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ESTROGENS, CORTICOSTERONE, CALCIUM AND FREE FATTY ACIDS
IN THE FEMALE TURKEY (MELEAGRIS GALLOPAVO)

DISSERTATION

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

By

Devendra Prasad Bajpayee, B.V.Sc. and A.H., M.S.

* * * * * *

The Ohio State University
1968

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   Adrenal Function in the Female Bird
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INTRODUCTION AND STATEMENT OF THE PROBLEM

During the past two decades interest in the measurement of estrogens in body fluids has increased considerably. This has been due largely to the widened scope of research in reproductive physiology. Subjects such as ovulation, metabolism of estrogens, ovarian steroidogenesis, effects of gonadotrophins on ovarian function, and estrogen effects on physiologic processes have been studied.

Work concerning the relationship of circulating levels of estrogen corticosteroids, plasma free fatty acids, and calcium in birds is based either on indirect evidence, or on exogenous estrogen stimulation. There have been no reports in which circulating levels of estrogen, adrenal hormones, lipids, and calcium have been simultaneously studied. This has been due to the lack of reliable microanalytical methods for estrogen determination. Due to recent advances in methodology, determination of estrogens by chemical methods of high specificity and sensitivity are now available.

It is generally accepted that the ovary of the bird is the main source of estrogens, but the relative importance of this source during egg production is vague. In addition, specific and more detailed information is desired regarding the changes in circulating estrogen levels during the period the bird is lighted for production
to understand the mechanism initiating the changes in lipid and calcium metabolism. Although it is well known that the onset of laying in the bird is accompanied by marked increases in the levels of plasma lipids, (Sturkie, 1965), and after exogenous estrogen administration (van Tienhoven, 1961), relatively little is known about the basic metabolic features responsible for these major increases in plasma lipids. Similarly, recent work on calcium metabolism in the bird suggests that hypercalcemia induced through estrogen stimulation is mainly related to the transport of yolk proteins rather than to the mobilizing action of calcium for egg shell formation. However, it is known that anomalies in some specific physiological mechanisms of the ovary and oviduct—which, though not related to calcium metabolism directly, can lead to the production of soft shelled eggs.

Urist (1959) mentions that female frogs, fish, and snakes which do not lay eggs with calcified shells also exhibit hypercalcemia during their reproductive period. Thus he predicted that this phenomenon is related basically to the transport of yolk proteins to the growing follicle in the bird. Study concerning quantitative identification of endogenous estrogen values along with blood calcium should provide information concerning the mechanism by which transport of yolk proteins and thus the rapid ovarian follicular development in the bird takes place. It is also of importance for understanding egg shell formation.

The adrenal gland of the bird enlarges and increases in weight during the reproductive cycle and following exogenous
estrogen stimulation. There are several possible explanations.

(1) Estrogen inhibits the secretion of follicle stimulating hormone (FSH) through a negative feedback mechanism and this results in a shift to more adrenocorticotropic hormone (ACTH) secretion. (2) Estrogens may mimick the action of ACTH directly on the adrenal. (3) A direct stimulatory action of estrogen on adrenal steroidogenesis.

This has already been shown in vitro in rat adrenals by Kitay (1961d). Van Tienhoven (1961a) has shown the possible involvement of the adrenal in the premature ovulatory mechanism in the chicken. Although his work was concerned with massive exogenous doses of ACTH a definite possibility exists that the adrenal may play a decisive role in ovulation in the bird coming into egg production.

To obtain some of the answers to questions raised in the foregoing discussion the following objectives were developed as the major focus of this study.

(1) To develop and evaluate a chemical method based on fluorometry for estimating blood estrogens in female turkeys.

(2) To determine the circulating levels of blood estrogens at regular intervals throughout the initial three week lighted (14L, 10D) period till the turkeys came into production and compare these estrogen values with changes in plasma calcium and plasma free fatty acids (FFA) estimated from the same blood samples.

(3) To analyze the circulating levels of corticosterone when turkeys are brought into production by light, and to correlate these changes with changes in blood estrogens, plasma calcium, and plasma free fatty acids.
CHAPTER I
REVIEW OF LITERATURE

The presently accepted system of steroid nomenclature follows the rules of the International Union of Pure and Applied Chemistry (1960). This system will be followed closely throughout this work. However, the hormones and some of their key intermediates and metabolites listed in Table 1 will be referred to by their trivial names.

A. Biosynthesis of Estrogens in Birds

It has been known for a long time that the bird produces estrogens (Gustavson, 1931, Marlow and Richert, 1940, and Hurst, Kuksis and Blendell, 1957). However, the first chemical evidence for the presence of estrone, estradiol-17\(^\alpha\) and estriol in the ovarian tissue of a laying hen was presented by Layne, Common, Maw and Fraps (1958). Biochemically, it has been clearly demonstrated that estrogens can arise from (1) acetate, (2) by conversion of both cholesterol and progesterone and (3) by aromatization of neutral C\(_{19}\) and C\(_{18}\) steroids. Ryan and Smith (1961 a, b, c, d) have presented data to demonstrate that the above three pathways do not operate independently, but constitute a single pathway for the biosynthesis of estrogens.

Nathanson and Towne (1939) first postulated the involvement
<table>
<thead>
<tr>
<th>Trivial Name</th>
<th>Systematic Names</th>
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<tr>
<td>Estrone</td>
<td>1,3,5(10)-estratriene3-3-17-one</td>
</tr>
<tr>
<td>Estradiol-17ß</td>
<td>1,3,5(10)-estratriene-3, 17ß -diol</td>
</tr>
<tr>
<td>Estriol</td>
<td>1,3,5(10)-estratriene-3,16α 17ß -triol</td>
</tr>
<tr>
<td>16-epiestriol</td>
<td>1,3,5(10)-estratriene-3,16ß 17ß -triol</td>
</tr>
<tr>
<td>17-epiestriol</td>
<td>1,3,5(10)-estratriene-3,16α 17α -triol</td>
</tr>
<tr>
<td>16,17-epiestriol</td>
<td>1,3,5(10)-estratriene-3,16ß 17α -triol</td>
</tr>
<tr>
<td>Progesterone</td>
<td>4-pregnene-3,20-dione</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>5-pregnene-3ß 01-20-one</td>
</tr>
<tr>
<td>17ß hydroxypregnenolone</td>
<td>5-pregnene-3ß, 17ß -dol, 20-one</td>
</tr>
<tr>
<td>Testosterone</td>
<td>4-androstene-17ß -o1-3one</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>5-androstene-3ß -o1-17one</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>4-androstene-3,17-dione</td>
</tr>
<tr>
<td>Androstenediol</td>
<td>4-androstene-3ß,17ß diol</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>4-pregnene-11ß,21-diol3,20dione</td>
</tr>
</tbody>
</table>
of testosterone as a possible precursor for estrogens. Both in vivo and in vitro experiments carried out by Bagget, Engel, Savard, and Dorfman (1956) have shown that labelled testosterone increases yields of estrone and estradiol-17\(^\beta\). Thus, in the sequence of biogeneration of estrogens, both the progestins and androgens are key intermediates. A general biosynthetic route for estrogens is acetate \(\rightarrow\) cholesterol \(\rightarrow\) 20\(^\alpha\) hydroxycholesterol \(\rightarrow\) pregnenolone \(\rightarrow\) progesterone \(\rightarrow\) 17\(^\alpha\) hydroxyprogesterone androstenedione \(\rightarrow\) testosterone \(\rightarrow\) estrone or estradiol-17\(^\beta\).

Although no in vivo or in vitro studies have been made in the avian system to elucidate the pathway for estrogen synthesis, recent studies by Breuer and Ozon (1965), Ozon (1966), and Gottfried (1964) have shown that steroidalogenesis in birds follows essentially the same pathway as in mammals.

Key enzymes involved in the various steps of estrogen synthesis have recently been shown by histochemical techniques to be present in avian ovarian tissue. For instance, Wyburn and Baillie (1966) have demonstrated histochemically the presence of 3\(^\beta\), 17\(^\beta\), and 20\(^\beta\) hydroxysteroid dehydrogenases in both the granulosa and theca interna of the fowl's ovarian follicle. The existence of these enzymes is a very good indication for biosynthesis of progesterone, androstenedione, and testosterone, key intermediates in estrogen biosynthesis and for interconversion of estrone and estradiol-17\(^\beta\) in the hen ovarian tissues. When sections of follicles were incubated with pregnenolone, dehydroandrosterone and androstenediol as substrates intense 3\(^\beta\)-hydroxysteroid
dehydrogenase activity was readily observed. Since pregnenolone can be converted to progesterone; dehydroandrosterone to androsterone (and subsequently can generate testosterone) and androstenediol to testosterone, it is reasonable to assume that these intermediates could be utilized subsequently for estrogen biosynthesis by the hen ovary. The most interesting observation of Wyburn and Baillie was the intense enzymic activity they encountered when sulphates of pregnenolone and 17 hydroxy pregnenolone were used as substrates. The biological importance of steroid sulphates has received considerable attention recently (Calvin and Lieberman, 1964, Calvin, Van de Wide and Liberman, 1963, and Baülieu et al., 1965) and will be referred to later in this study. However, two studies are worth mentioning in this regard. A soluble enzyme system has been prepared by Payne and Mason (1965) from bovine ovary that, in the presence of ATP, is capable of sulfurylating estradiol 17β, and Burstein and Dorfman (1963) have mentioned a sulphatase activity in the mammalian ovary.

The presence of 17β hydroxysteroid dehydrogenase activity in ovarian follicles obtained from the hen has been reported by Wyburn and Baillie (1965) and Chieffi and Botte (1965). While the former workers have studied this enzyme using estradiol-17β and testosterone as substrates, the latter group has shown two different enzymes (NAD and NADP dependent 17β hydroxysteroid dehydrogenases). This activity was specifically localized in the granulosa of the growing and atretic follicles. This observation led Chieffi and Botte to assume that estrogen metabolism in the hen ovary may be
confined to the granulosa cells only. Recently, the ability of the avian female gonad to produce estrogens very early in life has been ably demonstrated by Weniger (1965a, b, 1966). By growing embryonic (seven day old) ovaries in a synthetic medium for 24 hours and then extracting the medium for estrogens he presented both biological and chemical evidence for the presence of estrone and estradiol-17β, in the medium.

B. Metabolism of Estrogens in the Bird

There are numerous published reports on the excretion of estrogens and their metabolites in birds. Most of this work has been carried out in the female domestic fowl. MacRae, Zaharia and Common (1969) obtained the crystalline form of estradiol-17β from the droppings of laying hens. From urine obtained from birds with exteriorized uretal openings, Ainsworth and Common (1962) crystallized estrone. Studying the possible urinary conversion products of estrone, Ainsworth, Carter and Common (1962) obtained radioactive estriol, 16-epiestriol, 17-epiestriol, 16-oxoestradiol-17β, estradiol-17β, and 16-oxoestrone from a 24 hour urine sample of a laying hen injected intravenously with estrone-16-C14. MacRae, Layne and Common (1959) obtained radioactive estriol from the laying hen urine after intravenous injection of estradiol-17β-16-C14. This was not surprising since Mitchell and Hobkirk (1959) had obtained labelled estriol after incubating radioactive estradiol-17β with chicken liver slices. Subsequently, Hertelendy and Common (1965) obtained all four estriol epimers after injection of labelled estradiol-17β. However, when MacRae et al. (1960) injected estriol-16-C14 intravenously only
radioactive 16-oxoestradiol-17\(^\alpha\) and 16-epiestriol were excreted through urine. Recently, Mathur and Common (1967) have shown chromatographic evidence for the presence of estriol and 16, 17-epiestriol in the urine of laying hens.

Since 16-oxoestrone is an important urinary conversion product of injected estrone, the laying hen resembles the human female in the luteal phase as described by Slaunwhite and Sandberg (1956). In addition, the laying hen also resembles the female rhesus monkey (Flickinger and Wu, 1967) in its capacity to convert estradiol-17\(^\alpha\) to estriol and 16-epiestriol. Studying the metabolism of tritiated estradiol-17\(^\alpha\) in various tissues of the laying hen, Hawkins and Taylor (1967) found that the greatest proportion of free radioactive steroid (81%) was in the follicle wall, and the greatest (81%) conjugated radio-active steroid was found in the bile. This work also demonstrated that blood plasma contained equal amounts of free (50%) and conjugated (47%) radioactive steroid. In addition the plasma fraction had a significant amount of labelled estrone. This latter observation finds support from the work of Velie (1963) which presented evidence that isolated red blood cells from the hen are capable of converting estradiol-17\(^\alpha\) to estrone. A somewhat similar study undertaken by Jonsson and Terenius (1965) showed that two hours after an injection of tritiated estradiol-17\(^\alpha\) the radioactivity encountered in various tissues in order of decreasing intensity were, liver > adrenals > ovary > plasma > erythrocytes > heart and muscle. However, extremely high activities were present in the gall bladder, cloaca and small intestines. These high radioactivities
in parts of the alimentary canal could be derived from the conjugation of estrogens in the liver and their ultimate removal through bile. This observation is supported by a report of Hopwood and Gassner (1962) who have shown that ninety-nine percent of the radioactivity in the bile after treatment of the chicken with labeled synthetic estrogen was water soluble. In a later report, Hopwood et al. (1963) demonstrated conjugation of labeled synthetic estrogen by chicken liver slices in vitro. It is clear from the above reports that the liver is the chief organ in the bird for inactivating estrogens by conjugation which are ultimately excreted as water soluble compounds via bile.

The important function of avian liver in estrogen metabolism has been made more clear by the recent reports of Ozon and Breuer (1965), Renwick and Engel (1967) and Raud and Hobkirk (1967). Ozon and Breuer (1965) reported the presence of 17α-estradiol dehydrogenase activity in the chicken liver. This study prompted Renwick and Engel (1967) to investigate the avian liver and they were subsequently able to partially purify 17β- and 17α-estradiol dehydrogenase activity from chicken and turkey liver. Both of these enzymes catalyzed the reversible oxidation of estradiol epimers to estrone. The apparent cofactor required by the enzyme system was NADP. Both enzyme systems were localized in the soluble fraction. A partial separation of the activities of the two enzymes was accomplished on Sephadex G-200, which confirmed the existence of two distinct enzymes. Both enzymes are linked to pyridine nucleotides and demonstrate a marked preference for NADP. The product of both
dehydrogenations was estrone which was formed in an amount equivalent to the pyridine nucleotide reduced. Ainsworth et al. (1962) were repeatedly unsuccessful in finding estradiol-17α among the conversion products from urine of the laying hen. In light of Renwick and Engel's work it is possible to postulate that most of the estradiol-17α is converted to estrone in the liver of the hen prior to excretion via feces or urine. The demonstration of Raud and Hobkirk (1967) that estriol and 16-epiestriol are interconvertible by the avian liver lends general support to the work of Ainsworth et al. (1962), MacRae and Common (1960) and MacRae, Layne and Common (1959).

C. Effect of Female Sex Hormones on Lipid Metabolism of Birds

A distinctive feature of the lipid metabolism of the bird is its response to estrogen stimulation. The classical studies of Lorenz and coworkers (Entenman, Lorenz and Chaikoff 1938; Lorenz, Chaikoff and Entenman 1938; and Entenman, Lorenz and Chaikoff 1940) have demonstrated that the experimental stimulation of the ovaries with pregnant mare serum (PMS) can more than double the lipid content of the blood of immature birds. This rise in blood lipids also follows the injection of ovarian hormones (estrogens). These effects (estrogenic) are not confined to the female since a response also is observed in the male bird. In addition, elevated blood lipid content can be observed as early as twelve hours after introduction of estrogens, although it requires several weeks for PMS to produce a similar rise. Entenman, Lorenz and Chaikoff (1940) observed that 48 hours after estrogen injection into immature birds the oviduct weight had increased approximately eight times. There
was also a significant increase in blood lipids. The correlation between oviduct weight and blood lipids was highly significant ($r = 0.93$). These workers (Lorenz and Chiakoff, 1940) found that estrone was approximately six times as potent as a lipogenic agent as estradiol-17β.

Studying this unique influence of estrogens on avian lipid metabolism, Baum and Meyer (1956) attributed the lipemia observed in chickens to increased lipid synthesis rather than its increased mobilization. The discovery by Dole (1956) that lipids were transported from adipose tissue to the liver and other organs in the form of long chain unesterified (free) fatty acids (FFA) bound to serum albumin made it possible to re-examine the role of hormonal control of lipid metabolism. Thus, Heald and Rookledge (1964) observed that estrogens do cause increases in plasma FFA even though it is accompanied by increased lipid deposition. Intramuscular injection of estradiol depropionate into pullets (10-11 week old) increased the levels of the plasma FFA and total lipids within twenty-four hours. Since administration of gonadotrophins of mammalian source induced a parallel increase of plasma FFA along with an increase in blood lipids, and since mammalian gonadotrophins do induce extensive development of ovarian follicles of mature laying birds, the results of Heald and Rookledge suggest that the increase in the plasma FFA is a consequence of follicular stimulation and not a direct action of pituitary hormones. The fat mobilizing factor found in the pig and sheep pituitary or a purified ACTH injection were not effective in inducing changes in plasma FFA or total blood
lipids in chickens. This observation is of significance since the adipokinetic action of ACTH is well known in a wide variety of mammals (Engel and Lebowitz, 1966). Since Woods, Freeman and Kellner (1962) have reported plasma FFA increase by continuous infusion of mammalian ACTH, a rapid destruction of mammalian ACTH in the avian system cannot be ruled out. Heald and Rookledge also studied the effects of fasting upon plasma FFA. Here, a peculiar difference was found depending upon the physiological state of the bird. In the immature pullet, fasting led to a conspicuous rise in levels of plasma FFA and a decrease in plasma lipids, but such an increase was not evident in the laying bird, in which fasting led to a rapid drop in levels of plasma FFA and lipids with a parallel decrease in mean oviduct weight. This possibly indicated a lower output of estrogens from the ovary of the laying bird. This argument is supported by the observation that injection of exogenous gonadotrophins or avian pituitary powder maintained both oviduct weight and egg production. Since treatment of fasting laying birds with avian pituitary maintained the levels of plasma FFA and total lipids the experiments suggested that pituitary maintenance of normal ovarian function had failed in fasting condition of the adult female bird. In contrast, Gibson and Naibandov (1966) suggested that pituitary powder acted directly on the adipose tissues rather than through the target organ.

Studying the pattern of the changes in plasma FFA as the chicken came into lay, Heald and Badman (1963) observed that the levels of plasma FFA began to rise as the birds approached the
point of lay but fell sharply when laying commenced. This observation led Heald and Rookledge (1965) to study plasma FFA during the ovulatory cycle in the domestic fowl. They found no direct association between variations in plasma FFA concentration and the ovulatory cycle. The marked changes in the quantities of plasma FFA which occur at the onset of laying did not affect the relative proportions of individual fatty acids (palmitic, palmitoleic, oleic and linoleic acids). This proportion remained constant, which led Heald and coworkers (1964) to interpret that the FFA in the bird are derived from a single major source (depot fat) or that the stimulus giving rise to the increase in plasma FFA affects all the sources to the same degree. Recently, Heald, McLachlan, and Rookledge (1965) and Heald (1965) presented evidence that glucagon acts directly upon avian adipose tissues and subsequently accelerates the release of plasma FFA. Insulin, on the other hand, also increased plasma FFA, a finding which contrasts with similar studies in mammals where it depresses plasma FFA (Engel, 1962). Thus, Heald et al. (1965) suggested that insulin acts indirectly by promoting an increase in the release of glucagon from the pancreas which then acts directly on the depot fat. This finding is supported by work of Goodridge and Bull (1965) who have shown that insulin had no effect in vitro upon the rate of lipolysis in abdominal adipose tissue of the pigeon. However, Lepkovsky et al. (1967) using depancreatized and intact chickens presented evidence which is in conflict to the interpretations made by Heald et al. (1965) and Heald (1966). In both depancreatized and intact male and female birds, glucagon-free insulin
preparations produced a significant increase in plasma FFA, suggesting that insulin has a direct action on adipose tissue to increase plasma FFA. Due to the results obtained, Løpskovsky et al. (1967) cautioned that release of FFA in adipose tissues does not represent an isolated biological process; on the other hand, the release of FFA is integrated with glycogenolysis, maintenance of blood sugar levels and with body temperatures—all of which are important for survival of the animal. The same hormones which accelerate the release of plasma FFA also participate in thermogenic and glycolytic mechanisms. However, these mechanisms which involve estrogens, glucagon and insulin in the bird are yet to be elucidated.

D. Hormonal Control of Calcium Metabolism in the Bird

It has been known for a long time that estrogens increase blood calcium levels in a wide variety of avian species. Estrogen increases blood calcium levels in cocks (Zondek and Marx, 1939), cocks and drakes (Landauer, 1940), pigeons (Riddle and Dotti, 1938; and Pfeiffer and Gardner, 1938), sparrows (Pfeiffer, Kirschbaum and Gardner, 1940), and quail (Baldini and Zarrow, 1952).

The calcium in avian blood is present in two forms: (1) non diffusible egg calcium bound to phospholipoproteins, and (2) diffusible (ultrafilterable) calcium. It was demonstrated by Urist et al. (1958) that there was a tenfold increase in the total serum calcium following injection of 125 mg. of diethylstilboestrol. Parallel with this rise of blood calcium was an increase in the concentration of a specific phosphoprotein complex, which was found normally in the serum of laying hens but was absent from normal
rooster serum. Through schlieren pattern recordings in the ultracentrifuge at 50,740 rpm these workers separated this phosphoprotein complex into two different components viz. $X_1$ (a phosphoprotein) and $X_2$ (a phospho-lipid lipoprotein). This exogenous estrogen injection also increased chylomicrons and betalipoproteins while alpha lipoproteins were decreased. When the total serum calcium was elevated to 100 mg. percent, beta lipoproteins appeared to have bound approximately ten mg. percent of the calcium; albumin five mg. percent and the two phosphoproteins ($X_1$ and $X_2$) seventy-nine mg. percent of the total calcium. Six mg. percent of the total serum calcium in both the control and the estrogenized roosters was present in the diffusible form. Since this portion of serum calcium did not change during estrogen challenge it was concluded that the increase of serum calcium was entirely in the nondiffusible fraction. Urist speculated that since the major portion of serum calcium was present as an undissociated complex with the phosphoproteins and, since this phosphoprotein complex ($X_1$ and $X_2$ fractions) was deposited in the granules of egg yolk in the ovarian follicles of the laying hen, the major role of estrogen induced hypercalcemia observed in birds is for the transport of yolk proteins to the ovarian follicles. Studies by Polin and Sturkie (1957) had shown that a decrease occurred in the diffusible calcium level only at the time of shell deposition, although total calcium showed no change. Their data indicated that the diffusible calcium level was not constant in laying hens and shell deposition in early hours significantly altered the level of this calcium fraction. However,
after parathyroidectomy a marked drop occurred in total calcium level. Both calcium fractions (bound and diffusible) were significantly below the levels present in sham operated hens. Soon after the extirpation of the parathyroids Polin and Sturkie also observed that eggs present in the uterus were prematurely expelled with little or no shell deposition. Later, pursuing this problem, Polin and Sturkie (1958) showed that the ability of estrogen to augment blood calcium response was dependent on the action of parathyroid hormone to regulate the diffusible calcium levels. In other words, parathyroid hormone maintains diffusible calcium levels so that estrogens can increase the nondiffusible calcium levels. Thus, estrogens failed to show an increase in total blood calcium when the birds were parathyroidectomized. Polin and Sturkie's work (1957, 1958) is in conflict with the work of Winget and Smith (1958) who found just the opposite results. They observed that there was a decrease in the total plasma calcium associated with shell formation and that this decrease was almost wholly due to a decrease in the nondiffusible fraction, since the diffusible calcium remained fairly constant. Urist, Deutsch, Pomerantz, and McLean (1960) have interpreted an additive effect of parathyroid and estrogen upon serum calcium rather than a synergistic effect of these hormones. Hertelendy and Taylor (1961) attempted to clarify the situation with respect to the changes in blood calcium which occur during the laying cycle by following these changes in individual birds. These workers observed a definite fall in total plasma calcium in birds before and during the
calcification process. This fall was related to the actual process of calcification, and not due to a reduction in the rate of calcium absorption from the intestines. Maintaining the birds on calcium low diet did not influence this relationship. It was interesting that the birds on a low calcium diet maintained the total plasma calcium level at approximately twenty mg. percent. Thus, virtually all the calcium for egg shell formation must have come from the skeleton, suggesting that the mechanism controlling this mobilization of the bone material was extremely sensitive. Though birds used by Hertelendy and Taylor came from a highly uniform genetic strain they still demonstrated a considerable variation individually in plasma blood calcium at any particular stage. This may have been the basic reason why the studies of Polin and Sturkie (1957) and Winget and Smith (1958) gave completely different results. In a later study on diffusible plasma calcium, Taylor and Hertelendy (1961) cautioned that the pH of the plasma of laying hens increases approximately by 0.3 units when exposed to air for more than three hours which also decreased the diffusible calcium level by 1-2 mg. They also observed that shell calcification was always accompanied by a fall in the diffusible plasma calcium level which was also associated with a decrease in the total plasma calcium levels.

This high calcium requirement at the time of egg shell calcification process is met in class aves by a system of secondary bone which grows from the endosteal surfaces in the form of fine interlacing spicules providing a highly labile store of calcium. The formation of this specialized bone, known commonly as
medullary bone, is dependent on estrogens (Urist, Deutsch, Pomerantz and McLean, 1960) or estrogens and androgens (Taylor, 1966; Bloom, Domm, Nalbandov and Bloom, 1958; and Bloom, McLean and Bloom, 1941). According to Bloom et al. (1958) the amount of intramedullary bone in the laying hen is variable. When the egg is in the uterus, large numbers of osteoclasts appear between the trabeculae, which Taylor, Williams and Kirkley (1965) call the "bone destroying phase." On the other hand, in the "bone forming phase" the cell population is dominated by osteoblasts, and this phase occupies the periods between the calcification of successive eggs. Urist et al. (1960) speculated that the cell population in the medullary bone of the hen may change in character without any corresponding change in the volume of the osseous tissue. They also suggested that a fall in estrogen levels in the blood may be responsible for complete disappearance of the medullary bone at the time of termination of a normal ovulatory cycle in the hen, a stage when the ovary output of estrogens may have decreased significantly.

E. Adrenal Function in the Bird

In the past ten years, extensive work has been reported concerning the measurement of the adrenal output of glucocorticoids in the chicken (Phillips and Chester Jones, 1958; Newcomer, 1959; Nagra Baum Meyer, 1960; Nagra et al., 1963; deRoos, 1960, 1961; Urist and Deutsch, 1960; Sander, Lamoureux and Lantheen, 1963; Resko, Norton and Nalbandov, 1964; Frankel, Graber and Nalbandov, 1967a,b) and in the turkey (Brown 1960, 1961, 1964, 1967, 1968). These reports have either been confined to the adult male fowl
or immature birds.

This choice of the male was influenced by: (1) the adrenal vein of the male bird is easier to canulate, (2) lipemia in the adult female bird interfered with the older techniques for corticosterone analysis and made more exacting purification techniques necessary, and (3) the left adrenal of the female fowl is often imbedded in the ovary (Biswa1, 1954) making it difficult to surgically remove or canulate the gland.

F. Biosynthesis of Glucocorticoids in the Bird

Work concerning the biosynthesis of adrenal steroids by the avian adrenal has been carried out mostly in vitro. Sander and Lanthier (1963) and Sander et al. (1963) using progesterone-4\textsuperscript{14}C as a precursor reported 18-hydroxycorticosterone, corticosterone, aldosterone, 11-desoxycorticosterone and cortisol as major transformation products by duck adrenal slices. Repeating these experiments on ducks and chickens, and using two labelled precursors in the incubation medium (progesterone-4\textsuperscript{14}C and pregnenolone-7\textsuperscript{3}H) Sander et al. (1963) confirmed their earlier findings with duck adrenals except that in the adrenals of the domestic chicken the major product was corticosterone and not 18-hydroxycorticosterone. They also observed that progesterone was a more important precursor than pregnenolone for both duck and chicken adrenal systems. Recently Sander et al. (1965) using goose and duck adrenals demonstrated by using acetate-1\textsuperscript{14}C that cholesterol, corticosterone, aldosterone and 18-hydroxycorticosterone were the main products of transformation, suggesting that the
metabolic pathways leading to corticosteroids are probably identical with those described for mammals by Grant (1962).

G. Regulation of the Avian Adrenal Gland

The avian adrenal is unique in that it differs from its mammalian counterpart regarding its relationship with the pituitary. Brown, Brown and Meyer (1958), finding little difference in adrenal weights between intact and hypophysectomized birds, concluded that the avian adrenal does not require a large amount of hypertrophy to go from a relatively inactive to a functional secretory state. They suggested that the adrenal glands of the hypophysectomized birds function at a relatively higher level, indicating that the adrenal is relatively autonomous. Adrenal weight in chickens was maintained following hypophysectomy as shown by the works of Nalbandov and Card (1943) and Baum and Meyer (1956). Newcomer (1959a) supported the view that the avian adrenal is autonomous. Ma and Nalbandov (1963), on the basis of experiments on hypophysectomized male birds, suggested that the adrenals of birds, in contrast to the testes and thyroids, were somewhat independent of hypophyseal support regarding their ability to increase in weight, even though it was perfectly obvious that a pituitary gland in situ had a greater growth stimulating effect on the adrenals than an autotransplanted adenohypophysis, and that the latter causes heavier adrenals than those found in hypophysectomized birds. Although no direct metabolic activity in the adrenals was studied, data for $^{32}$P uptake by the adrenals suggested that the pituitary gland in its normal location might
play an inhibitory role on the function of the hypothalamus or vice versa. They also suggested that in birds there may be an extra hypophyseal, perhaps a hypothalamic, source of ACTH which remained unaffected by hypophysectomy. Frankel, Graber and Naibandov (1967\textsuperscript{a}) recently presented evidence that the adrenal gland in adenohypophysectomized birds maintained corticosterone production at 37 percent of the control. Similar results were obtained in \textit{vitro} by them using adrenal slices from adenohypophysectomized cockerels. Metopirone treatment caused a precipitous decrease in corticosterone production in both intact and hypophysectomized birds. Similarly, dexamethasone phosphate eliminated corticosterone from both groups of cockerels. These results suggested that adrenal function in adenohypophysectomized cockerels is not autonomous as previously thought but is supported by an extrahypophyseal ACTH. Their experimental evidence also supported the view that the classical ACTH feedback is not at the pituitary level in cockerels.

Continuing investigation of this problem, Frankel, Graber and Naibandov (1967\textsuperscript{b}) obtained opposite effects upon adrenal function of intact and hypophysectomized cockerels by placing electrolytic lesions in the ventral tuberal area of the hypothalamus. They found decreased corticosterone output in intact birds and an increase in adenohypophysectomized birds. The area where the lesions were placed in the hypothalamus was claimed as specific for adrenal function. Histologically, interenal tissue cells at the periphery in the lesioned but intact birds showed decreased activity and an increase in amount of chromaffin tissue; in contrast, the
internal tissue at the periphery in the adenohypophysectomized birds appeared stimulated and exhausted of its granulation. Although these data appear to support the view of the role of the hypothalamo-hypophyseal axis in regulation of adrenal function in intact cockerels they did not indicate that the extrahypophyseal support for the adrenal function in adenohypophysectomized cockerels was localized in the hypothalamus. Their data also failed to explain the peculiar adrenal function obtained in cockerels which, without an anterior pituitary, could be stimulated to produce more corticosterone by lesions placed in the hypothalamus.

H. Sex Dimorphism in the Adrenal and Relation to Sexual Organs in the Birds

Sauer and Latimer (1930) observed that the female fowl had about 30 percent more interrenal tissue that the male bird. This sexual dimorphism in the bird's adrenal interrenal tissue is similar to that in mammals where it is well known that the gland is generally larger in the female (Chester Jones, 1957). This difference seems to be dependent upon gonadal steroids; estrogens cause an increase in adrenocortical size in the ovarectomized female and in normal and castrated males. Testosterone, on the other hand, diminished the enlarged adrenal of the castrate male and the gland of normal males and females. This relationship seems to be true in a wide range of vertebrates as indicated by Stanworth's (1953) results on fishes and those obtained in humans (Hartman and Brownell, 1949). Kar (1947) demonstrated that castration of the male fowl causes hypertrophy of the
interrenal and the sex difference in the gland disappears after removal of the testes. However, this hypertrophy of internal tissue following castration was shown to be prevented by the administration of testosterone.

Recently, Nagra, Sauers and Wittmaier (1965) demonstrated that testosterone lowered the amount of corticosterone in adrenal effluent blood of stressed cockerels, but testosterone was effective only in castrated birds. Since this reduction in hormone production was also observed in in vitro studies using adrenal tissue with various concentrations of testosterone, they suggested that androgen acted directly on the adrenal gland.

Similar work concerning the effects of estrogens on the adrenal gland in the female bird is scanty. However, Riddle (1923) had shown, in the pigeon, that the adrenals hypertrophy during ovulation. The mean increase was found to be 40 percent. This observation is supported by the work of Riddle and Minoura (1933) who reported that repeated subcutaneous transplants of whole adrenals into female doves hastened sexual maturity. This role of the adrenal is shared by mammals and several of the lower vertebrates alike. Mandl (1954) suggests that corticosteroids enter into normal ovarian physiology at the cellular level. Thus in fishes, the possibility of a gonado-adrenal dependence is suggested by observations of adrenal hypertrophy and hyperactivity associated with female gonadal development and spawning (Stanworth, 1953; Robertson and Wexler, 1957, 1959). Robertson (1957), and Phillips, Holmes and Bondy (1959) have shown that the amounts of
corticosteroids in the blood of fish increased progressively to the high level of 70-80 ug.% at spawning, then fall to lower levels during the postspawning period.

The most significant contribution to the study of the mechanism by which estrogens influence adrenal function is contained in a series of papers by Kitay and coworkers (1961a, b, c, d, 1963a, b, c, 1964, 1965). Although no similar studies are known in aves a generalized, though brief, picture of this estrogen action on adrenal function in the female mammal is warranted.

In female rats Kitay (1963) has shown that adrenal secretion of glucocorticoids as well as pituitary ACTH are reduced after oophorectomy. These changes are reversible following replacement therapy with a depot form of estradiol-17β. This stimulatory effect of estradiol-17β on ACTH is demonstrable in adrenalectomized, oophorectomized rats. Thus, the stimulatory effect of estradiol-17β on adrenal corticosterone production may be secondary to increased ACTH release. However, when Kitay (1963b) added estradiol-17β directly in vitro to adrenal slices obtained from oophorectomized rats, increased corticosterone production occurred. Kitay et al (1965) presented additional evidence concerning this direct effect of estradiol-17β on adrenal function. It was demonstrated by him that adrenal homogenates obtained from previously oophorectomized rats produced a significantly lower amount of corticosterone in vitro than homogenates obtained from intact or estradiol-17β treated oophorectomized rats. This drop in corticosterone production was
reversed by the addition of estrogen to the medium. This decrement in adrenal glucocorticoid production occurred even with the addition of either NADP and glucose-6-phosphate or NADPH alone. The defect also persisted after unilateral adrenalectomy (a procedure for chronic stimulus of endogenous ACTH release) or when large doses of ACTH were administered \textit{in vivo} before measuring corticosterone production \textit{in vitro}. In contrast however, a stimulated corticosterone production occurred from adrenal homogenates obtained from hypophysectomized, oophorectomized rats given estradiol-17\textsubscript{\text{\( \beta \)}}. The magnitude of this direct action of estrogen remained unclear owing to a concomitant and an apparently independent stimulatory effect on pituitary ACTH and its effect on hepatic metabolism of corticosterone shown by Kitay (1963\textsuperscript{c}). Haynes and Berthet (1957) advanced a theory that the availability of NADPH is the major rate-limiting factor in the enzymatic biosynthesis of corticosterone from cholesterol. The generation of NADPH is accomplished through a series of steps starting from ACTH stimulated formation of cyclic adenosine-3\'5\' monophosphate which in turn activates the enzyme phosphorylase. This enzyme increases the availability of glucose-1-phosphate from the glycogen stored in the adrenal gland. Glucose-1-phosphate is converted by phosphoglucomutase to glucose-6-phosphate which ultimately serves as the substrate for glucose-6-phosphate dehydrogenase—the enzyme responsible for the reduction of NADP to form NADPH. Voluminous literature concerning this theory has been reviewed in detail by Hill (1965). However, the unique estrogen action cannot be explained on the basis of this enzymatic
mechanism since in their (Kitay et al., 1965) work adrenal stores of glycogen were increased after oophorectomy, and adrenal phosphorylase and hexokinase activities did not change while glucose-6-phosphate dehydrogenase activity increased.
Theoretically, there are two ways to judge the activity of an endocrine gland. (1) By changes in the release of its hormones or (2) by changes in the metabolism or the content of the chemical substances of the gland in question. The quantity of hormones released may be determined by blood and/or urine analysis or by their effect on other organs. When it is a question of ovarian hormones, quantitative detection in the urine has traditionally been used to interpret the characteristic changes in the release of these hormones from the ovary. However, Parkes (1955) has pointed out that estrogens in the urine may be merely what the organism does not want, and the information desired is circulating levels of estrogen. Dorfman (1965) also emphasises the physiologic importance of measuring estrogens in the blood.

Only recently have attempts been directed to quantitate estrogens in avian blood. Layne et al. detected a compound identical with estrone in the conjugated fraction from 650 ml.
of blood in the laying hen. O'Grady (1965) and O'Grady and Heald 
(1965), using a double isotope derivative technique, were successful 
in detection of estrone and estradiol-17\(^\circ\) from the blood plasma of 
the laying hen. Ozon (1965) subsequently reported quantitative 
detection of estrone, estradiol-17\(^\circ\) and estriol from the blood 
of adult pullets and found higher concentration of these estrogens 
in the blood of twenty-one day old female birds. Of all the above 
reports, the double isotope derivative technique as described by 
O'Grady and Heald (1965) and O'Grady (1965) had a degree of sensi­
tivity, specificity and accuracy which was not previously available. 
Although remarkable in its sensitivity and reproductibility, this 
method is extremely complicated and expensive, and not adaptable 
to routine analysis of a large number of samples.

The object of this chapter is to describe a procedure 
capable of detecting submicrogram levels of estrogen that is adap­
table to routine analysis of a large number of samples. This 
method is based on preliminary purification of the blood and 
separation of the three classical estrogens by alumina adsorption 
column chromatography and subsequent extraction of Kober chromogens 
into an organic phase.
Method

A. Reagents

(1) Reagent grade ethyl ether was purified by shaking with 0.3 M ferrous sulphate in 0.4 M sulfuric acid. The acidity was removed by washing with distilled water. The ether was subsequently distilled over sodium or potassium hydroxide. The ether was used as it distilled.

(2) Benzene, toluene and heptane were repeatedly washed with new portions of concentrated sulphuric acid until the acid layer remained colorless after repeated shaking. The solvents were then rinsed with distilled water to remove acidity. They were passed through a plug of anhydrous sodium sulphate and finally were distilled at 80°C, 110°C and 98°C respectively. By this procedure, fluorescence-free solvents were obtained which exhibited similar or even less fluorescence than the "fluorometric grade" solvents obtained commercially.

(3) Reagent grade chloroform was shaken several times with concentrated sulphuric acid, washed with distilled water to remove acidity and finally, kept in the freezer (-20°C) overnight to "freeze out" the remaining water. The bottom layer (chloroform) was siphoned into a clean bottle and this was passed through a plug of anhydrous sodium sulphate. Absolute chloroform obtained this was was stored at -21°C.
B. Glassware was kept meticulously clean. The method recommended by Bush (1961) was followed, except that the two final cleaning solutions were made in 95% ethanol rather than in methanol. These were (a) 1:4 glacial acetic acid:95% ethanol (b) 95% ethanol with a trace of acetic acid.

C. Collection of blood

(1) Heparanized blood samples were obtained from laying turkeys by two different techniques, (a) Through a cardiac puncture, and (b) From posterior vena cava via a catheter. For the latter the turkeys were anaesthetized by pentobarbital sodium and laid on their right side. An incision, 8 cm. long, was made medially, parallel to the vertical axis of the right thigh and about four centimeters below the coxofemoral or hip joint. The fascia was removed and the femoral vein exposed. The vein was punctured by a 15 gauge hypodermic needle and the heparinized catheter (30 cm. long) (polyethylene tubing PE 90; having a size B plastic tubing adapter for syringe, Clay-Adams, Inc., New York) was pushed through this needle to a premarked point (17 cm.) on the catheter. This point made it possible to place the inner end of the polyethylene tubing in the posterior vena cava slightly anterior to the junctions of the ovarian veins. The position where the catheter entered the femoral vein was ligatured tightly to prevent its slipping out. The muscular tissue was sutured back and the remaining catheter (13 cm) was made into a loop and held with sutures on the skin. Three turkeys were successfully catheterized by this procedure. Blood was collected and pooled samples from catheterized
birds stored in the freezer until extracted.

(2) Pooled blood samples from the heart were collected from 10 castrated male turkeys.

D. Extraction

A complete flow sheet showing the various steps for extraction and purification of blood samples for subsequent chromatography is presented in Figure 1. The pH of turkey blood ranged from 7.3 - 7.8. Thus, in this study all blood samples were acidified to pH 6. The extractability of estriol by ether is greater in an acid medium than in a basic medium. This statement is supported by the work of Doisy and Thayer (1931) who encountered estriol in the biologically active fraction from human pregnancy urine that resisted extraction by ether from a weakly alkaline solution. Also, in an extensive survey on this point Engel, Slaunwhite, Carter and Nathanson (1950) have shown that recovery of estriol increases when the pH of the medium is decreased. This difference in extractability is evidently due to the greater hydrophilic character of estriol over estrone or estradiol-17\(^{\alpha}\), rather than their differences in acidic strength, because the PKa of estrone and estriol (9.36 and 9.11 respectively; Feiser and Feiser, 1959) are only slightly different.

In step #1 of Figure 1, 50 ml glass stoppered centrifuge tubes were used for ether extraction. A 100 ml sample is distributed equally in five 50 ml centrifuge tubes and ether extraction carried out. This aids in good recovery of the top ether layer since the tubes are centrifuged each time after extraction with fresh portions of ether. Most of the procedures for estrogens require that the crude
(1) Blood (100 ml.)
adjusted to pH 6.0 with .05N H₂SO₄ extracted
3x with equal volumes of ether.

Aquous phase
discarded

(2) Ether phase
evaporated to dryness in N₂ stream, picked
up in warm 70% methanol (3x10 ml.) strained
through a plug of glass wool, kept at -21°C
overnight, and then centrifuged for 30
minutes at -10°C. Supernatant transferred
to new tube, warmed to room temperature and
finally shaken with 10 ml. n-heptane.

n-Heptane
layer discarded.

(3) Methanol solution evaporated in vacuum oven or
forced air drying oven.

(4) Dried residue dissolved in toluene (30 ml.).
Acidic and phenolic fraction extracted with
1.0 N KOH. (2x10 ml., 1x15 ml.).

Toluene layer
discarded.

(5) Aquous layer backwashed once with 10 ml. of toluene.
The aquous layer was adjusted to pH 3-3.5 with
65% H₂SO₄.

(6) Acidified aquous layer is extracted 4x10 ml. of ether.
The ether layer is reduced to a final volume
of approx. 20 ml. This is shaken with 2 ml.
of 6% NaHCO₃, and the bicarbonate layer is
discarded. The ether is washed with 2 ml. of
distilled water, passed through anhydrous sodium
sulphate plug, and finally, evaporated to dryness.

Residue for chromatography.

Figure I. Flow sheet for extraction and purification of blood.
ether extract be shaken with a sodium bicarbonate solution. This was not possible with turkey blood, which always formed a white emulsion which was difficult to break, even after centrifugation. This was obviously due to extensive amounts of lipids, phospholipoproteins and other non steroidal "gunk" which is present in avian blood. Thus, to eliminate these huge amounts of extraneous material, the dried ether extract was dissolved in warm 70% methanol, (step #2, Figure 1) which precipitated a yellow slimy material that was easily separated by straining the methanol solution through glass wool. The methanol solution was then kept overnight in a freezer (-21°C) to precipitate the lipids. The precipitate obtained at -21°C was almost white. This "freezing out" technique in 70% aqueous methanol has been used to purify estrogens from tissues by Diczfalussy, Cassmer, Alonso and Montserrat (1961) and Mitchell and Davies (1954). It has also been used as a preliminary step for purification of other steroids in the blood by Yannone, McComas and Goldfien (1964), and many others. A partition between 70% aqueous methanol and n-heptane transferred the remaining lipids to the heptane layer which was discarded. After partitioning acidic and neutral portions by the use of toluene and 1.0 N KOH, the aqueous layer was acidified to pH 3-3.5 and extracted with ether. It was in step #6 of the extraction procedure that the ether extract was shaken with 6% NaHCO₃. This concentration of sodium bicarbonate removed the maximum amount of interfering fluorescent materials with a minimum removal of estriol. Florkin and Stotz (1963) have shown distribution of estrogens in ethyl ether and various aqueous
phases made up with acid, sodium bicarbonate, sodium carbonate and sodium hydroxide. It was shown by them that 3%, 10% sodium carbonate and 0.1 N sodium hydroxide removed estriol from the ether phase. However, 5% sodium bicarbonate did not. Mellin (1965) had shown that 9% sodium bicarbonate did not remove estriol from the ether phase. In the present study, 6% sodium bicarbonate has been used to remove the pigments effectively without removing estriol from the ether phase. After a final wash with distilled water (10% by volume), the ether is passed through a plug of anhydrous sodium sulphate and evaporated to dryness. A "visibly clean" tube is obtained when the extraction procedure is carried out as described.

E. Chromatographic separation of estrogens

Column chromatography was carried out by a modification of the procedure of Nakao and Aizawa (1956). Glass columns were made by cutting the top off 25 ml. delivery pipettes and placing a small glass wool plug at the tapered end. The columns were then stoppered below by corks, filled with 8 ml. of benzene and subsequently packed with 0.6 gm. of aluminum oxide (neutral of Activity 1, Brinkmann Instruments Inc., Long Island, N. Y.) which was poured from the top and was allowed to settle. The corks were then removed and the benzene allowed to flow. Frequent tapping of the stem of the column by a pencil aided in formation of a good column. After benzene flows through, the sample is placed on the column (2 ml. in benzene). The column was washed subsequently with 6 ml. of 1% ethanol in benzene (V/V). Estrone, estradiol-17β and estriol are then successively eluted with 4 ml.
portions of 1%, 5% and 30% ethanol in benzene (V/V) and the eluates collected separately in 15 ml. glass stoppered centrifuge tubes. Into the fractions, 4 ml. of 5% hydroquinone solution is added (delivering 20 mg. of hydroquinone) and the fractions evaporated in a vacuum oven. Two aqueous blanks, two standards and two equivalent volume aliquots of a pooled blood sample from castrated male turkeys were run in parallel columns along with the blood samples.

The flow rate of the columns was such that it took approximately 9 minutes for 1 ml. of eluate to pass through the column. Thus, each of the three eluates (4 ml. portions of 1%, 5% and 30% ethanol in benzene) took approximately 36 minutes to flow through. Since packing and washing of the column required an extra 16 ml. of solvent generally about 5 hours were required to complete the chromatographic step.

The columns must always be kept covered with solvent. Usually, the solvents are run through the column until their surface is just above (2 cm.) that of the top of the adsorbent, and then the next solvent is added.

F. Fluorescence reaction

The procedure for induction of fluorescence and its subsequent extraction into an organic phase as described by Mahesh (1964) was adopted for this study. Although the procedure was similar, the technique differed slightly. The Kober reaction was carried out by heating the dried extracts containing 1 ml. of 65% sulphuric acid at 100°C in a sterilizer for 45 minutes. The samples were
rapidly cooled in a freezing brine (-15°C) bath and 1.7 ml. of chilled distilled water added to each tube. The rack containing the tubes was then shaken. To each tube 3.5 ml. of pre-cooled 2% p-nitrophenol solution in 1% ethanol in chloroform (V/V) was added. The tubes were stoppered and shaken vigorously for 20 seconds. The tubes then were centrifuged for 3 minutes at -10°C, the aqueous layer removed and the tubes set again in the brine bath until the fluorescence was read. The fluorescence was read in a Turner Fluorometer (Model 110) equipped with a high sensitivity kit and 110-851 lamp, using a combination of Wratten 2A (passing wavelength longer than 415 mu.) and 61 (dominant wavelengths 533 mu.) as primary filters; and 23A (passing wavelength longer than 570 mu.) as a secondary filter.

G. Calculations

The quantity of each estrogen present in whole blood was calculated by the formula:

\[
\text{ug of estrogen/100 ml of blood} = \frac{\text{sample reading} \times \text{ug of standard} \times 100}{\text{standard reading} \times \text{volume of blood}}
\]

The values reported were uncorrected for losses.

H. Bioassay

The intravaginal tetrazolium method of Martin (1960) was used to estimate the biological potencies of the three fractions separated chromatographically. For this, 1.575 liters of heart blood was extracted and purified in 100 ml. aliquots and the chromatographic fractions pooled.

Mice were ovariectomized at 6-7 weeks of age by bilateral flank incisions, the ovaries removed by grasping the ovarian fat and cutting
the uterine horn, a little below its tip. The incisions were closed by suture clips. Two weeks later they were primed by a subcutaneous injection of one microgram of estrone. Mice were screened for a positive response by checking the vaginal smears stained with methylene blue. Only those mice which responded positively with cornified or nucleated cells were used for assay. This bioassay is based on the reduction of water soluble 2,3,5-triphenyltetrazolium chloride to a water insoluble reduction product--formazan, which is soluble in organic solvents. This reduction in the vagina of a mouse has been shown to be specific for estrogens (Martin, 1960) and forms the basis of the highly sensitive estrogen assay developed by him. The method calls for a single intravaginal injection of estrogen and the unknown in solution so that the required dose/mouse was contained in .01 ml. of the solution. Each mouse was injected with .5 mg. of 2,3,5-triphenyltetrazolium chloride in .01 ml. of water 23.5 hours after the estrogen injection, and 30 minutes later the mice were sacrificed. The vaginas were removed, cut open, washed with distilled water to remove the excess tetrazolium chloride, and dried on filter paper. They were then placed in 4.5 ml. of 3:1 ethanol:tetrachlorethylene solution. The reduction product was estimated spectrophotometrically at 500 μm using a Backman DU spectrophotometer equipped with a Gilford absorbance kit. All intravaginal injections were given using an "Alga" micrometer syringe assembly (Burroughs and Wellcome, Tuckhow, N. Y.) which delivers .01 ml. of liquid in one complete revolution with a claimed accuracy of ± 0.05 μl. The needle was a special
intravaginal needle (gift from Dr. L. Martin's laboratory, University of Sidney, Australia) which consists of a 23 gauge hypodermic needle, the end being blocked with solder and has a hole cut in the side 1/16 inch from the distal end.

I. Statistical analyses

The bioassay was a 2 x 3 factorial assay based on 3 dosage levels spaced at equal intervals using 6 mice per point. The transformation $100 \log_{10} (1000 \times \text{optical density})$ was performed and the log transformed data statistically treated by the method outlined by Bliss (1951, 1956).

This statistical treatment uses the orthogonal factorial coefficients and computational constants worked out by Bliss for analyzing a balanced bioassay. The index of precision (Lambda) was subsequently calculated, the magnitude of which determines the efficiency of a given response as a method for assaying estrogens. The smaller the value of Lambda, the greater the inherent precision of the assay technique.
Results

Exploratory studies were undertaken to detect estrogens in blood obtained from the heart and from the indwelling catheter in the posterior vena cava. These early studies employed a different procedure for purification of the crude ether extract of blood than that finally adopted and outlined in the section on methodology. To precipitate the lipo-protein complex from the crude ether extract, 95% ethanol was used instead of the 70% methanol. The latter, however, was incorporated in the purification procedure finally adopted. The 95% ethanol was used because it evaporated much faster than 70% ethanol. However, since the miscibility of n-heptane with 95% ethanol is very high, it was not possible to carry out the n-heptane-alcohol partition in these earlier studies. Due to the absence of the n-heptane-alcohol partition, the ethanol solution remained yellow when it was subjected to evaporation. This pigment added to the fluorescence due to estrogens. When this dried residue was dissolved in toluene, and a toluene-1 N KOH partition carried out to separate acidic and phenolic materials from the neutral components, the aqueous layer was always opaque white. The latter was obviously due to the extraneous material still present in the ethanol solution before evaporation. When the final ether extract in step #6 (Figure 1) was shaken with 6% sodium bicarbonate, the latter turned yellow, although this did not completely remove the yellow pigment because it was still visible when the dried residue was transferred onto the alumina column.
The chromatographic step in these earlier studies was exactly as described by Nakao and Aizawa (1956), except that the column was made by using a 1 ml. pipette which was attached to a drying tube with teflon tubing. The volume of eluting solvents was reduced from 5 ml. to 4 ml., which, in this technique, was adequate to separate the three estrogens without tailing. After charging the column with the dried blood extract dissolved in two ml. of benzene, estrone, estradiol-17β and estriol were eluted successively with 1%, 5%, and 30% ethanol in benzene.

Table 2 presents data obtained from pooled blood samples collected from the heart and from the posterior vena cava via a catheter. The results show that there was a significant difference (P < .01) for all three estrogens between values obtained from heart blood and from blood collected from the vena cava. Blood from the vena cava had a higher concentration of estrone and estradiol-17β, and lower levels of estriol. The values from the heart blood, however, indicate just the opposite. Here, estriol was in the highest concentration followed with lesser amounts of estradiol-17β and estrone.

To validate that the materials isolated as described above had estrogenic activity, a bioassay (Martin, 1964) was performed. Table 3 summarizes the bioassay data obtained from 1.575 liters of heart blood and assayed for estrone, estradiol-17β and estriol. In terms of precision the estrone (λ = .07) and estradiol (λ = .06) bioassays were good. Statistically, these were valid assays, since variance for the combined slope for both estrone and estradiol-17β were significant (P < .01). Deviations from parallelism for the estrone
TABLE 2

ESTROGEN CONCENTRATIONS IN POOLED BLOOD SAMPLES OBTAINED FROM HEART AND VIA A CATHETER IN POSTERIOR VENA CAVA

<table>
<thead>
<tr>
<th>Source of Blood</th>
<th>Estrone*</th>
<th>Estradiol-17*</th>
<th>Estriol*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catheter</td>
<td>.24 ± .01</td>
<td>.23 ± .02</td>
<td>.16 ± .01</td>
</tr>
<tr>
<td>(9 determinations)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart</td>
<td>.15 ± .01</td>
<td>.16 ± .01</td>
<td>.29 ± .002</td>
</tr>
<tr>
<td>(20 determinations)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Difference highly significant P < .01.

(a) Mean estrogen values ± standard error.
assay were well within the experimental error ($s^2$). Although variance for the slope for estriol was significant ($P < .01$), the slope was less steep, which resulted in poor precision ($\lambda = .18$). The bioassay showed that the isolated material had estrogenic activity. Comparison of estrogen concentration obtained by bioassay with those obtained with chemical procedures were criticized by Paulsen (1965). In this preliminary study, the concentration of estrogens obtained with bioassay (Table 3) were approximately 1/8th the values obtained by chemical means (Table 2).

Because of this large discrepancy between values obtained with the bioassay technique and the chemical procedure, further studies to improve the specificity of the chemical procedure were instituted. It was reasoned that the blood from castrated male turkeys (6 weeks old) should contain little or no estrogens. Analysis with the above chemical technique showed .018 ug percent estrone, .018 ug percent estradiol-17\(^\beta\), and .025 ug percent estriol. Because this indicated the method to be very non-specific, the entire purification and chromatographic procedure was re-investigated. First, it was found that 70% methanol precipitated lipoproteins as did 95% ethanol. However, when 70% methanol was stored in the freezer overnight, an additional precipitate was formed. With the use of 70% methanol, it became possible to perform a simple partition between the 70% methanol and n-heptane which almost completely transferred the yellow pigment into the n-heptane layer, which was discarded. The column of Nakao and Aizawa (1956) was replaced by the alumina column used by Touchstone and Murawec (1965). This column requires
TABLE 3
BIOASSAY CHARACTERISTICS OF ESTROGENS ISOLATED FROM BLOOD OF LAYING TURKEY HENS (Martin's Mouse Bioassay)

<table>
<thead>
<tr>
<th>Dose of Estrogens (nanograms)</th>
<th>Mean optical density</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Estrone</td>
<td>Estradiol-17β</td>
</tr>
<tr>
<td>0.0</td>
<td>.003</td>
<td>.002</td>
</tr>
<tr>
<td>.5</td>
<td>.015</td>
<td>.025</td>
</tr>
<tr>
<td>1.0</td>
<td>.026</td>
<td>.036</td>
</tr>
<tr>
<td>2.0</td>
<td>.031</td>
<td>.050</td>
</tr>
</tbody>
</table>

Equivalent volume of blood (ml.)

<table>
<thead>
<tr>
<th></th>
<th>Estrone</th>
<th>Estradiol-17β</th>
<th>Estrifol</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.25</td>
<td>.021</td>
<td>.037</td>
<td>.030</td>
</tr>
<tr>
<td>10.50</td>
<td>.036</td>
<td>.052</td>
<td>.031</td>
</tr>
<tr>
<td>21.00</td>
<td>.045</td>
<td>.063</td>
<td>.041</td>
</tr>
</tbody>
</table>

Ug/100 ml.

<table>
<thead>
<tr>
<th></th>
<th>Estrone</th>
<th>Estradiol-17β</th>
<th>Estrifol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>.021</td>
<td>.020</td>
<td>.030</td>
</tr>
</tbody>
</table>

a. Error term ($s^2$) 21.330 8.000 19.880

a. Slope (b) .58.55 b 44.00 b 24.86 b

a. Lambda ($s^2/b$) .070 .064 .180

**a.** Values obtained after 100 log$_{10}$ (1000 x optical density) transformation.

b. Variance highly significant ($P < .01$).
1 gm. of activated alumina which is washed with 30 ml. of 1% ethanol in benzene. Estrone and estradiol-17\(^\gamma\) are then eluted with 10% ethanol in benzene and estriol with 50% ethanol in benzene. This column, although efficient, was abandoned on two grounds: (1) 30 ml. of 1% ethanol in benzene required approximately 9 hours to elute, (2) Even 20 ml. of 10% ethanol in benzene was not sufficient to elute estrone completely with the brand of alumina used in the present study. The latter problem may have been due to difference in activity grades of alumina. Thus, preliminary experiments were conducted in which 0.6 gm. of alumina was used, and 6 ml. of 1% ethanol in benzene was arbitrarily chosen to wash the column. Surprisingly, both conditions were found to be near optimal for the subsequent elution of estrone, estradiol-17\(^\gamma\) and estriol. These column modifications were finally retained. The eluting characteristics of estrone, estradiol-17\(^\gamma\) and estriol were studied in the columns thus modified which then formed the basis for the final column standardization. All columns in this study were washed with 6 ml. of 1% ethanol in benzene after 0.1 ug. of estrone, estradiol-17\(^\gamma\) or estriol was transferred on to separate columns.

For eluting estrone the next 4 ml. of 1% ethanol in benzene was collected in 0.5 ml. aliquots from the estrone column and the fluorescence reaction carried out as described in the methodology section. Figure 2 presents the eluting characteristics of estrone from the column. It is clear from the figure that the last 4 ml. of 1% ethanol in benzene recovered almost all the estrone, the recovery being 98.6 percent.
FIGURE 2. Standardization of column. I. Eluting pattern of estrone.

FIGURE 3. Standardization of column. II. Eluting pattern of estradiol-17β. The broken portion in the line indicates the column was washed with 6 ml. of 1 percent ethanol in benzene.
The column for estradiol-17β was similarly washed with 6 ml. of 1% ethanol in benzene and then an additional 4 ml. of the same solvent collected in one tube. Subsequently, 4 ml. of 5% ethanol in benzene was added to the column and 0.5 ml. aliquots collected. Fluorescence was determined as previously described. Figure 3 shows the eluting characteristics of estradiol-17β. The results show that estradiol-17β was eluted completely by 3.5 ml. of 5% ethanol in benzene, indicating that estradiol-17β eluted in a more compact band than estrone. This is also indicated by the high fluorescent activity found in the second, third, and fourth 0.5 ml. aliquots. No fluorescence was detected in the 4 ml. of 1% ethanol in benzene collected prior to elution of estradiol-17β, indicating good separation from estrone. Similarly, no fluorescent activity was found in 4 ml. of 30% ethanol in benzene added after elution of estradiol-17β, indicating good separation from estriol.

To study the eluting characteristics of estriol, the column was washed with 6 plus 4 ml. of 1% ethanol in benzene, and then 4 ml. of 5% ethanol in benzene was added, and this was collected in one tube. No fluorescence was found in this tube, indicating that estriol did not tail with estradiol-17β in this modified column. Estriol was finally eluted by 4 ml. of 30% ethanol in benzene which was collected in 0.5 ml. aliquots. Figure 4 shows the eluting characteristics of estriol. Again, as in the case of estradiol-17β, 3.5 ml. of the eluting solvent was adequate to recover all of the fluorescence from the column. The recovery in this case was 95%.
FIGURE 4. Standardization of column. III. Eluting pattern of estriol. The broken line indicates the column was washed with 10 ml. of 1 percent ethanol in benzene.
The recoveries obtained in the modified chromatographic procedure for estrone, estradiol-17β and estriol (98.6, 97.5 and 95 percent) are excellent. This is not surprising, since column chromatography on active alumina had given similar recoveries in the work reported by Touchstone and Murawec. The columns so standardized are an improvement over the columns of Nakao and Aizwa for two reasons: (1) The use of 6 ml. of 1% ethanol in benzene as a preliminary wash reduces the size of the blank. This may be due to some contamination of alumina itself, which is eliminated by washing with a comparatively more polar solvent than benzene alone. (2) The column more efficiently eliminates interfering fluorogenic contaminants and contributes to the eliminating of the interfering non-estrogenic fluorescence from blood obtained from castrated male turkeys. This is evident from exactly the same fluorescent response repeatedly observed in aqueous blanks and blood obtained from castrated male turkeys (Table 6).

Recoveries of estrogens added to pooled heart blood from castrated male turkeys, using the modified purification and chromatographic procedure:

To test the precision, accuracy and sensitivity of the entire modified procedure as outlined in the section on methodology, a series of recovery experiments were performed by addition of 0.1, 0.05 and 0.01 microgram of estrone, estradiol-17β and estriol to 85 ml. aliquots of a pooled blood sample obtained from castrated male turkeys. Duplicate blanks consisting of equal volumes of water and blood in which no estrogens were added were carried through the procedure at the
TABLE 4
RECOVERIES OF ADDED ESTROGENS FROM THE BLOOD OF CASTRATED MALE TURKEYS

<table>
<thead>
<tr>
<th>Estrogen added Microgram</th>
<th>Estrone (a) Percentage Recovery (b) Coefficient of Variation</th>
<th>Estradiol-17B (a) Percentage Recovery (b) Coefficient of Variation</th>
<th>Estriol (a) Percentage Recovery (b) Coefficient of Variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>.1</td>
<td>94.66± .76</td>
<td>91.70± .60</td>
<td>91.13±1.25</td>
</tr>
<tr>
<td>.05</td>
<td>95.79±1.90</td>
<td>90.79±1.90</td>
<td>91.60±1.91</td>
</tr>
<tr>
<td>.01</td>
<td>87.50±3.70</td>
<td>92.82±3.20</td>
<td>90.00±3.69</td>
</tr>
</tbody>
</table>

(a) Mean percentage recovery ± standard error.
Each value represents 12 determinations.

(b) Expressed as percentage. (Snedecor, 1955).
same time. Extraction, purification, chromatography and fluorescence reaction were conducted as described above. The recovery data is presented in Table 4. Recoveries ranged from 87.5 - 95%. The average percentage recoveries for 0.01 ug. of each estrogen although similar to those achieved in 0.1 ug. to 0.05 ug. range, had a higher percentage coefficient of variation (12% to 14%). This undoubtedly is due to low fluorescence and resultant errors due to instrumentation. However, it would be correct to emphasize that since the blank values obtained for both water and blood to which no estrogens were added were exactly the same, the fluorescence obtained by as little as 0.01 ug. is specifically due to estrogens, demonstrating the high sensitivity and specificity of the method. Considering the blank = 65 units (when matched against a dummy) the fluorescence due to .01 ug. of estrone, estradiol-17β and estriol are 11%, 10% and 8% respectively, above the fluorescence found in the blank.

To attain the highest sensitivity in any method based on fluorometry, the fluorescence of a given amount of the material should be high, and that of the blank - low. This would allow the maximum excitation light of the instrument to be used. In this method, 0.1 ug of estrone gives 65 - 70 units of fluorescence even at the lowest excitation light setting. This response depends upon many physio-chemical characteristics of the sulphuric acid-estrogen complex when it is extracted into an organic phase (2% p-nitrophenol in 1% ethanol in chloroform). Strickler, Wilson and Grauer (1961) using this same procedure had shown that the stability of the complex deteriorated with a rapid decrease in
fluorescing energy with time. These workers also noted that the amount of fluorescent activity within the sample varied with the temperature of the solution, the specimen's fluorescence giving a higher value at temperatures below 0°C. In this study constant conditions with regard to time and temperature were rigorously maintained. Since fluorescence remains constant below 0°C, the tubes were held in a -5°C bath prior to reading.

B. Results obtained from individual samples

Table 5 lists typical values accumulated from analyzing 100 ml. of heart blood of individual turkey (laying) hens. The table includes figures for the fluorometric readings obtained, to illustrate the differences between estrone, estradiol-17β and estriol in terms of their fluorescing power. The data clearly show that of the three classical estrogens, estrone is in the highest concentration in the laying hen.

A standard technique adopted for all the estrogen analysis reported in this study included two blanks, two standards (comprising 0.1 ug. each of estrone, estradiol-17β and estriol) and two 100 ml. aliquots from a pooled sample of castrated male turkey blood (see Table 6). These were extracted and purified as described in Figure 1 and run on a chromatographic column parallel with blood samples to be analyzed for estrogens. Table 6 includes the fluorometric values obtained for 0.1 microgram of estrone, estradiol-17β and estriol. When blood from castrated male turkeys underwent the procedures of extraction, purification, and chromatography as outlined in the methodology section, it always gave fluorometric values equal to
that of blanks. This has been repeatedly observed in this laboratory, indicating the high specificity of the method.
TABLE 5.
TYPICAL VALUES OF ESTRONE, ESTRADIOL-17[6] AND ESTRIOL
OBTAINED FROM BLOOD OF ADULT LAYING TURKEY HENS

<table>
<thead>
<tr>
<th>Hen Number</th>
<th>Estrone (ug./100 ml. blood)</th>
<th>Estradiol-17</th>
<th>Estriol (ug./100 ml. blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>537</td>
<td>.020 (13)*</td>
<td>.011 (7)*</td>
<td>.018 (9.5)*</td>
</tr>
<tr>
<td>637</td>
<td>.030 (20)</td>
<td>.025 (15)</td>
<td>.006 (3)</td>
</tr>
<tr>
<td>606</td>
<td>.027 (18)</td>
<td>.008 (5)</td>
<td>.009 (5)</td>
</tr>
<tr>
<td>582</td>
<td>.037 (25)</td>
<td>.008 (5)</td>
<td>.011 (6)</td>
</tr>
<tr>
<td>575</td>
<td>.015 (10)</td>
<td>.020 (11)</td>
<td>.021 (11)</td>
</tr>
<tr>
<td>589</td>
<td>.042 (28)</td>
<td>.022 (13.5)</td>
<td>.011 (6)</td>
</tr>
<tr>
<td>604</td>
<td>.013 (9)</td>
<td>.010 (6)</td>
<td>.004 (3)</td>
</tr>
<tr>
<td>602</td>
<td>.030 (20)</td>
<td>.008 (5)</td>
<td>.009 (5)</td>
</tr>
<tr>
<td>606</td>
<td>.015 (10)</td>
<td>.018 (11)</td>
<td>.009 (5)</td>
</tr>
<tr>
<td>601</td>
<td>.030 (20)</td>
<td>.022 (13)</td>
<td>.012 (7)</td>
</tr>
<tr>
<td>540</td>
<td>.050 (33)</td>
<td>.018 (11)</td>
<td>.007 (4)</td>
</tr>
<tr>
<td>600</td>
<td>.042 (28)</td>
<td>.025 (15)</td>
<td>.011 (6)</td>
</tr>
</tbody>
</table>

Average: .029, .016, .01
Range: .013-.05, .008-.025, .006-.021

* Figures in parenthesis refer to fluorometric units.
<table>
<thead>
<tr>
<th>Fluorometric units</th>
<th>Estrone</th>
<th>Estradiol-17</th>
<th>Estriol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood (castrated male)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Blood (castrated male)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Standard</td>
<td>66</td>
<td>60</td>
<td>53</td>
</tr>
<tr>
<td>Standard</td>
<td>66</td>
<td>59</td>
<td>51</td>
</tr>
</tbody>
</table>

**TABLE 6**

Fluorometric Response of Blood from Castrated Male Turkeys and 0.1 Microgram of Estrone, Estradiol-17β, and Estriol After Setting Blank at Zero
Discussion

By all criteria used, it was concluded that the method described (which is based on extensive purification, chromatographic separation and an improved fluorescence reaction) measures estrogens in turkey blood with good accuracy. To achieve this, elimination of almost all interfering fluorogenic contaminants is necessary. It has been experienced in this laboratory, that the A.C.S. grade reagents are not acceptable unless they are purified. Quenching of fluorescence by foreign substances in the solvents and in the chromatographic material is very important since they introduce a source of error in biological determinations where purification is never perfect to begin with. Moreover, the reagent blank gives only illusory security if the fluorogenic materials change each time the determinations are made. For this reason extreme care was taken to assure the purity of solvents used in this study.

There are numerous reports describing separation of estrogens by paper chromatography, (Mellin, 1965; Mellin, Erb and Estergreen, 1965; Mahesh, 1964; and Bush, 1961). However, column chromatography has the advantage in that the fractions are quantitatively collected directly into a collecting tube. Tait and Tait (1954) have shown that paper chromatographic techniques tend to result in high and variable blanks which lowers the sensitivity of the method. In contrast, alumina adsorption (column) chromatography has the advantage of producing low and consistent blank values. The key to consistent results with column chromatography is the purification of solvents.
These problems with paper chromatography are well illustrated by Mahesh (1964).

The estrogen values obtained in Table 5 are comparable to those calculated from the bioassay. These are truer values than those shown in Table 2. Ozon (1965) using a fluorometric method after partition chromatography has reported values of 1 ug., 0.16 ug., and 0.15 ug. for estrone, estradiol-17β and estriol respectively in 60 ml. of adult chicken plasma in two individual samples reported by him. Data in this study was exclusively on turkeys. However, preliminary data using the methodology outlined here, have shown average values of .09, 0.18 and .006 percent for estrone, estradiol-17β, and estriol respectively. These observations obtained from laying chickens by this method agree with the findings of O'Grady and Heald (1965) and O'Grady (1966). These workers, using a double isotope derivative technique and multiple chromatographic steps had to find radioactivity in the presumptive estriol fraction. Individual samples (whole blood from the heart of laying chickens) analyzed by the methodology outlined here have also shown negligible (majority of the samples reading the same as banks) amounts of fluorescence in the estriol fraction.

Ittrich extraction of Kober chromogens (Ittrich, 1058) as described by Mahesh (1964) has been used in this method to detect estrogens fluorometrically. Since it is based on the methodology of Kober reaction (Kober, 1931) it is claimed that this is a useful extension. More of the possible nonestrogenic but potentially interfering fluoroescing contaminants which are hydrophilic are
left in the aqueous layer giving a final "wash" before final readings are made. The sensitivity is high enough to detect in the neighborhood of .01 micrograms of estrogen.

The choice of bioassay was made on the basis of high sensitivity and specificity. The sensitivity of this bioassay is claimed to be $1 \times 10^{-6}$ ug. Progesterone, testosterone and cortisol at 1000 times highest dose levels of estrone did not increase the vaginal reduction of tetrazolium (Martin, 1960).

In this study the mice were primed with 1 ug. of estrone and only those mice showing positive characteristics of vaginal smears were used. A period of 10 days was allowed to lapse before the sensitive mice were used for the bioassay. The optical density response was lower than that reported by Martin (1964). This may be due to Martin's use of mice within one week after priming. Martin's technique leaves the possibility that mice may have been used even a day or two after priming, resulting in a "carry over" of the estrone injected when the mice were used for bioassay, resulting in higher optical density and more inconsistency.

Secondly, in this study optical density determinations were made in 4.5 ml. of the solvent in contrast to 1 ml. used and recommended by Martin. Also, ethanol was used as a vehicle to deliver all the injections, while Martin used water as a vehicle with very low concentration of ethanol. However, the slopes of the curves for estrone and estradiol-17\(\alpha\) are approximately those which Martin (1965, 1964) has obtained.

In the present study blood was acidified to pH 6 before
preliminary extraction of estrogens with ether. The importance of this was explained in the section on methodology. However, another point worth mentioning is that hydrolysis of the sulphate conjugated steroids is usually accomplished by solvolysis as described by Burstein and Lieberman (1958) or by continuous ether extraction at pH of 1 or less, (Lieberman and Dobriner, 1948). Estrogen sulphates appear to be important intermediates in the metabolism of estrogens. Aldercreutz (1962) and Purdy et al. (1961) have mentioned particularly the importance of estrone sulphate which may be biologically active and could be of great physiological importance. Sulphurylation appears to be an important step in the metabolism of steroids in the chicken. This contention finds support from the recent work of Wyburn and Baillie (1965) who have demonstrated 3 B-hydroxysteroid dehydrogenase activity in the granulosa cells and their intraovular processes and theca interna of the fowl ovarian follicles using pregnenolone sulphate and 17B-hydroxypregnenolone sulphate as substrates. Low acidity was also demonstrated using dehydroepiandrosterone sulphate. These reactions were claimed to be faster than those observed with free steroids. Since Siiteri and MacDonald (1963) have demonstrated that dehydroepiandrosterone sulphate can be metabolized to estrogens, the possibility exists in the avian system for a similar biosynthetic pathway for estrogens. However, until further studies are conducted, no definite conclusions can be made about the true role of these conjugates.
Summary

A method for detection of submicrogram quantities of estrone, estradiol-17β and estriol is described. The method incorporates ether extraction of blood and purification of the dried ether extract by precipitation of lipids and lipid-like material by 70% aqueous methanol in the cold. Pigments are removed by simple solvent partition with n-heptane and with 6% sodium bicarbonate. The three estrogens are then chromatographically separated by alumina adsorption column chromatography. Ittrich extraction of the Kober chromogens is finally used to detect estrogens fluorometrically.

This method has been used to detect estrone, estradiol-17β and estriol in laying turkeys. The data show that estrone is the main estrogen present in heart blood of laying turkeys, with lesser amounts of estradiol-17β and estriol. The method was partially biologically validated by the mouse bioassay.

In conclusion, it is claimed that this method is highly specific, relatively simple, and suitable for routine analysis of turkey blood.
CHAPTER III

EFFECT OF LIGHT ON THE CIRCULATING LEVELS OF ESTROGENS, CORTICOSTERONE, CALCIUM AND FREE FATTY ACIDS, IN ADULT FEMALE TURKEYS

Since Rowan's (1925) initial discovery that increasing day length artificially in late fall and winter induced premature gonadal activity in migratory birds, it has been demonstrated repeatedly that day length exerts a major influence on the reproductive function of birds. This photosexual reflex is one of the most essential mechanisms in the control of avian pituitary gonadotrophic activity, and has been used effectively in controlling reproduction in domestic birds. Adequate general reviews on photoperiodism are available for domestic birds (Fraps, 1959), and for other birds (Farner, 1959 and Wolfson, 1959). Experiments devoted to effects of various light regimes on preconditioning and on subsequent reproduction of turkey hens have been conducted by Harper and Parker (1957, 1960, 1962), Marr et al. (1956), Leighton and Shoffner (1961 a,b), Wilson, Ogasawara and Asmundson (1962), Marsden, Cowen and Lucas (1962), McCartney et al. (1961) and

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Marsden and Lucas (1964). Benoit and Assemacher (1959) have tried to elucidate the physiological mechanism of this peculiar though complex action of light on birds, and have shown that light stimulates the gonadotropic function of the anterior lobe of the avian pituitary by a direct action on the hypothalamus, and also by acting via an oculohypothalamic pathway. A neurohumor secreted and released by the activated hypothalamus is carried out by the hypophyseal portal plexus to activate the gonadotropic function of the anterior pituitary.

Proper reproductive function in birds is completely dependent on an intact hypothalamus, pituitary, hypophyseal portal system and parseminens (Opel and Nalbandov, 1961a,b; Lepkovsky and Yasuda, 1966; Hill, Corkill and Parkes, 1934; and Hill and Parkes, 1934). Thus, gonads of birds, like those of mammals, are responsive only when tropic hormones are released from the anterior pituitary.

Marshall (1960) discussing the avian sexual cycle, has described three successive phases, viz. regeneration (reflected in a sudden loss and subsequent recovery of breeding function); acceleration (characterized by sex hormone production and gametogenesis) and culmination (involving ovulation and insemination). Since this study is confined to the three-week period before the onset of laying, wherein turkeys are stimulated into production (14L, 10D), and which is equivalent to the acceleration phase as described by Marshall, a short description follows. For most species of birds, the accelerator of the sexual cycle is light. In this phase, the bird has attained a physiological condition in which the neuroendocrine apparatus becomes active and secretion of
gonadotrophins begin. These, in turn, activate the gonads and cause the flow of sex hormones which is accompanied by gametogenesis, gross hypertrophy of the oviduct and development of accessory sexual characteristics. Towards the end of the acceleration phase, the testes in the male contain massive numbers of spermatozoa, and the female is ready to begin the final rapid development of follicles that will end in ovulation and ovoposition. Recently, Bacon, Cherms and McShan (1966a,b) have studied the gonadotrophin concentration in pituitaries of turkey hens reared in different light regimens and at various phases of growth and reproduction. Their work shows that when female turkeys come into production, the levels of gonadotrophins are drastically reduced (ten-fold reduction). A more complete picture depicting the changes in the internal physiology of the bird at this stage requires measurement of estrogens. In addition to their profound action on the reproductive process, estrogens play a pivotal role in the regulation of lipid and calcium metabolism in the female bird in this particular period of high metabolic demand.

The involvement of the adrenal cortex in regulating intermediary metabolism, mineral metabolism, stress, reproduction and gonadal interactions suggests its participation in the physiological adaptations of birds to periodic fluctuations (cycles) in the environment. These periodic fluctuations in birds would constitute superb examples of the efficacy of the phenomena denoted by Witschi (1958) as correlative adaptations in the avian species at the time of reproduction. There is a paucity of information
concerning possible cycles of adrenal activity in birds. However, the coincidence of increased adrenocortical activity and gonadal activity is supported by Hohn's (1947) work on the Mallard, (Anas platyrhynchos). Similarly, the adrenal cortex of the Starling (Sturnus vulgaris) has an annual period of cortical hypertrophy which, in males, is concurrent with spermatogenesis (Burger, 1938). Bulbring (1937a,b) studying the sensitivity of adrenalectomized drakes to adrenocortical extracts, found variable amounts of cortical extracts required for survival throughout the year. The requirement for cortical extract to maintain life for a definite time in the spring period of sexual activity was found to be much greater than during the nonbreeding seasons.

The purpose of this study is to correlate possible light-induced variations in circulating estrogens with changes in physiological circulating levels of corticosterone, calcium and total free fatty acids which will contribute to an integrated picture of the over-all changes induced by light. The relative adaptive significance of these responses will be discussed in terms of their physiological importance.
MATERIALS AND METHODS

A. Experimental Design

One hundred twenty eight Williams Bronze female turkeys reared under restricted light conditions (6L, 18D) were distributed equally (16 birds/pen) in 8 pens, and the light increased (14L, 10D) at 30 weeks of age. The first day under the latter light regime constitutes day 1. However, a day before the turkeys were put on the stimulatory light regime (day 0), heparinized blood samples from the heart were collected (approximately 100 ml. or more/bird) from the 16 birds constituting one pen (Pen Number 1). Subsequent batches of blood samples were collected serially from Pen 2, 3, 4, 5, 6, 7, and 8 and on day 3, 6, 9, 12, 15, 18 and 21 respectively.

Thus, in this experimental design, (albeit from different turkey hens) blood samples were available on every third day for estrogen, corticosterone, plasma calcium and plasma free fatty acids determinations for the entire 21 day period before onset of laying.

All blood samples were collected between 1:00 - 2:00 p.m. From each blood sample, a 10 ml. portion was centrifuged and the
plasma was divided into separate tubes for corticosterone (2 ml.), calcium (1 ml.), and free fatty acid (1 ml.) analysis. All blood and plasma samples were kept at -21°C until analyzed.

B. Estrogen Determination

Was as described in Chapter II.

C. Corticosterone Determination

McLaughin et al. (1958) method for quantitative estimation of corticosterone as modified by Brown (1968) for turkey plasma was used. This method entails extraction of 2 ml. of plasma with 15 ml. of absolute chloroform. After the chloroform solution is shaken with 1 ml. of 0.1 N sodium hydroxide, (removing phenolic and acidic contaminants), a 10 ml. portion from this chloroform layer is transferred onto a silica gel column. The column is washed by 20 ml. of 1.8% ethanol in chloroform (1.8 : 98.2) and corticosterone is then eluted by 40 ml. of 5% ethanol in chloroform (5 : 95). Four ml. of concentrated sulfuric acid is then added to the 40 ml. eluent, and the tubes shaken for one minute. After a lapse of thirty minutes, fluorescence is measured, using a Turner Model 110 Fluorometer, equipped with a high sensitivity Kit, No. 110-853 lamp, and using combinations of Turner nos. 3, 48 and a 10%. transmission filter as primary, and Corning #4-94 and Turner 2A-15 as secondary filters.

This method gives values for corticosterone in chicken blood similar to those found recently by the extended method of Frankel et al. (1967). However, unlike their method, the method of Brown (1968) is relatively simple.
D. Determination of Plasma Calcium

A completely automated method employing an Auto Analyser (Technicon Instruments Division, Chauncey, N.Y.) was used for plasma calcium determinations. This method has been described in detail by Kessler and Wolfman (1964). The method depends on primary separation of calcium from interfering materials by dialysis under acid conditions. The dialyzed calcium is then determined colorimetrically, using cresolphthalein complexone in alkaline medium, with absorption measured at 580 mu. At this wavelength response due to magnesium is minimal. The possibility of magnesium interference in avian plasma, like that in humans, is not significant, since Quinn and White (1967) have shown that most of the magnesium ion in both these species is concentrated in erythrocytes rather than in plasma.

For analysis of calcium by this method, samples of turkey plasma were diluted 1:1 with deionized distilled water. Figure 5 shows typical responses obtained with standard solutions containing 5, 7.5, 10, 12.5, 15, 17.5 and 20 milligrams percent calcium, and with five different aliquots from a pooled turkey plasma sample diluted 1:1 with deionized distilled water. The last three peaks are from the same plasma samples except that this time it was diluted 1:1 with a standard solution containing 10 mg. percent calcium. The average deviation from the mean is 0.10 mg. calcium per 100 ml., and the recovery 96 percent. All samples were analyzed in duplicate.
FIGURE 5. Recordings for calcium determination. The numbers on top of the first 7 peaks indicate calcium concentration in milligram percent.
E. Determination of Free Fatty Acids

A slightly modified method for microdetermination of plasma free fatty acids by colorimetry, as described by Kram, Schmidt, Riggio and Gallo (1964) was used to analyze free fatty acids in turkey plasma. The method depends upon primary extraction of free fatty acids in the plasma by the technique of Dole (1956). The dried residue is taken up in 5 ml. of absolute chloroform, 2.5 ml. of cupric nitrate reagent is added, and the tubes are shaken and centrifuged. The upper aqueous layer is aspirated, using a capillary pipette having a fine but slightly bent tip for aspiration. Care was taken to remove the aqueous layer and the disk of protein precipitate completely. After the addition of 0.1 ml. of 0.1% sodium diethylidithio-carbamate and shaking, the absorption was determined at 435 μm, using a Beckman DU Spectrophotometer. Figure 6 shows a typical standard curve obtained after carrying out the entire procedure with 0.2, 0.3, 0.4, 0.5, and 0.6 micromole of stearic acid. Although only small deviations in optical density were observed from day to day, a standard curve was made with each separate analysis, using the above concentrations (in duplicate) of a standard stearic acid solution. All plasma samples were analyzed in duplicate.
FIGURE 6. Typical standard curve for free fatty acid determination.
RESULTS AND DISCUSSION

A. Estrogens

Table 7 presents mean values of estrone, estradiol-17β and estriol obtained in the 21 day period before onset of lay. It is obvious from the data that the concentration of estrone in turkey hens gradually rose from an almost negligible amount (.008 ug.% at day 0) to an average value of .032 ug.% at day 21. The concentration of estriol, in contrast, dropped from .027 ug.% on day 0 to .013 ug.% on day 21. These changes are highly significant \( P < .01 \).

In the case of estradiol-17β however, there was a gradual decrease in concentration to day 9, after which the blood concentration increased. This increase almost paralleled the rise in estrone concentration thereafter (Figure 7). Figure 7 also shows that all three estrogens peaked at day 12, after which there was a significant decrease \( P < .05 \) in all three, to day 15.

The ratio of estrone : estradiol-17β : estriol has changed from 1.0 : 2.3 : 3.4 (day 0) to 1.0 : 0.6 : 0.4 at day 21 after turkeys were subjected to stimulatory light regimes. This change
### TABLE 7

**BLOOD ESTROGEN LEVELS IN THE 21 DAY LIGHTING PERIOD**

<table>
<thead>
<tr>
<th>Time in Days After Lighting</th>
<th>Number of Birds</th>
<th>Estrone</th>
<th>Estradiol-17$^\beta$</th>
<th>Estriol</th>
<th>Estrone + Estradiol-17$^\beta$ + Estriol</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16</td>
<td>.008 ± .0008 $^a$</td>
<td>.018 ± .0016 $^a$</td>
<td>.027 ± .0023 $^a$</td>
<td>.053 ± .0034 $^a$</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>.015 ± .0018</td>
<td>.016 ± .0018</td>
<td>.028 ± .0027</td>
<td>.059 ± .0044</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>.017 ± .0015</td>
<td>.015 ± .0018</td>
<td>.022 ± .0012</td>
<td>.053 ± .0030</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>.019 ± .0019</td>
<td>.013 ± .0018</td>
<td>.012 ± .0013</td>
<td>.044 ± .0042</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>.028 ± .0032</td>
<td>.025 ± .0023</td>
<td>.014 ± .0012</td>
<td>.068 ± .0052</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>.018 ± .0027 $^b$</td>
<td>.014 ± .0013 $^b$</td>
<td>.011 ± .0012 $^b$</td>
<td>.043 ± .0040 $^b$</td>
</tr>
<tr>
<td>18</td>
<td>16</td>
<td>.026 ± .0023</td>
<td>.016 ± .0013</td>
<td>.011 ± .0007</td>
<td>.053 ± .0027</td>
</tr>
<tr>
<td>21</td>
<td>16</td>
<td>.032 ± .0026</td>
<td>.017 ± .0014</td>
<td>.013 ± .0008</td>
<td>.062 ± .0036</td>
</tr>
</tbody>
</table>

---

$a$) Mean ± Standard Error.

$b$) Significantly different from day 9 to day 12 group (P < .05).
FIGURE 7. Effect of light on estrogen concentration in turkey hens.
in the pattern of estrogens can be interpreted in light of the work done by Chieffi and Botte (1965). These workers have shown the presence of a specific enzyme (DPN dependent 17B hydroxysteroid dehydrogenase) in the granulosa of only the growing and atretic ovarian follicles from the hen's ovary. This enzyme catalyzes dehydrogenation of estradiol-17\(^\beta\) to estrone. The data reported in this study supports and amplifies the findings of Chieffi and Botte on two counts. First, it has been demonstrated for the first time that blood concentrations of estrone rise in the period (21 days) of rapid ovarian development. Secondly, within treatments there is a highly significant \((r = 0.33; \ P < .01)\) correlation between estrone and estradiol-17\(^\beta\) concentrations. The latter implies that, regardless of light regime, the concentrations of estrone and estradiol-17\(^\beta\) are closely related. On the basis of the above observations, it is tempting to predict that the selective increase in estrone concentrations may be due to higher enzymic (DPN dependent 17B HSDH) activity during this period. The above statement finds support from the work of Hawkins and Taylor (1967), who studied the metabolism of estradiol-17\(^\beta\) in the laying hen, and found significant amounts of labeled estrone only in blood plasma after injecting the hen with tritiated estradiol-17\(^\beta\). This rapid conversion of estradiol-17\(^\beta\) to estrone could occur in the ovary, liver or blood.

The role of the liver in this biotransformation becomes important since Ozon and Breuer (1965) first reported the presence of 17\(^\alpha\) and 17\(^\beta\) estradiol dehydrogenase activity in the liver of
laying chickens. Since then, these enzymes have been partially purified from both chicken and turkey livers (Renwick and Engel, 1967). Both of these enzymes catalyze the reversible oxidation of estradiol epimers to estrone, and require NADP⁺ as a cofactor. A possibility exists then, that when birds come into production this enzymic activity increases under the influence of gonadotrophins released from the anterior pituitary, thus ultimately favoring an increased synthesis of estrone as shown in this study. Similar arguments can be advanced for the role played by blood in the biotransformation of estradiol-17β to estrone in birds lighted to production, since the work of Velle (1963) has shown that isolated erythrocytes from the laying hen convert estradiol-17β to estrone.

The dramatic decrease in concentration of estriol can be explained by considering the pathways through which it can arise. In birds, recent data (Ozon and Breuer, 1965; Ozon, 1965; Breuer and Ozon, 1965) support the growing contention that estriol in the blood arises through estrone (estradiol-17β) transformation. The reaction B in this scheme is a three-step reaction and proceeds as estradiol-17β → epiestriol → 16-oxoestradiol-17β → estriol. If this is the chief pathway for estriol biosynthesis in birds, then the explanations provided for increases in estrone biosynthesis in this study fit this scheme well. The diminishing amount of estriol can be accounted for by either inhibition of 16-oxoreductase in the last step of reaction B which consequently, decreases estriol concentration, or due to the poor availability of estradiol-17β per se (the main precursor for
for estriol in this pathway) because the increased 17β hydroxysteroid dehydrogenase activity (as found by Chieffi and Botte, 1965) favors more estrone biosynthesis, (Reaction A).

Partial support for both of these explanations comes from the work of Common and his group (MacRae, Zaharia and Common, 1959; Ainsworth and Common, 1962; and Hertelendy and Common, 1964) who have obtained crystalline amounts of estradiol-17β, estrone, and 16-epiestriol from feces and urine of laying chickens, but failed to obtain crystallized form of estriol from these sources. Future experiments studying enzymic activity as they are influenced by light regime could possibly resolve this problem. Another pathway, not investigated in the avian system so far, that could explain the high concentration of estriol in the blood of birds which are in a restricted light regime and are reproductively quiescent, utilizes free or the sulphate conjugate of dehydroepiandrosterone (DHA), a weak androgen, to produce estriol via (a) estradiol-17β or (b) directly through 16-hydroxyintermediates (Siiteri and MacDonald, 1963). The series of reactions in this scheme can be pictured as shown in Figure 8.

![Figure 8](image.png)

**FIGURE 8.** Scheme for estriol biosynthesis.

The possible presence of this pathway in the bird is supported by the following data. First, Wyburn and Baillie (1966) have shown
that DHA is utilized by granulosa cells of the fowl ovarian follicles. Secondly, in the reproductively quiescent period when the demand for more potent estrogens (estrone and estradiol-17β) is negligible, this pathway can be physiologically important because it circumvents the formation of more potent estrogens during this period by formation of a weak estrogen, viz. estriol, directly through the 16-hydroxy-intermediates. Estriol is also reported to be mildly antiestrogenic, (Miyake, 1964). Thirdly, in the laying period, the bird may, and probably does, secrete an appreciable quantity of androgen for the proper synthesis and secretion of egg albumin. Indirect evidence for this is based on the characteristic comb response (a very specific action of androgens) which a bird shows when it comes to lay. Since DHA may be a key intermediate for the ultimate synthesis of a more potent androgen (testosterone), this pathway may be a physiological mechanism by which the bird keeps its internal environs characteristic of the reproductive phase it is in.

During the reproductive quiescent period it eliminates the formation of more potent estrogens and androgens by synthesizing comparatively large amounts of estriol directly via 16-hydroxyintermediates. When birds are lit for production, increasing the demands for both highly potent androgens and estrogens, this pathway via 16-hydroxyintermediates "shuts off" and a low concentration of estriol results. Partial support for this hypothesis comes from the work of Common et al. (1965) who have shown that daily urinary estrone excretion by the hen is relatively low before laying begins, attains maximal values shortly before on the day of the
first oviposition, falls to somewhat lower levels and drops to extremely low levels immediately on cessation of laying.

B. **Corticosterone**

The effect of light on plasma corticosterone concentration is presented in Table 8 and Figure 9. Analysis of variance shows that during the 21 day period there was a significant change \((P < .01)\) in corticosterone concentration. A significant increase in corticosterone concentration occurred during the first three days of 14 hours light regime which returned to almost base values (values at day 0) by the sixth day. This drop was statistically significant \((P < .05)\) when compared with the preceding mean value at day 3, (Tuckey's method for multiple mean comparison, Snedecor, 1956). When mean values in Table 8 are plotted against the time in days that turkeys were subjected to the stimulatory light regime (Figure 9), it is apparent that after day 6, the concentration of corticosterone has gradually risen, reaching a high value of 2.78 ug. percent by day 21, when the experiment was terminated.

The increase in corticosterone in the blood may be attributed to stress which is obviously inherent in extended activity due to longer lighting periods. To explain this sudden rise and fall on the basis of stress, it can be postulated that the initial rise of corticosterone is due to an increased secretion of ACTH, which subsequently declines by the end of the third day once the bird becomes adapted to the longer day. The subsequent rise in circulating levels of corticosterone may be attributed to the
action of estrogens. Separate correlation analysis between corticosterone and the three individual estrogens show a highly significant positive correlation \((r = 0.31; \ P \ll 0.01)\) between corticosterone and estrone. In laboratory rodents, Kitay and coworkers (Kitay et al., 1965; Kitay, 1963) have demonstrated that estrogen acts directly on the adrenal to stimulate steroidogenesis. Phylogenetically, most of the members of the lower vertebrate class, which include birds, show heightened secretory activity of interrenal glands at the time of rapid yolk deposition, ovulation and spawning (Miller, 1955; Robertson, 1957; Phillips and Chester Jones, 1958; Phillips et al., 1959; and Robertson and Wexler, 1957).

Also, in pigeons, Riddle (1923) demonstrated a 40 percent increase in weight of interrenal tissue at the time nearing ovulation. Thus, it is very likely that increases in corticosterone secretions in turkey hens in the face of rising levels of estrone demonstrate this direct interrelationship of ovary and adrenal activity at the level of organ interactions. However, in interpreting the data of this study on the basis of the above explanation, it must be borne in mind that peripheral levels of corticosterone, as determined in this study, are only indirect evidence that increased synthesis and secretion of adrenocorticosteroids from the adrenal occurred due to estrogen action. Holzbauer (1957) has mentioned that adrenal (glandular) concentration of corticosterone constitutes an adequate index of secretory activity. This study cannot give concrete evidence that increased synthesis and secretion of corticosterone occurred. Future studies conducted by measuring corticosterone
output from the adrenal or by measuring the half life of corticosterone will be necessary to support the contention that light has a significant effect on the adrenal gland by increasing secretion of corticosterone from the adrenal cortex. This contention also finds support by the work of Yates, Herbst and Urquhart (1958), Herbst et al. (1960) and Glenister and Yates (1961) in rats. These workers have shown that peripheral plasma adrenocorticoid concentrations could reflect processes other than secretion, and could be subjected to modification by peripheral mechanisms. In humans the 17-hydroxycorticosteroid increase due to estrogen stimulation is related to a decrease in metabolic transformation, decreased excretion, and increased plasma protein (transcortin) binding of 17-hydroxycorticosteroids, although adrenal secretion of these steroids may have actually been depressed (Cohen et al., 1958; Mills et al., 1960; Peterson et al., 1960; and Robertson et al., 1959).

Transcortin is a specific corticosteroid-binding protein in the plasma. Since it binds corticosterone in equimolar amounts (Seal and Doe, 1962) its binding capacity is an indication of its concentration in plasma. The overall altered steroid (estrone and corticosterone concentration) dynamics found in this study when turkey hens were subjected to prolonged photoperiods, may have increased this specific corticosterone binding protein resulting in a high concentration of corticosterone in the blood. Future studies may help clarify this problem by separate measurements of free and bound corticosterone in the female bird.
C. Plasma Calcium and Plasma Free Fatty Acids

Table 8 presents values for plasma calcium and plasma free fatty acids (FFA). Analysis of variance shows that in the 21 day period of extended light regime, both plasma calcium and FFA changed significantly ($P < .01$). In both cases, a significant difference was apparent from day 3 and onwards. On day 12 however, both plasma calcium and FFA concentrations dropped significantly ($P < .05$).

FFA reached a peak value of 2.43 micromoles percent on day 15 and thereafter registered a gradual drop, which was significant ($P < .05$) by the time the experiment was terminated. The concentration of plasma calcium in contrast, after registering a fall similar to that of FFA on day 12, maintained a gradual rise, reaching a high value of 29.36 milligrams percent on day 21. Figure 9 shows these trends clearly.

Correlations between plasma calcium and FFA ($r = .45$), calcium and estrone ($r = .40$) and FFA and estrone ($r = .40$) were highly significant ($P < .01$) during the 21 day period of gonadal stimulation by increased day length.

In any interpretation of the calcium and FFA data of this study, it should be noted that this experiment was not conducted with the birds already in egg production. The main purpose of this experiment thus, was to study calcium and lipid metabolism by responses observed in plasma calcium and FFA concentration, due to a photosexual reflex action of light on birds coming into production.

The highly significant correlation obtained between FFA and plasma calcium can be attributed to either of two causes: (a) A
TABLE 8
EFFECT OF LIGHTING ON PLASMA CALCIUM, FREE FATTY ACIDS AND CORTICOSTERONES

<table>
<thead>
<tr>
<th>Time (days after lighting)</th>
<th>Number of Birds</th>
<th>Weight (Kg)</th>
<th>Calcium mg. percent</th>
<th>Free Fatty Acids Micromoles/ml.</th>
<th>Corticosterone mcg. percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16</td>
<td>8.64±.13</td>
<td>12.86±1.59</td>
<td>1.14±0.20</td>
<td>1.52±0.14</td>
</tr>
<tr>
<td>3</td>
<td>16</td>
<td>8.92±.18</td>
<td>13.65±1.37</td>
<td>1.13±0.07</td>
<td>2.24±0.15</td>
</tr>
<tr>
<td>6</td>
<td>16</td>
<td>9.25±.15</td>
<td>22.52±1.35</td>
<td>1.70±0.12</td>
<td>1.56±0.09 a)</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>8.85±.15 a)</td>
<td>27.62±1.78</td>
<td>2.39±0.06</td>
<td>1.72±0.17</td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>9.22±.17</td>
<td>24.69±.77 a)</td>
<td>1.80±0.15 a)</td>
<td>2.13±0.10</td>
</tr>
<tr>
<td>15</td>
<td>16</td>
<td>9.11±.14</td>
<td>26.39±0.131</td>
<td>2.43±0.13</td>
<td>2.22±0.19</td>
</tr>
<tr>
<td>18</td>
<td>16</td>
<td>9.40±.17</td>
<td>26.64±0.96</td>
<td>2.32±0.02</td>
<td>2.20±0.15</td>
</tr>
<tr>
<td>21</td>
<td>16</td>
<td>9.72±.12</td>
<td>29.36±0.96</td>
<td>2.15±0.11 a)</td>
<td>2.78±0.17</td>
</tr>
</tbody>
</table>

a) Significantly different from the preceding group (P < .05).
FIGURE 9. Effect of light on plasma corticosterone, calcium and free fatty acids of turkey hens.
characteristic dual action of estrone in mobilizing skeletal calcium and adipose tissue lipids and (b) A hyperphagic condition created by the extended light regime. The latter condition, again, could be caused by hormonal increases of estrone and corticosterone. A discussion follows.

The findings of Urist et al. (1958) demonstrated a ten-fold increase in serum calcium when birds (both male and female) were injected with high doses of estrogens. Along with this rise in blood calcium was an increase in a specific phospholipoprotein complex in blood. Studying the nature of the calcium present in the serum of these estrogenized birds, these workers demonstrated that almost 80 percent of the calcium concentration was bound to this phospholipoprotein complex. Since these lipoproteins were ultimately deposited into ovarian follicles, these workers concluded that the estrogen stimulated increase in blood calcium plays an important part in the transportation of yolk proteins from sites in the liver to ovarian follicles. The method for chemical analysis of plasma calcium used in the present study determines total plasma calcium. Thus, the highly significant positive correlation ($r = 0.45, \ P \leq 0.01$) found between plasma calcium and FFA in this study may indicate the relationship which Urist and coworkers first reported. If this is the case, then the present study gives this relationship a more sound foundation because the simultaneous determinations of physiological levels of estrogen as carried out in this study also have shown a highly significant ($P \leq 0.01$) positive correlation between estrone and plasma calcium and
estrone and FFA separately. This also agrees with the findings of Heald and Rookledge (1964) who observed increases in FFA in birds by estrogen stimulation.

The extreme rise of total plasma calcium as reported in this study cannot be attributed solely to the explanation put forth above on the basis of a highly positive correlation between calcium and FFA. This is so because of a peculiar occurrence of bone structure (medullary bone) in almost all birds, which ultimately is a highly labile source of calcium for the bird at the time of egg laying. This bone formation is dependent on estrogens. Since at this phase of gonadal stimulation the birds are in the process of secreting high levels of estrone, it is highly probable that a heightened formation of medullary bone is also taking place in preparation for the onset of egg laying. What is the ultimate source of this high calcium concentration? The answer is not readily available, although previous work has established that estrogens favor a positive calcium balance in birds. A possible source could be due to photoperiodically induced hyperphagia. Using experimental design similar to that reported in this study, Farner and coworkers (King and Ferner, 1956, 1959; McGreal and Farner, 1956; Farner, 1960; and Farner et al., 1961) and King (1961) have shown a hyperphagic condition in _Z. gambelii_ resulting in fat deposition and weight increase. Thus, the source of high levels of blood calcium may be dietary calcium since the turkey hens in this study also showed a highly significant weight increase (_P < .01_) during the 21 days. The possible effect of estrogens in lipid metabolism, together with an increase in protein anabolism and food efficiency
makes the physiological role of estrogen in the female bird a very important one, and this may be the basis of their use in the poultry industry. Thayer, Jaap and Penquite (1944) also have reported a hyperphagic condition due to an exogenous estrogen treatment in chickens.

The overall pattern in FFA observed in this study support the data of Heald and Badman (1963) in chickens. These authors reported an increase in plasma FFA concentration before onset of egg laying which ultimately dropped to significantly lower levels at the time the first egg was laid. The values reported in this study on turkeys agree with the pattern found by Heald and Badman in chickens.

The sudden drop in FFA concentration just prior to, and at the time of egg laying, does not follow the steady pattern of increase observed in estrone and corticosterone concentration which makes the interpretation of FFA data difficult solely on the basis of increased concentration of these two hormones. However, it should be noted that glucagon is a potent mobilizer of plasma FFA in chickens (Lepkovsky et al., 1967; Heald, 1965). The advent of immunoassays and the availability of glucagon in crystallized state makes it tempting to study the levels of this pancreatic hormone in birds lighted for production in an attempt to clarify the hormonal regulation of lipid and nitrogen metabolism.

Since the caloric requirement of birds probably increases during this period of 21 days before onset of egg lay, as is demonstrated by significant weight increase and high level of fat deposition as reported by other workers mentioned above, it is likely that the
rate of estrification of FFA taken up by the tissues also increased. Rate of esterification is an important determinant of the metabolic fate of FFA because competition may exist between the enzyme system catalyzing esterification and that catalyzing oxidation to supply the caloric requirements of the bird. Future work to study the rate of esterification of FFA by the surviving tissues in birds at various time intervals of an extended light regime would thus help interpret the drop observed in FFA concentration at the time egg laying commenced.

A partial explanation for the drop observed in FFA levels may be due to the fact that the bird, during lay, deposits extensive amounts of fat in the abdominal cavity. Lepkovsky et al. (1967) have shown an inverse relationship between FFA levels and blood glucose concentration in the laying bird. A rise in FFA levels decreases blood glucose concentration significantly and vica versa. Dole (1956) has shown that FFA levels in mammals can be decreased by administration of glucose. Also, it is an established fact that when adipose tissue uses carbohydrate actively the release of FFA is slowed (Felts, 1964). This is thought to be due to the ability of various hormones to alter either esterification reactions or lipolytic activity within the adipose tissue. Epinephrine is known to increase blood glucose concentration in chickens (Sturkie, 1965), while as observed in the case of mammals, it fails to mobilize avian adipose tissue lipids. The work of Wurtman (1966) and Wurtman et al. (1966, 1967) has shown that adrenal corticoids regulate the synthesis and secretion of epinephrine. Data in this study show that
corticosterone levels increase when turkey hens come to lay. This implies that a simultaneous increase in epinephrine concentration probably occurs, which will result in increased blood glucose levels, and thus a resultant increased esterification and decreased mobilization of FFA. This probably means that an enhanced esterification occurs in the laying bird which may give rise to decreased oxidation of FFA because more carbohydrates are available for energy sources. This increased esterification should promote deposition of triglycerides and ultimately decrease rate of free fatty acid mobilization, resulting in low values of FFA as reported by Heald and Badman (1963) and also found in this study.
SUMMARY

One hundred and twenty eight turkey hens reared in a restricted light regime were lighted for production (14L, 10D) at thirty weeks of age. Blood concentration of estrogens (estrone, estradiol-17β and estriol), corticosterone, calcium and total free fatty acids were determined in 16 birds on day 0, 3, 6, 9, 12, 15, 18 and 21 of the extended light regime. In the period of 21 days before start of lay, there was a highly significant (P < .01) increase in estrone, estradiol-17β, corticosterone, calcium and free fatty acids. In the same period however, blood concentration of estriol showed a highly significant (P < .01) decrease. The ratio of estrone : estradiol-17β : estriol changed from 1.0 : 2.3 : 3.4 at day 0 to that of 1.0 : 0.6 : 0.4 on day 21. A highly significant (P < .01) positive correlation was found between estrone and estradiol-17β (r = .33); estrone and calcium (r = .40); estrone and plasma free fatty acids (r = .40); and calcium and plasma free fatty acids (r = .45). Correlation between corticosterone and free fatty acids (r = .20) was also significant (P < .05).
LITERATURE CITED


Bulbring, E. 1937b. "Relation between size of testes and requirement of cortical extract in adrenalectomized drakes," J. Physiol. 91:18P.


Newcomer, W. S. 1959. "Adrenal and blood $\Delta^4$-3-ketocorticosteroids following various treatments in the chick," Amer. J. Physiol. 196:276-278.


Robertson, O. H. and B. C. Wexler. 1959. "Hyperplasia of the adrenocortical tissue in pacific salmon (genus Oncorhynchus) and rainbow trout (Salmo gairdnerii) accompanying sexual maturation and spawning," Endocrinology. 65:225.


Sandor, T., J. Lamoureux, and A. Lanthier. 1963. "Adrenocortical function in birds. In vitro biosynthesis of radioactive corticosteroids from pregnenolone-7-H3 and progesterone-4-C14 by adrenal glands of the domestic duck (Anas platyrhynchos) and the chicken (Gallus domesticus)," Endocrinology. 73:629-636.


