERIMENT III - PUROMYCIN + RESTRAINT BIRDS

Animal No.	Acidophil Count	E µg/100 mg	NE µg/100 mg	E/NE Tissue	E µg/liter	NE µg/liter	E/NE Plasma	Bk µg/100 ml	PNMT Units
1	14	85.52	35.33	2.42	10.16	4.66	2.18	1.20	1.546
2	15	101.46	48.15	2.11	8.52	3.74	2.28	1.08	1.661
3	11	63.43	24.39	2.60	9.75	3.18	3.07	3.19	1.589
4	19	86.31	41.38	2.09	14.12	5.58	2.53	2.63	1.498
5	21	96.80	44.87	2.16	10.50	3.57	2.94	2.21	1.916
6	13	146.17	57.96	2.52	11.28	3.64	3.10	1.42	2.954
Mean	15.5	96.61	42.01	2.32	10.72	4.06	2.68	1.96	1.861
± S.E.	1.5	11.27	4.68	0.09	0.77	0.36	0.17	0.35	0.227

TABLE XXV

EXPERIMENT III - CORTICOSTERONE + PUROMYCIN + RESTRAINT BIRDS

Animal No.	Acidophil Count	E µg/100 mg	NE µg/100 mg	E/NE Tissue	E µg/liter	NE µg/liter	E/NE Plasma	Bk µg/100 ml	PNMT Units
1	21	80.03	33.98	2.36	8.36	3.51	2.38	18.61	1.219
2	13	78.41	36.05	2.16	6.72	1.91	3.52	15.32	1.372
3	10	132.34	84.13	1.57	9.21	2.55	3.61	22.05	2.478
4	16	114.46	44.67	2.56	7.54	2.59	2.91	13.97	1.447
5	9	81.70	36.76	2.22	8.67	3.85	2.25	25.61	1.863
6	15	71.72	33.59	2.14	8.43	3.83	2.20	26.28	1.955
7	--	105.93	53.88	1.97	7.79	2.92	2.67	19.59	1.369
Mean	14.0	94.94	46.15	2.14	8.10	3.02	2.79	20.20	0.169
±S.E.	1.8	8.61	6.90	0.12	0.31	0.28	0.22	1.79	0.169

TABLE XXVI

α-META METHYL TYROSINE EXPERIMENT

Group (n=10)	Treatment	Acidophils 0.09 mm^3 blood	Epinephrine µg/liter	Norepinephrine µg/liter
I	Normal Control	14.2 ±0.8[1]	4.45 ±.75	1.72 ±.25
II	Restraint for three hours	25.1 ±2.3 (.01)[2]*	12.15 ±1.36 (.01)*	4.18 ±.52 (.01)*
III	α-meta-methyl tyrosine[3]	13.1 ±1.4 (.01)+	5.52 ±.65 (.01)+	1.44 ±.22 (.01)+
IV	α-meta-methyl tyrosine + restraint[4]	15.7 ±0.9 (.01)+	6.14 ±1.18 (.05)+	2.00 ±.41 (.05)+

[1]Standard error of the mean.

[2]Number in parentheses indicates value of P.

[3]Birds were injected with 50 mg 1P of α-meta-methyl tyrosine 15 hours prior to autopsy.

[4]Birds were injected with 50 mg 1P of α-meta-methyl tyrosine 12 hours prior to restraint.

*Compared to normal control group (I).

+Compared to restrained control group (II).

TABLE XXVII

DEXAMETHASONE EXPERIMENT

Group (n=10)	Treatment	PNMT Units	±S.E.
I	Normal Control	1.261	0.121
II	Dexamethasone[1]	2.005 (0.05)[2]*	0.229

[1]Dexamethasone-treatment consisted of administering 5 mg of dexamethasone IP 2.5 hours prior to autopsy.

[2]Number in parentheses indicates value of P.

*Compared to normal, control group (I).

VITA

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