STEROID ESTROGEN, PROGESTERONE AND ANDROGEN CONCENTRATIONS IN THE PLASMA OF THE DOMESTIC FOWL IN RELATION TO THE OVULATION CYCLE

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Andrew James Peterson

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A.J. Peterson

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ABSTRACT

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STEROID ESTROGEN, PROGESTERONE AND ANDROGEN CONCENTRATIONS IN THE PLASMA OF THE DOMESTIC FOWL IN RELATION TO THE OVULATION CYCLE

Concentrations of progesterone, estrone, estradiol, androstenedione and of total androgen in the peripheral blood plasma of laying hens were measured by appropriate saturation analyses. The concentrations of all these steroids attained peak values some two to eight hours before ovulation. There were indications of an earlier minor peak of estrogen at 18 to 22 hours prior to ovulation. None of these peaks of plasma concentration were demonstrable on days when ovulation did not occur. The observed concentration of progesterone ranged from 0.5 to 12.5 ng/ml; estrone from 14 to 242 pg/ml; estradiol from 36 to 364 pg/ml; androstenedione from 98 to 954 pg/ml; and total androgens from 150 to 1100 pg/ml.

RESUME

Doctor of Philosophy Chimie agricole

Andrew James Peterson

LES CONCENTRATIONS DE L'OESTROGENE STEROIDE, DE LA PROGESTERONE ET DE L'ANDROGENE DANS LE PLASMA SANGUIN DE LA POULE EN RELATION AU CYCLE OVULAIRE

On a dosé les concentrations de l'oestrone, de l'oestradiol, de l'androstènedione et de l'androgène totale dans le plasma du sang périphérique de la poule pondeuse par des méthodes convenable de 'saturation analysis'. Les concentrations de tous ces steroïdes atteignaient des valeurs maximales entre deux et huit heures avant l'ovulation. Il y avait des indices d'une valeur maximale de l'oestrogène entre dix-huit et vingt-deux heures avant l'ovulation. Aucune de ces valeurs maximales n'etait démontrable aux jours ou une ovulation n'avait pas lieu. Les concentrations de la progestérone avaient une gamme de 0.5 jusqu'à 12.5 ng/ml; l'oestrone, de 14 jusqu'à 242 pg/ml; l'oestradiol, de 36 jusqu'à 344 pg/ml; et de l'androgène totale, de 150 jusqu'à 1100 pg/ml.

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GLOSSARY

(a) Systematic Nomenclature of the Steroids Mentioned in Either a Trivial or Abbreviated Form in This Thesis.

Trivial	Systematic Name
cholesterol	cholest-5-en-3β-ol
corticosterone	11β,21-dihydroxypregn-4-en-3,20-dione
pregnenolone	3β-hydroxypregn-5-en-20-one
17α-hydroxypregnenolone	3β,17α-dihydroxypregn-5-en-20-one
progesterone	pregn-4-en-3,20-dione
17α-hydroxyprogesterone	17α-hydroxypregn-4-en-3,20-dione
20α-hydroxyprogesterone	20α-hydroxypregn-4-en-3,20-dione
20β-hydroxyprogesterone	20β-hydroxypregn-4-en-3,20-dione
pregnanedione	5β-pregnan-3,20-dione
allopregnanedione	5α-pregnan-3,20-dione
androsterone	3α-hydroxy-5α-androstan-17-one
dehydroepiandrosterone	3β-hydroxyandrost-5-en-17-one
androstenediol	androst-5-en-3β,17β-diol
5β-androstanedione	5β-androstan-3,17-dione
androstenedione	androst-4-en-3,17-dione
11β-hydroxyandrostenedione	11β-hydroxyandrost-4-en-3,17-dione
testosterone	17β-hydroxyandrost-4-en-3-one
11-ketotestosterone	17β-hydroxyandrost-4-en-3,11-dione
19-hydroxytestosterone	17β,19-dihydroxyandrost-4-en-3-one

Trivial	Systematic Name
dihydrotestosterone	5α-androstan-17β-ol-3-one
etiocholanolone	3α-hydroxy-5β-androstan-17-one
estrone (E_1)	3-hydroxyestra-1,3,5(10)-trien-17-one
6α-hydroxyestrone (6α-OHE_1)	3,6α-dihydroxyestra-1,3,5(10)-trien-17-one
15α-hydroxyestrone (15α-OHE_1)	3,15α-dihydroxyestra-1,3,5(10)-trien-17-one
16-ketoestrone (16ketoE_1)	3-hydroxyestra-1,3,5(10)-trien-16,17-dione
estradiol (E_2)	-
estradiol-17α ($E_2$17α)	estra-1,3,5(10)-trien-3,17α-diol
estradiol-17β ($E_2$17β)	estra-1,3,5(10)-trien-3,17β-diol
16-ketoestradiol-17β (16keto$E_2$17β)	3,17β-dihydroxyestra-1,3,5(10)-trien-16-one
estriol (E_3)	estra-1,3,5(10)-trien-3,16α,17β-triol
16-epiestriol (16epiE_3)	estra-1,3,5(10)-trien-3,16β,17β-triol
16,17-epiestriol (16,17epiE_3)	estra-1,3,5(10)-trien-3,16β,17α-triol
17-epiestriol (17epiE_3)	estra-1,3,5(10)-trien-3,16α,17α-triol

(b) Abbreviations Used in This Thesis.

CBG	corticosteroid-binding globulin
CPB	competitive protein-binding
FSH	follicle-stimulating hormone
FSH-RF	follicle-stimulating hormone releasing factor

GLPC	gas-liquid partition chromatography
LH	luteinizing hormone
LH-RF	luteinizing hormone releasing factor
RIA	radioimmunoassay
SHBG	sex hormone binding globulin
TLC	thin-layer chromatography

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GENERAL INTRODUCTION

The elucidation of the mechanisms which control the ovulatory cycles of female birds is one of the more interesting problems in comparative endocrinology today. Many observations and experiments have indicated that the hypothalamo-pituitary-gonadal axis is one of the primary control mechanisms in the reproduction of all vertebrates. The common domestic hen is, however, the only avian species for which there is a reasonably coherent perspective of the processes involved in ovulation.

Although the exact relationships have not been resolved, it is almost certain that ovulation in the domestic hen is contingent upon the release of LH from the pituitary, this release being mediated by the hypothalamus. The gonadal hormones, that is, the androgens, estrogens and progestins, have been postulated as being of major importance in the control of this LH release. When the present author began his research, no information was available as to the circulating levels of these gonadal steroids during the ovulatory cycle.

The present thesis is concerned with the extension of knowledge regarding the variations in plasma concentrations

of ovarian hormones that occur throughout the ovulatory cycle of the domestic hen. This thesis, therefore, includes the following three studies:-

(a) A study of the concentration of progesterone in peripheral plasma of the laying hen in relation to ovulation.

(b) A study of estrone and estradiol concentrations in peripheral plasma during the ovulatory cycle of the domestic hen.

(c) A study of plasma levels of total androgen and also of androstenedione in the domestic hen throughout the ovulation cycle.

PART I

HISTORICAL REVIEW

CHAPTER I

THE NATURE AND OCCURRENCE OF OVARIAN STEROIDS IN THE NON-MAMMALIAN TETRAPODA

I.1. General Introduction

Comparative studies, whether they concern physiology, anatomy or endocrinology, often lead to a fuller understanding of the origin, and subsequent evolution, of many animal systems. The complex organization of birds and mammals, for example, is almost certainly the outcome of decisive evolutionary steps which occurred at some early stages of vertebrate history. To some extent, one may investigate these important changes by examining the "lower" vertebrates of today; and there is a sense in which the organization of the "higher" vertebrates cannot be fully understood without some understanding of the organization of the "lower" forms.

These comparative studies, however, are fraught with difficulty when one takes account of the extremely wide variety of animal organization. Adaptive specialization, parallel and convergent evolution, homologous and analogous systems, and the development of homeothermy are only a few of the phenomena which further complicate the study. Only by careful judgement can one hope to disentangle those

systems which are of primary significance from those which have been added as later refinements.

In the past, the comparative endocrinologist based his studies on the manner in which the "lower" vertebrates resembled the mammals. This was due primarily to the relative lack of data on the endocrine systems of these "lower" forms as compared to mammals. The bias resulting from this original approach can be misleading; for if one accepts current concepts of the evolution of mammals from fish, then one should likewise compare the endocrinological developments, rather than the reverse. That is to say, one should not concentrate solely on annotating the endocrinological similarities between the mammals and the remaining vertebrata, but also on the dissimilarities, because the dissimilarities are quite possibly more relevant to the evolution of the endocrine systems.

This literature review is restricted to an examination of the comparative function of the gonadal hormones in the reproduction of the non-mammalian Tetrapoda. The mammals are excluded, mainly because of the vastness of the subject if they were so included, and also to reduce any comparative bias. In the interests of brevity, the review is further restricted to the nature and occurrence of gonadal hormones

and their relationship to ovulation.

It is hoped that such a review may contribute to a better understanding of the evolution of the reproductive processes from amphibia to birds. It is also hoped that the review will permit a greater insight into the hormonal control of ovulation in the domestic hen, the subject of the thesis.

I.2. The Nature and Occurrence of Ovarian Steroids in Amphibia

I.2.1. Introduction

The comparative endocrinology of the amphibia has been confined largely to studies on the hormonal control of metamorphosis. A recent analytical survey of the literature on the endocrinology of the axolotl, Ambystoma mexicana, from 1876 onwards, has revealed some 227 articles (270). No less than 126 of these are devoted to the inter-relationship between metamorphosis and the thyroid gland. Only four accounts deal with steroid hormones of any sort, and of these two are concerned with the effect of androgens and estrogens upon experimental metamorphosis, and the other two deal with the relationship between gonadal hormones and sexual differentiation in the embryo. While this example pertains to only one species, and this unique in the animal world, it does

indicate the general trend of research on the comparative endocrinology of amphibia.

Most of the available information concerning the ovarian steroids of amphibia has been gained over the past ten years. Various steroids have been reported as present in amphibia, but the exact identification of some is questionable. Not all authors have applied sufficiently stringent chemical criteria before claiming the identity of an isolated compound. This review will distinguish, therefore, between those reports in which the identification seems reasonably certain and those where the identification is doubtful or at least tentative. Several reviews on this topic are available (16,210,227,288).

I.2.2. Steroid Estrogens

Knowledge concerning the steroid estrogens of amphibia has not advanced much since van Tienhoven (288) stated that the evidence for their presence was fragmentary and largely indirect. Most of the information concerns the estrogens of the ovary. The plasma estrogens have not been studied to the same extent.

The first report of an estrogenic substance extracted from an amphibian ovary was that of Grant (110). This worker did not give any reference to his analytical technique and

his finding must be accepted with reservation.

The estrogens $E_2$17β and E_1 were detected in extracts of whole ovaries of Xenopus laevis by Gallien and LeFoulgoc in 1960 (101). Their identification procedure included celite-column chromatography and fluorometric measurement of the fractions thus separated. The validity of this technique is questionable, however, because it has been pointed out recently that an identical method overestimates the steroid estrogens in the blood of the domestic hen (254).

Chieffi and Lupo (42) detected $E_2$17β, E_1 and E_3 in ovarian extracts of Bufo vulgaris. Their identification is provisional, having been based on paper chromatography of acetylated derivatives in only one solvent system.

Gallien and LeFoulgoc (101) mentioned briefly that the total steroid estrogen concentration in the blood of adult female X. laevis was 4 μg/100 ml. Cedard and Ozon (36) presented evidence for the presence of estrogens in the blood of Rana temporaria. The concentrations reported by them for E_1 (0.1 μg/100 ml), $E_2$17β (0.3-0.5 μg/100 ml) and E_3 (0.3-0.7 μg/100 ml); and the value of Gallien and LeFoulgoc cannot be regarded as other than tentative, being based on the rather unspecific method of celite chromatography and sulphuric acid fluorescence. There is no doubt that Gallien and LeFoulgoc were measuring estrogenic activity as evidenced by their

application of the Allen and Doisy (11) bioassay to their extracts. The absolute identification, however, and the concentrations of the steroid estrogens in X. laevis, B. vulgaris and R. temporaria remain doubtful.

Dale (67) reported finding $E_2 17\beta$ in the urine of R. sylvatica and of R. pipiens larvae. The chemical evidence for the presence of $E_2 17\beta$ was fairly definite, having been based on paper chromatography in two solvent systems and on a positive reaction to the Kober reagent.

Since the report of Chieffi and Lupo (42) there have been four publications concerning the estrogens of amphibia. Polzonetti-Magni et al. (236) have reported the presence of $E_2 17\beta$ and E_3 in the plasma of female R. esculenta during three successive animal cycles. The identity of the estrogens was established by the coincidence of gas-liquid partition chromatographic (GLPC) times with those of standards and their acetates. The amounts of E_3 varied from 1.1 to 3.6 μg/10 ml and those of $E_2 17\beta$ from 1.1 to 2.3 μg/10 ml plasma. The levels of both estrogens were so reduced after ovariectomy that they were no longer detectable. Estriol and $E_2 17\beta$ have also been stated as being present in the newt, Triturus cristatus (237).

Recently Di Prisco et al. (238) studied the ovary and

the associated fat bodies in T. cristatus carnifex. Using GLPC to measure the steroids isolated by TLC and also GLPC of acetylated and oxidative derivatives, they were unable to detect any estrogen in the ovary but measured E_1 in the fat body at a concentration of 4 μg/62 g. More recently Ozon et al. (229), using electron capture gas-liquid chromatography, have reported $E_2 17\beta$ at a concentration of 0.06 μg/100 ml in the plasma of Pleurodeles waltlii.

It is obvious from the foregoing survey that our knowledge of the steroid estrogens in amphibia is very limited. In extremely few instances has the identity of the steroid been established with reasonable rigour. This situation represents a gap in our understanding of comparative endocrinology and offers an excellent opportunity for further intensive research.

I.2.3. Progestins

If the data concerning the amphibian estrogens are fragmentary, then our knowledge of the progestins found in amphibia is almost non-existent. Chieffi and Lupo (42) reported the detection of progesterone in B. vulgaris. Since only one solvent system was used in the paper chromatographic identification, the reported concentration of progesterone of 16 μg/kg ovary must be regarded as purely tentative.

Progesterone is implicated in ovarian steroido-genesis and it is also becoming increasingly evident that progesterone plays an important role in amphibian ovulation. These two topics will be considered in later sections. This indicates indirectly that progesterone, or at least progestins, is most likely present in the ovary and blood of amphibia but as yet there has been only one unequivocal report of its existence.

Di Prisco *et al.* (238) measured progesterone in the ovary and fat body of *T. c. carnifex* by GLPC and adequate derivativization. They detected 2 μg of progesterone in 210 g of ovarian tissue and 10.1 μg in 62 g of fat body.

Recently Thornton (286), using a bioassay (285), provided evidence for a progesterone-like factor in the blood of gonadotrophin-primed females of *B. bufo*. The bioassay was based upon the stimulation of the meiotic division of *Xenopus* oocytes *in vitro* and although the method lacks absolute specificity, it would seem to permit detection of progestins in the plasma.

I.2.4. Androgens

The evidence for the presence of androgens in amphibia is based primarily on *in vitro* metabolic studies and will be discussed in the relevant section. Until recently, there was no direct evidence concerning the elucidation of C_{19}

steroids in amphibia although 17-ketosteroids had been tentatively identified in the excreta of larval frogs (67). In 1971, however, Di Prisco *et al.* (238), reported the detection of androstenedione in the ovary of *T. c. carnifex* at a concentration of 3 μg/210 g. They also found dehydroepiandrosterone and testosterone in the associated fat bodies at a concentration of 7.5 μg and 2.0 μg per 62 g, respectively.

I.2.5. Steroid Biosynthesis and Metabolism in the Amphibian Ovary

As mentioned previously, *in vitro* studies on the ovarian steroids of amphibia have provided indirect evidence for the presence of a particular steroid in the intact ovary. Two related lines of research indirectly implicate the presence of various steroids. One is the *in vitro* incubation of various radioactive steroids with ovarian tissue and the subsequent determination of the metabolites formed. The other approach is based on the visualization of the hydroxysteroid dehydrogenase enzymes, thereby permitting histological studies on the sites of steroid synthesis. Reviews of this subject are available (16,26,210).

It must be kept in mind that the *in vitro* incubation of ovarian tissues with certain radioactive steroids does not necessarily demonstrate normal secretory products nor intact

biosynthetic pathways. Before a given radioactive metabolite is unequivocally identified as a normal secretory product of the ovary, it must be identified in the venous return blood of the intact animal. Likewise the incorporation of radioactive precursors, and their observed metabolites, into the normal pathways of ovarian steroidogenesis can be concluded only when the exogenous steroids can be shown to occur endogenously. Needless to say, none of the above criteria have been applied in the studies on amphibia. The various experiments described below, however, do afford some indication of ovarian steroid biosynthesis and metabolism as it may occur in some amphibian species.

The biosynthesis of androgens from progesterone was first examined by Callard and Leathem (33). They incubated ovarian fragments from R. pipiens and Necturus maculosus with ^{3}H-pregnenolone, ^{14}C-progesterone and ^{14}C-testosterone and determined the metabolites by chromatography and crystallization to constant specific activity. Progesterone could not be identified in the incubates of either amphibian species when pregnenolone was used as a precursor, but 17α-hydroxyprogesterone was tentatively identified in the incubate from R. pipiens. Testosterone and androstenedione were formed from progesterone by the ovaries of both animals, however testosterone was the predominate product in R. pipiens

and androstenedione the major metabolite in N. maculosus. Only the incubates of N. maculosus, using ^{14}C-progesterone as the precursor, yielded 17α-hydroxyprogesterone; it was not detected in R. pipiens. The 5α-reduction pathway was favoured by N. maculosus, the 5β pathway by R. pipiens. With ^{14}C-testosterone as the precursor, 5β-reduced steroids were again characteristic of R. pipiens, whereas the 5α pathway predominated in N. maculosus.

The foregoing observations supplemented those of Ozon et al. (224), who incubated testosterone with supernatants of homogenized ovarian tissue from the frog R. temporaria. Three metabolites were identified, viz., androstenedione, 5α-androstanolone and 5α-androstanedione. In contrast to R. pipiens, the 5α-reduction pathway predominated in R. temporaria.

In 1967 Ozon (227) carried out a detailed study of the in vitro biosynthesis of C_{19} steroids in ovarian homogenates of P. waltlii. After incubation with ^{14}C-progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone were definitely identified in the medium. Rao et al. (243) studied the metabolism of ^{14}C-17α-hydroxyprogesterone by ovarian tissue of X. laevis at three stages of climactic metamorphosis. The only C-19 metabolite found was androstenedione, and it was found in all three cases. In spite of an

extensive search, no testosterone could be detected.

Xavier and Ozon (300) have shown recently that ovarian tissue of the viviparous anuran Nectophrynoides occidentalis was able to metabolise in vitro pregnenolone-^{3}H to progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone. Moreover they showed, by incubation with both pregnenolone-^{3}H and ^{14}C-17α-hydroxyprogesterone and comparison of the $^{3}H/^{14}C$ ratios, that the principal route of C_{19} steroid formation was via the Δ^{4}-3-ketosteroids. The metabolites were characterised adequately by derivative formation and also by crystallization to constant specific activity. Although estrogens were tentatively identified when ^{3}H-testosterone and ^{14}C-androstenedione were used as substrates the low rate of aromatization (0.2%) precluded absolute identification of E_1 and $E_2$17β.

After incubating androstenedione-^{14}C with ovarian tissue of X. laevis, Redshaw and Nicholls (246) were able to detect androstenedione, testosterone, 5α-androstane-17β-ol-3-one, 5α- and 5β-androstane-3,17-dione, E_1 and $E_2$17β. The estrogens were produced in almost equal amounts. When ^{14}C-progesterone was used as the precursor, the yields of estrogen were too low to measure, although 17α-hydroxyprogesterone, androstenedione, and testosterone, as well as several

unidentified metabolites were detected. When the donor toads were pretreated with pregnant mare's serum gonadotrophin, however, and the ovaries then incubated with ^{14}C-progesterone, the yield of estrogen was some 0.2 per cent.

Recently Di Prisco *et al.* (238), incubated acetone powder prepared from ovaries of *T. c. carnifex*, with ^{14}C-cholesterol and ^{14}C-testosterone. The metabolites were characterised by crystallization to constant specific activity. When cholesterol was used as the precursor, the metabolites were progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone. When ^{14}C-testosterone was used as precursor, the metabolites were 11-ketotestosterone, androstenedione, 11β-hydroxyandrostenedione and E_1.

The synthesis *in vitro* of both E_1 and $E_2$17β from testosterone was demonstrated by Ozon and Breuer (223) when they incubated testosterone with the 1000 x g supernatant of an ovarian homogenate of *R. temporaria*. The rate of aromatization was 0.27%, a value of the same order as that obtained with human ovaries under similar experimental conditions (223). Ozon (227) incubated ^{14}C-testosterone with an ovarian homogenate of *P. waltlii* and observed that the rate of formation of radioactive $E_2$17β was 0.4 per cent. 19-Hydroxytestosterone was tentatively identified as an intermediate, and this suggested that estrogen biosynthesis may

be similar in both amphibian and mammalian ovaries (257).

Rao *et al.* (242) have recently demonstrated that 17-day old larvae of *X. laevis* can metabolise $E_2 17\beta$ *in vitro* to several hydroxylated products. The metabolites found were E_1, E_3, 6α-hydroxy E_1, and 15α-hydroxy E_1; these last two represent the first report of 'non-classical' estrogens in amphibia.

From an histological demonstration of the key enzymes involved, it is possible to deduce which various regions of the vertebrate ovary may be concerned with steroidogenesis. This relatively new technique is based upon the incubation of tissue sections with suitable hydroxysteroid substrates, relevant cofactors and with a tetrazolium salt as the final hydrogen acceptor. If the suspected enzyme is present, the tetrazolium derivative is precipitated as coloured crystals of formazan within the region of enzymatic activity. For a complete examination for the presence of the relevant enzymes, 3β-, 11β-, 17β- and 20β-hydroxysteroids should be used as substrates. To date, however, only 3β-hydroxysteroid-dehydrogenase (3β-HSDH) has been sought in the amphibian ovary (210).

The observations obtained by using this histochemical technique for the detection of the enzymes involved in ovarian

steroidogenesis present a confusing picture. One of the problems is that there is no agreement on the anatomy of the various tissues found in the amphibian ovary. For example, there is still doubt whether a theca interna is present (16).

Pesonen and Rapola (232) applied this technique to the ovaries of X. laevis and B. bufo. Chieffi and Botte (43) used a similar method to study the ovary of R. esculenta. Joly (142) examined the ovaries of Salamandra for enzymatic activity. Both Pesonen and Rapola and Chieffi and Botte were unable to detect any 3β-HSDH activity in any region of the ovaries studied. Joly reported that the primary oocytes of Salamandra gave no reaction, whereas the follicular epithelium of the oocyte in its secondary growth phase did show some slight activity.

The apparent absence of 3β-HSDH from X. laevis and B. bufo may be related to the fact that neither Ozon et al. (224) nor Callard and Leathem (33) could find such enzymatic activity in R. temporaria, R. pipiens or Necturus maculosus. Another factor which may be pertinent concerns the use of the steroid sulphate, rather than the free steroid itself, as the substrate for the incubation. It has been shown that in the ovary of the domestic hen, 3β-HSDH action was far more rapid with pregnenolone sulphate and 17α-hydroxypregnenolone sulphate (299). The nature and occurrence of steroid conjugates

in amphibia, however, are entirely unknown.

The reported lack of 3β-HSDH activity is difficult to reconcile with the report of Di Prisco *et al.* (238) which demonstrated the formation of 3-ketosteroids from cholesterol in the ovary of *T. c. carnifex*, thus indicating the presence of a 3β-HSDH. Furthermore, as will be discussed later, a 3-keto-Δ^4 steroid is explicitly implied in the *in vitro* ovulation of *R. pipiens* (297). Recently, however, Redshaw and Nicholls (246) have shown that 3α-, 3β-, 17α- and 17β-hydroxysteroid dehydrogenases are present in the ovary of *X. laevis*. Enzymatic activity appeared in the thecal layers of the larger vitellogenic or postvitellogenic oocytes, suggesting that steroid synthesis is restricted to the follicular layers of mature oocytes and to the short-lived 'post-ovulatory follicles'. Until more information is forthcoming, the site of steroid synthesis in the amphibian ovary and the enzymes involved, will remain somewhat obscure.

I.3. THE NATURE AND OCCURRENCE OF OVARIAN STEROIDS IN REPTILES

I.3.1. Introduction

Almost all the literature on reptilian reproduction deals with general life history studies (289) and the endocrinology of reproduction in reptiles has been largely

neglected. The available evidence for the presence of various ovarian steroids, either in the ovary or in the blood, is in most cases indirect. So far as is known, there has been no identification of steroid estrogens either *in vivo*, or after *in vitro* incubations. The data concerning the progestins and androgens are fragmentary and based upon studies on only nine species. Consequently, it may be prudent to present the available information with a minimum of comment.

I.3.2. Steroid Estrogens

To the author's knowledge, there has been no identification of any steroid estrogen in any reptilian species. Evidence that the reptilian ovary secretes estrogen is based upon the effect of ovariectomy, or of estrogen injection, on the oviduct (34,85). The effects of exogenous estrogens on the composition of the blood, which may mimic the changes observed in the intact animal as it approaches sexual maturity, also constitute evidence for the presence of estrogens in reptiles (288).

I.3.3. Progestins

The presence of progestins in reptiles is better established than is that of estrogens. This stems from the proposal that there may be a correlation between the presence of progestins and ovoviviparity or viviparity in reptiles (31).

Porto (ex 24) reported the presence, based on bioassay, of progestin in an extract of 150 corpora lutea of two species of ovoviviparous snakes, Bothrops jararaca and Crotalus terrificus. Bragdon et al. (24), studying two more ovoviviparous snakes, Thamnophis radix and Natrix sipedon sipedon, determined "progesterone-like" activity in the plasma by the Hooker-Forbes (127) bioassay. They reported low progestin levels (0.3-1.0 μg/100 ml) in non-pregnant females of Natrix sipedon sipedon with an increase during pregnancy to 8 μg/100 ml at full term.

Cooper and Ferguson (52) have provided indirect evidence that the bright orange spots developed by gravid female collared lizards, Crotaphytus collaris collaris, may be under the influence of a progestin and/or an androgen. Daily progesterone and testosterone injections induced significant increases in orange-spotting within ten days in non-gravid females, suggesting that these or related steroids are present in the gravid female.

Recently Chan et al. (38) have identified progesterone unequivocally in the plasma of Natrix sipedon pictiventris by the use of competitive protein-binding (CPB) assay and gas-liquid chromatography. There was no significant difference between the values obtained by the two methods. The plasma progesterone levels were found to reflect the reproductive

stage of the animal, being lowest in non-pregnant females (1 to 3 μg/100 ml), increasing to a peak during mid-pregnancy (5.0 μg/100 ml) and declining towards term.

I.3.4. Androgens

There is no direct evidence concerning the ovarian androgens of reptiles. As mentioned above, there are some indications that androgens may be present in gravid females of Crotophytus collaris collaris. In vitro incubations of ovarian tissue with labelled C_{21} steroid precursors do lead to androgen formation, from which observations the presence of androgens in the intact ovary may be inferred. The data are discussed in the following section.

I.3.5. Steroid Biosynthesis and Metabolism in the Reptilian Ovary

The experimental procedures for elucidating the possible steroid biosynthetic and metabolic pathways of the reptilian ovary have been almost identical to those applied to the amphibia. The arguments against inferring too much from the available data are, therefore the same as those advanced above in the discussion of in vitro incubations of the amphibian ovary.

In 1964 Callard and Leathem (30) incubated ovarian slices from the snakes Natrix sipedon pictiventris and Coluber

constrictor constrictor with ^{3}H-pregnenolone. Detailed chemical identification revealed the presence of progesterone in the incubates. The same authors supplemented the foregoing information by incubating either ^{3}H-pregnenolone, ^{14}C-progesterone or ^{14}C-testosterone with ovarian fragments from the snakes Coluber constrictor constrictor, Natrix sipedon pictiventris, Thamnophis sirtalis sirtalis and Natrix taxispilota (31). Rigorous chemical criteria were applied to the identification of the radioactive products. When ^{3}H-pregnenolone was the substrate, progesterone was identified in the incubates of all the species studied. In addition to progesterone, 17α-hydroxyprogesterone and testosterone were identified in the incubates of Thamnophis sirtalis sirtalis. Only tissue from Natrix sipedon pictiventris was incubated with steroid precursors other than ^{3}H-pregnenolone. When ^{14}C-progesterone was used as precursor, 17α-hydroxyprogesterone, androstenedione and testosterone were identified, whereas incubation with ^{14}C-testosterone yielded androstenedione and either androstanedione or etiocholanedione. No other androgen metabolites were found.

It is remarkable that no estrogens were detected by Callard and Leathem but this does not preclude their presence. The rate of aromatization of the neutral steroids may be so low that the yields of estrogen could well be below the

sensitivity of the methods used.

Callard (32) extended the above observations when he examined the ovaries of Natrix sipedon pictiventris for 3β-HSDH activity. This enzyme was demonstrable in the ovarian theca and granulosa of pregnant and non-pregnant snakes; and, in addition, it was present in the "corpora lutea" of pregnant animals. The role of the "corpus luteum" in the synthesis of progesterone is doubtful because the per cent conversion of ^{3}H-pregnenolone to progesterone was similar in pregnant and non-pregnant snakes.

I.4. OVARIAN STEROID HORMONES OF BIRDS

I.4.1. Non-domestic Species

I.4.1.1. Steroid Estrogens

In 1963 Dyrenfurth and Hohn reported the presence of E_1 and $E_2 17\beta$ in the ovaries of red-winged blackbirds (Steganopus tricolour) and Wilson's phalaropes (Oxyechus vociferus) (74). Previous to this the presence of estrogens in wild birds had been inferred from the results of estrogen administration into non-breeding, immature or castrate animals, inducing effects identical to those observed during the breeding season. For example, the beak colour of the red-bill weaver finch (Quelea quelea) and, in the case of love birds (parakeets, budgerigars) the cere over the base of the beak

(230), have been found to be determined by estrogens (294). The induction of oviducal growth has been shown to be influenced by estrogens in many birds, including the domestic pigeon (286), the sparrow Passer domesticus (156) and the canary (124). It is now well-known that estrogens profoundly influence many behavioural and physiological systems in birds and extensive reviews on this subject are available (231,268,287,288).

Hohn and Cheng (126) extended the study of gonadal hormones in wild birds, when, in addition to Wilson's phalaropes and red-winged blackbirds, they studied pigeons, mallards and killdeer. They detected E_1 and $E_2 17\beta$ in the ovaries of all species examined. Estriol was measured in all species except the killdeer. The concentrations of estrogens found in the ovaries are shown in Table 1. No data are available on the nature and occurrence of estrogens in the blood of wild birds.

I.4.1.2. Progestins

A definite role for progestins in wild birds has been systematically investigated only in the reproductive behaviour of doves (167) and the reproductive cycle of canaries (123). The presence of progestins in birds is inferred mainly from their ability, upon administration thereof, to elicit

Table 1. Steroids in the ovaries of certain birds, measured in micrograms per 100 gram body weight; after Hohn and Cheng (126).

Species	Wilson's Phalarope	Red-winged Blackbird	Pigeon (Breeding)	Domestic Hen (Laying)	Mallards (Breeding)	Killdeer
No. of birds in sample	97	24	2	1	8	21
Testosterone	0.43	0.14	1.11	0.19	0.27	0.27
Androstenedione	1.02	0.28	0.38	0.50	1.46	0.34
Progesterone	0.47	0.15	n.m.*	n.m.*	1.39	0.12
Estrone	0.40	0.32	0.79	n.f.**	0.57	0.47
Estradiol-17β	1.13	1.15	2.20	1.50	1.37	0.02
Estriol	0.40	0.29	0.03	n.m.*	0.26	n.m.*

*Not measured

**None found

the appearance of several naturally occurring reproductive processes. These include pre-ovulatory development of the oviduct (124,166), brood patch development (143) and incubation and sexual behaviour (304).

The occurrence of progestins in wild birds was first reported in 1967 (126). Progesterone was detected in the ovaries of Wilson's phalaropes, red-winged blackbirds, mallards and killdeer (Table 1). As in the case of estrogens, information on the progestins in the blood of wild birds is lacking.

I.4.1.3. Androgens

In many species of wild birds there is evidence that the female secretes androgens, the ovary being implicated as the site of secretion (288). Physical characters, such as bill, shank and plumage colours, are controlled by androgens in some instances (231,294). Behavioural responses in the females of some species also are influenced by androgens (177). Indeed, in the Wilson's phalarope, which exhibits behavioural and secondary sex characterization indicative of a degree of sex reversal, it has been found that the testosterone content of the ovary exceeds that of testicular tissue of the same species (126).

Testosterone and androstenedione are present in the

ovaries of Wilson's phalaropes, red-winged blackbirds, mallards and killdeer (Table 1). In addition, the *in vitro* incubation of ovarian tissue from Wilson's phalaropes with ^{3}H-pregnenolone, ^{14}C-17α-hydroxyprogesterone and ^{3}H-17α-hydroxypregnenolone demonstrated the formation of dehydroepiandrosterone, androstenedione and testosterone (81).

The specific source of these androgens is still in doubt although the ovarian interstitial cells, arising from the connective tissue cells of the stroma, have been suggested (287).

I.4.2. Steroid Estrogens of Domestic Species

I.4.2.1. Introduction

Early work on the effects of estrogens on the domestic fowl showed that hypertrophy and hyperplasia of the oviduct, characteristic of puberty in the hen, could be stimulated in immature chickens by injection of human placental extracts (147). The earliest report of the presence of estrogens in the domestic hen was in 1931, when estrogenic activity, tested by bioassay, was detected in extracts from the faeces (111). The presence of estrogens in the ovary was shown, also by bioassay, by Marlow and Richert in 1940 (175).

I.4.2.2. Urine and Faeces

The first chemical identification of any estrogens in birds was reported in 1957 (128). Paper chromatography and subsequent colour reactions indicated that E_1, $E_2 17\beta$ and E_3 were present in the droppings of the domestic hen. However, in view of later work on the urinary estrogens of the domestic hen, this identification of E_3 is questionable. MacRae et al. (172) confirmed the presence of $E_2 17\beta$ in hen droppings when they isolated a small amount of crystalline material and identified it as $E_2 17\beta$ by melting-point and infra red absorption spectrophotometry. This was the first reported isolation of any estrogen in crystalline form from avian material. Subsequently E_1, 16epiE_3 and $E_2 17\beta$ have been isolated and characterized in crystalline form from laying-hens' urine (8,120,122). In addition E_3 and 16, 17epiE_3 have been identified by stringent chromatographic criteria (181).

The isolation and identification of E_1, $E_2 17\beta$ and 16epiE_3 enabled quantitative studies to be applied to laying-hens' urine. Common et al. (50) investigated Brown's (27) method for human urinary estrogens and determined that it was reasonably reliable for the measurement of E_1, but not of $E_2 17\beta$, in the urine of the laying hen. The presence of Kober-positive material, which appeared to be non-steroidal

in nature, interfered with the assay of $E_2 17\beta$. The initial investigation therefore, was limited to the study of urinary estrone excretion. It was found that the amount of E_1 excreted per day ranged between 0.7 and 1.2 μg for non-laying hens while for laying hens the amount ranged from 2.2 to 5.0 μg per day. The results also demonstrated an increase in daily urinary excretion of E_1 over a period of two to three weeks before laying began. Peak levels of E_1 excretion in the urine were reached three to five days before the oviposition of the first egg (50).

Mathur and Common (180) supplemented these results on daily E_1 excretion when they examined six-hour urine samples from two laying hens. They showed that E_1 excretion in the laying hen was not constant over 24 hours, but displayed a cyclic variation. The highest values tended to occur 8½ to 10½ hours before oviposition, and by inference, ovulation. Oviposition was always accompanied by falling E_1 excretion.

The relative amounts of E_1 and of the cis-estriols (16epiE_3 and 17epiE_3) excreted was next studied by Mathur et al. (179). They showed that the hens examined excreted about one-sixth to one-quarter as much 16epiE_3 plus 17epiE_3 as E_1 in their urine.

Mathur and Common (185) using a technique based on TLC of the methyl ethers of the phenolic urinary steroids,

which removed the interference due to Kober-positive non-steroidal material, examined the daily excretion of $E_2 17\beta$ and E_1. They observed that $E_2 17\beta$ was quantitatively the major estrogen in the non-laying bird, whereas in the laying bird, the amounts of E_1 and $E_2 17\beta$ tend to be nearly equal.

Tang et al. (282) examined the effects of different environmental temperatures on urinary estrogens of maturing fowls and reported daily levels of E_1 and E_3 considerably higher than those reported previously. Furthermore, the levels of $E_2 17\beta$ and 16epiE_3 were frequently below the sensitivity of the assay method, that of TLC of acetate derivatives and subsequent gas-liquid chromatography. Tang et al. suggested that the discrepancies between their results and those reported previously, may be due to the superior specificity of their procedure combined with its freedom from interference, as compared to methods based on the Kober reaction. Further work, however, is needed before these differences in reported concentrations can be resolved.

Recently Chan (37) made a detailed study of the four-hour urinary excretion of E_1 and $E_2 17\beta$ in a single hen, using a modification of Brown's (27) original method. He found that E_1 excretion for days of ovulation was significantly higher for the interval ten hours to six hours before

ovulation than for other four-hour periods. There were indications that a second, though lower maximum occurred around 22 hours to 18 hours before ovulation. Similar but statistically non-significant fluctuations were observed for the four-hour $E_2 17\beta$ excretion. Oviposition was always associated with falling estrogen excretion, a result in agreement with that reported previously (185).

I.4.2.3. Blood

Kornfield and Nalbandov (160), using a bioassay, demonstrated estrogenic activity in the blood of chicks less than 20 days old. Layne et al. (165) obtained chromatographic evidence for the presence of E_1 in laying-hens' blood. The first definite identification of any steroid estrogens in the blood was that of O'Grady and Heald (217) using a method involving double-labelled derivatives. They identified E_1 and $E_2 17\beta$ in plasma of the laying hen. Ozon (225), by celite column chromatography and fluorometric measurement of the fractions thus separated, found evidence for the presence of E_1, $E_2 17\beta$ and E_3 in whole blood. O'Grady (219) extended his previous work (218) when he determined the concentrations of E_1 and $E_2 17\beta$ in six plasma samples drawn from the domestic hen. His technique was a modification of the one previously used, and involved the use of

triple-labelled derivatives. Work presented later in this thesis indicates that the method of Ozon and O'Grady markedly overestimates the concentrations of steroid estrogens in the blood of the hen.

Recently Bajpayee and Brown (15), using whole blood obtained by heart-puncture, measured E_1 and $E_2 17\beta$ in the laying hen. Their method was based on alumina chromatography and a Kober-Ittrich fluorometric procedure. These workers also showed the presence of E_1, $E_2 17\beta$ and E_3 in the blood of the laying turkey hen.

During the course of his work, the author of this thesis published a report on the estrone and estradiol concentrations in the plasma of the domestic hen in relation to ovulation (235). The experimental details will be presented in Part II of this thesis. The reported concentrations of estrogens in the blood of domestic species are shown in Table 2.

I.4.2.4. Ovary

As mentioned earlier, bioassay studies showed the presence of estrogenic activity in the ovary (175). Greatest activity was found in the maturing follicular membrane. Fraps and Sykes (ex 161) determined, also by bioassay, the presence of estrogens in the ovary. They found also that

Table 2. Reported estrogen concentrations in the blood of domestic birds. Values expressed as pg/ml.

Reference Estrogen	DOMESTIC HEN				TURKEY HEN
	225 (Whole Blood)	219 (Plasma)	15 (Whole Blood)	235* (Plasma)*	15 (Whole Blood)
Estrone	16700	15200-31600	900	14-138	130-500
Estradiol	2700	8300-21400	1800	36-284	80-250
Estriol	2500	-	60	-	60-210

*The work on which these values are based is fully reported in Part II of this thesis.

greatest activity was associated with the walls of the maturing follicles. Furthermore, they demonstrated that estrogen activity of the ovary varied during the normal sexual cycle of the hen.

Layne et al. (165) obtained evidence, based on paper chromatography and ultraviolet absorption spectrometry, for the presence of E_1 and $E_2 17\beta$ and, more tentatively of E_3, in ovaries of laying hens. Later work has indicated that the fraction identified as E_3 was more likely to have been mostly 16epiE_3. Hohn and Cheng (126) have also reported finding $E_2 17\beta$ in the ovary of a laying hen.

I.4.3. Progestins of Domestic Species

I.4.3.1. Introduction

It has long been known that progesterone, either alone or in combination with estrogens and androgens, exerts a marked effect on the reproductive physiology of the domestic hen. The synergism between these three types of hormones in causing hypertrophy of the immature oviduct is well documented (287,288).

Progesterone alone appears to control the secretion of avidin by the mature oviduct and much interesting work has been on the mode of action of steroid hormones using this progesterone-avidin system (221). It has been observed that administration of progesterone causes cessation of laying with a concomittant induction of moult (119,148,265). There is also evidence that progesterone administration can cause greater egg production (5) and progesterone has been implied in the control of ovulation, an aspect which will be discussed later.

I.4.3.2. Urine and Faeces

No information is available on the endogenous progestins in the urine or faeces. Indirect evidence, based on *in vivo* metabolism of injected ^{14}C-progesterone indicating that progestins are normally present, will be considered later.

I.4.3.3. Blood

Fraps et al. (80) were the first to demonstrate progestational activity in any avian species. They examined the blood of laying hens for such activity by the bioassay of Hooker and Forbes (127) and attributed their results to progesterone. Their results, however, should properly be interpreted as indicating progestins, as later work has shown the bioassay used is not specific for progesterone (154). Fraps et al. (89) subsequently reported progestins in the blood of cocks and non-laying hens, but none were detected in capons.

Lytle and Lorenz (170) detected progesterone chromatographically in the ovarian blood of laying hens and by spectrophotometry estimated the concentration at 5 μg/100 ml. O'Malley et al. (220), by use of a double isotope technique in conjunction with electron capture gas-liquid chromatography, identified progesterone in blood from the vena cava of laying hens. They reported a concentration of 0.126 μg/100 ml and suggested the lower value as compared to that reported by Lytle and Lorenz, could be due to dilution by peripheral blood. In addition, they identified chromatographically the 20-hydroxy epimers of progesterone, with the 20β form predominating. Recently Furr (94) and Furr and Pope (98) have identified progesterone and the 20-hydroxy epimers

of progesterone in peripheral plasma of laying hens. The identification of these steroids was based on column, thin-layer and paper chromatography together with GLPC and mass spectrometry. Furr (95) has also reported that values of progesterone ranged from 0.96 to 17.1 ng/ml.

During the course of his work, the author of this thesis published a report on the progesterone levels in the peripheral plasma of the domestic hen during the ovulatory cycle (234). The experimental details will be presented in Part II of this thesis. Suffice here to say the levels of progesterone reported by the author ranged from 0.5 to 12.5 ng/ml.

Subsequent to this report, three papers have been published on the progesterone concentrations in peripheral plasma during the ovulatory cycle of the domestic hen. Arcos and Opel (14) by GLPC, reported that maximum concentrations of progesterone in peripheral plasma of laying hens ranged from 3.61 to 7.90 μg/100 ml. Arcos (personal communication) has since indicated that there was a misprint in the original report of Arcos and Opel and that the actual maximum values ranged from 3.61 to 7.90 ng/ml. Cunningham and Furr (55) using a radioimmunoassay (RIA) based on an antiserum induced in goats against progesterone-11-succinyl-bovine-serum-albumin, observed that the concentration of progesterone

ranged from 2.68 to 6.47 ng/ml. Kappauf and van Tienhoven using a competitive protein-binding (CPB) assay, found the level of progesterone varied from 0.31 to about 5.8 ng/ml (154). The reported concentrations are summarized in Table 3.

Table 3. Reported concentrations of progesterone (ng/ml) in the blood of the domestic hen.

Reference	Concentration
14	3.61 - 7.90
55	2.68 - 6.47
95	0.96 -17.1
154	0.31 - 5.8
170	50
220	1.26
234	0.50 - 12.5

I.4.3.4. Ovary

Layne et al. (164) reported chromatographic evidence for the presence of progesterone in extracts of ovaries of laying hens.

More recently Furr (94,95) and Furr and Pope (98) have identified progesterone, the 20-hydroxy epimers of

progesterone, and pregnenolone in the ovary of the domestic hen. The amount of progesterone varied according to the physiological state of the hen. Levels of 0.41 to 9.26 μg/g ovarian tissue were measured in laying hens and broody hens showed similar concentrations, whereas the amounts in moulting birds were barely detectable. Both Furr and Layne et al. found the greatest concentration of progesterone in the growing follicle; it was present also in the post-ovulatory follicle but only negligible amounts were found in the stroma.

I.4.4. Androgens of Domestic Species

I.4.4.1. Introduction

The hypertrophy of the comb in the prepuberal pullet is evidence that the domestic hen secretes androgen (48, 287). It has been shown that the ovary, at least in part, produces this androgen (107). The anabolic effect of androgens is well-documented for mammals and it has also been shown that suitable doses of androgen stimulate growth in the chicken (162). Androgen, in sufficient dosage, will cause a slight hypertrophy of the immature oviduct (162). Doses of androgens, however, which alone are without appreciable effect on the oviduct, will greatly enhance oviducal hypertrophy caused by a given dose of estrogen (48), an effect considered to be true synergism. Similar synergistic

associations between androgens and estrogens exist in birds with respect to medullary ossification (155), calcium and phosphorus retention (48,49), and to increased endosteal bone formation (146).

I.4.4.2. Urine and Faeces

No information is available on the androgens present in the urine or faeces of domestic birds.

I.4.4.3. Blood

O'Malley et al. (220) were the first to determine androgens in the blood of the domestic hen. Using either a double isotope technique or electron capture gas-liquid chromatography, they measured testosterone (0.056 μg/100 ml) and androstenedione (0.098 μg/100 ml) in pooled vena cava plasma from laying hens. Furr and Thomas (96) substantiated these results when they detected, by GLPC, levels of testosterone in laying-hens' plasma ranging from 20 to 120 μg/100 ml. In addition 5β-androstane-3,17-dione has been demonstrated in the plasma of laying hens (98). Recently using a CPB assay, testosterone has been measured in the plasma of laying and non-laying hens. There was no significant difference in the concentrations measured, being 960 pg/ml for laying and 950 pg/ml for non-laying birds (259).

I.4.4.4. Ovary

Hohn and Cheng (126), using paper chromatography in conjunction with spectrophotometry, have shown that testosterone and androstenedione are present in the ovary of the laying hen. They found concentrations, in micrograms per gram ovarian tissue, of 0.32 and 0.84 for testosterone and androstenedione, respectively. The only other androgen that has been identified is 5β-androstane-3,17-dione, detected in the free steroid fraction of the ovary (98).

I.5. OVARIAN STEROID METABOLISM IN BIRDS

I.5.1. Introduction

Most of the work on the metabolism of progestins, androgens and estrogens has been performed on the domestic hen; there are however, a few reports on wild birds. Some of the information has been obtained from *in vitro* incubation of either liver, ovary or oviduct with radioactive steroid precursors, and some have been derived from injection of labelled steroids, especially estrogens, and determining the metabolites excreted in the urine. In addition, the ovary has been examined for the presence of the various hydroxysteroid dehydrogenases that are active in the biosynthesis and metabolism of steroids.

The data available must be accepted with due

reservation. It has been remarked previously, the caution with which one must accept the results obtained from the incubation of various tissues; caution must also be exercised in the interpretation of the results gained from the *in vivo* injection of labelled steroids. The excretory products only demonstrate the fate of the injected material and possible metabolic pathways. They do not necessarily prove what happens normally in the bird, nor can the site of the various metabolic pathways be inferred from such experiments. Accepting these limitations, it is possible to give some indication of the various metabolic pathways of the ovarian steroids normally operating in the bird.

To give a more complete picture, this section will trace the onset of steroidogenesis in the embryo through to the source of the ovarian hormones in the adult and, finally, to the metabolism of these hormones.

I.5.2. Embryonic Steroidogenesis

The biosynthesis of steroid hormones by the embryonic gonads of birds has been reviewed recently (112) and only the salient features will be mentioned here. There is much evidence that steroidogenesis begins early in the embryonic gonads of both sexes. Chemical determinations have shown that estrogens are present in extracts of 10-day old ovaries

and also in the amniotic and allantoic liquids and in the peripheral blood of female embryos of the same age. The *in vitro* biosynthesis of E_1 and $E_2 17\beta$ from dehydroepiandrosterone occurs in 7½-day old ovaries, other products being androst-5-en-3β, 17β-diol, androst-5-en-3β, 17α-diol and etiocholanolone. When six-day old gonads were used, that is, before the histological differentiation of sex which is considered to occur at 6½ days, E_1 and $E_2 17\beta$ were still formed as were the androstenediols. No etiocholanolone was detected (112). Estrogen biosynthesis from either acetate or progesterone occurs as early as six days but is not observed prior to this stage (293).

The first signs of 3β-HSDH activity in the chick and also the quail occur before the morphological differentiation of the gonads (112), and possibly as early as two days old in the case of the chick (107). Enzymatic activity is confined to the interstitial cells arising from the medullary cords, no reaction being observed in the cortex during embryonic development (112).

The above observations suggest that the avian gonad has the potential to secrete sex hormones before morphological differentiation into either sex, and that the steroids produced may be important in the early development of sex

as proposed by Wolf and Gerlinger as early as 1935 (295).

I.5.3. The Source of Ovarian Steroids in Birds

The identification of the cell types involved in steroidogenesis is based mainly on the histological examination, either with or without visualization of hydroxysteroid dehydrogenase enzymes. Additional information has been obtained by extracting different parts of the ovary and determining the relative concentrations of the steroids present. It has been mentioned earlier that the greatest concentration of progesterone in the ovary of the laying hen occurred in the growing follicle, although it was present in the post-ovulatory follicle.

Until recently attempts to identify the ovarian cells involved in steroidogenesis were only partly successful (107). In a series of papers published over the last two years, however, Dahl (57-66) has resolved much of the confusion that has surrounded the site of steroid secretion in the ovary of the domestic hen. His work was based on a microscopic study of the various cell types in the ovary from which he was able to distinguish distinct steroid-secreting cells. These have a characteristic morphology, being laden with lipids, having abundant smooth endoplasmic reticulum and mitochondria with tubular, rather than lamellar

cristae (45). Dahl observed that most interstitial cells found in the theca interna were of this type and he has called them the thecal glands (57). A small number of cells, identical in morphology and size with those of the thecal glands were found in the inter-follicular stroma (58). These cells are probably homologous to the exfollicular glands of the rook (175).

The thecal glands were found to undergo alterations following the administration of different hormones and drugs. They were stimulated by gonadotrophins (62), whereas steroids caused morphological changes consistent with atrophy and decreased function (61), an effect most likely due to the feedback mechanisms via the anterior pituitary. Clomiphene also stimulated the thecal glands (63), in keeping with its known anti-estrogenic activity.

In contrast to the steroid-secreting activity of the theca interna, the granulosa cells were found to be primarily "nursing" cells for the growing oocyte and had the characteristic histology of protein-forming cells (64). There was little evidence that they could secrete steroids. The granulosa cells were stimulated by the administration of steroids even though the thecal glands of the same follicle showed decreased function (65). In spite of this stimulatory effect on the granulosa, all the follicles remained

small and did not mature, the whole ovary being smaller than normal and atrophic in appearance.

The administration of gonadotrophins also had a stimulatory effect on the granulosa cells as well as the thecal glands (66). Furthermore, the appearance of the granulosa cells stimulated by gonadotrophins and those stimulated by steroids were very similar. Because the granulosa cells are most likely not involved in steroid secretion, Dahl proposed that the gonadotrophin stimulated the thecal glands to produce endogenous steroids, and these in turn stimulated the protein-secretory activity of the granulosa cells (66). Dahl also suggested that gonadotrophins per se are not essential for the early growth of the granulosa cells, but that it is the steroids of the thecal glands which exert the primary stimulatory effect on these cells (64). Supporting this view is the observation that the thecal glands exist as morphological entities before sexual maturity and are not merely confined to the preovulatory stage of the follicle (60).

The results obtained from the visualization of the enzymes involved in steroidogenesis do not always agree with the observations of Dahl. The finding of 3β-HSDH activity in the interstitial cells of the theca interna and stroma of the domestic hen (44,211,299) and quail (258) does agree with

the notion that these cells are involved in steroidogenesis. Such activity has been found dependent on the physiological state of the bird, being prominent in the laying hen and much reduced in the moulting bird (23). The converse was observed for 17β-HSDH activity in the thecal and stromal glands being very intense during moulting, and virtually absent during laying (23).

Various reports have indicated, however, the presence of 3β-, 11β-, 17β- and 20β-HSDH activity in the granulosa cells (44,299). Such histological demonstrations of hydroxysteroid dehydrogenase activity must be interpreted with caution as they may overrate the functional capacity of the granulosa cells (299). What has been shown histochemically is that they possess the enzymes necessary for steroid secretion but not that they are, in fact, producing them at any one time.

In conclusion the remark of Young (302) is highly relevant. He suggests it may be futile and unrealistic to attempt to identify specific cell types as the source of hormones in the ovary. The various tissues involved in steroidogenesis are probably subject to various metabolic controls which change their hormone production either in rate or in kind. At the same time, the evidence as a whole makes it seem most likely that the thecal and stromal glands

are the predominant steroid-secreting cells in the ovary of the domestic fowl.

I.5.4. *In Vitro* Ovarian Steroid Metabolism in Birds

I.5.4.1. Liver

Much of the information on the *in vitro* metabolism of ovarian steroids in birds has been obtained from the incubation of liver slices or homogenates with radioactively-labelled hormones. Mitchell and Hobkirk (192) were the first to report on the *in vitro* metabolism of steroids by an avian tissue when they showed that liver slices from laying domestic hens were able to convert $E_2 17\beta$ to E_3.

Breuer and Ozon (25), comparing the metabolism of androgens and estrogens in the "lower" vertebrates, indicated that testosterone is metabolised by the avian liver to derivatives of 5β-androstane. These workers also incubated the following estrogens with liver slices from 10-20-day old chicks and immature pullets; E_1, $E_2 17\beta$, $E_2 17\alpha$, 16-keto-$E_2 17\beta$ and $16\alpha OHE_1$ (223). Using adequately stringent chemical characterizations they isolated various conversion products (Table 4). These results demonstrated the presence of the following enzymes in the liver of the domestic hen; 16α-, 16β-, 17α-, and 17β-hydroxysteroid oxidoreductases as well

Table 4. The conversion products formed by the *in vitro* incubation of various steroids with chick liver. (After Ozon and Breuer (223)).

Steroids Incubated	Steroids Identified in Medium
Estrone	Estradiol-17α Estradiol-17β
Estradiol-17α	Estrone Estradiol-17β
Estradiol-17β	Estrone Estradiol-17α 16-epi-Estriol Estriol
16-keto-Estradiol-17β	16-epi-Estriol Estriol
16α-hydroxyestrone	17-epi-Estriol Estriol

as 16α- and 16β-hydroxylases. It was not surprising that 16epiE_3 was the predominant metabolite considering that the 16β- and 17β-hydroxysteroid oxidoreductases and 16β-hydroxylase were the most active enzymes observed. When tritiated 16epiE_3 and E_3 were incubated separately with liver homogenates from laying hens, the conversion of E_3 to 16epiE_3 was found to be much greater than the conversion of 16epiE_3 to E_3. The major intermediate of these conversions was shown to be 16-keto$E_2$17β (244).

Other studies involving the simultaneous incubation of ^{14}C-E_1 and ^{3}H-E_1-SO_4 with chicken liver homogenates, have demonstrated that the liver is capable of reducing E_1 to $E_2 17\beta$ and E_1-SO_4 to $E_2 17\beta$-SO_4, the latter apparently without prior hydrolysis of the sulphate group. Furthermore, the conversion of E_1 to $E_2 17\beta$ was found to be more rapid than the conversion of $E_2 17\beta$ to E_1. There was no evidence of C-2 substituted compounds, and this indicated a lack of C-2 hydroxylating enzymes in the liver of the domestic hen (245).

The *in vitro* incubation of avian liver tissue with progestins has not been studied to the same extent and there has been only one report to date on the metabolism of progesterone in the liver of the domestic hen. Nakao *et al.* (208) incubated progesterone with chicken liver preparations for three hours and identified both 20α- and 20β-hydroxyprogesterone in the medium. They did not detect pregnanediols among the reaction products.

I.5.4.2. Ovary

Mention has been made of the ability of the embryonic ovary to metabolize a variety of radioactive steroids *in vitro*. This section examines the *in vitro* ovarian steroid metabolism in the bird after hatching.

The first report of the *in vitro* incubation of avian

ovarian tissue with ^{14}C and ^{3}H labelled precursors was in 1968 when Fevold and Pfeiffer studied the androgen production by phalarope gonadal tissue homogenates (81). Androstenedione and testosterone were identified as being formed from ^{3}H-pregnenolone, ^{3}H-17α-hydroxypregnenolone and ^{14}C-17α-hydroxyprogesterone, whilst dehydroepiandrosterone was formed from ^{3}H-pregnenolone and ^{3}H-17α-hydroxypregnenolone. Ovarian tissue from partially developed ovaries, obtained in the spring and weighing less than 150-200 mg, tended to synthesize more testosterone than androstenedione, while those weighing more than 150-200 mg appeared to form more androstenedione. This general pattern was observed regardless of the substrate used.

The only information available on the <u>in vitro</u> steroid formation by the ovary of the domestic hen concerns the incorporation of ^{14}C-acetate into progesterone, androstenedione, testosterone and $E_2$17β by ovarian slices in the presence of gonadotrophin (23). Distinct profiles of radioactive steroids formed were seen in the tissues of moulting, laying and broody hens. The highest conversion of acetate to steroid occurred in ovarian slices of moulting hens, the radioactivity distributed principally as androstenedione (54%), testosterone (7%) and $E_2$17β (40%) with little or no

progesterone. The incorporation of radioactivity into steroids was high also in the broody hen, but the distribution of radioactivity was different; progesterone (14%), androstenedione (28%), testosterone (9%) and $E_2 17\beta$ (35%). The ratio of androgens to estrogens suggested that relatively larger amounts of androgens were synthesized by the ovarian tissue of the moulting hen. In the laying bird, approximately equivalent incorporation occurs into progesterone, androstenedione and $E_2 17\beta$. These results suggest that the laying hen synthesizes equivalent amounts of progestins, androgens and estrogens while moulting hen synthesizes a relatively large proportion of androgens with little or no progestins. The broody hen synthesizes chiefly progestins and estrogens with a lesser proportion of androgens. The chemical analysis for progesterone in the ovary of the domestic hen also indicates that similar concentrations of progesterone are found in laying and broody hens but negligible amounts are found in moulting birds (95).

I.5.4.3. Other Tissues

The only other tissue that has been used for <u>in vitro</u> metabolic studies appears to have been chick embryo cartilage. When this tissue was incubated with ^{14}C-progesterone for one hour, radioactive 5β-pregnanedione, 5β-pregnane-3α-ol-20-one

and 5β-pregnane-3α-20β-diol were found in the medium (199).

I.5.5. *In Vivo* Ovarian Steroid Metabolism in Birds

I.5.5.1. Progestins

The study of the *in vivo* metabolism of progestins in birds, as for the estrogens, has been approached in two ways. The first has been the administration of the radioactive hormone followed by killing the birds at set time intervals and then determining the nature and distribution of radioactivity in various tissues. The second has been the injection of the radioactive steroid followed by the determination of the radioactive metabolites in the urine.

Morgan and Wilson (194) studied the metabolism of radioactive progesterone by the first approach. Following the intravenous administration of ^{3}H-progesterone, radioactivity was recovered within five minutes from the magnum, and at all time intervals up to one hour approximately 25% of the magnum radioactivity was localized in the nuclei. Although the metabolism of tritiated progesterone to a variety of compounds was rapid in all tissues studied, progesterone itself and its 5α-reduced derivative, allopregnanedione, were the principal components identified in the magnum cytoplasm and the only labelled steroids recovered from the

magnum nuclei. In all the tissues studied, allopregnanedione was recovered in a significant percentage only from the magnum, shell-gland, comb, and in one case from the brain (including the pituitary gland). Although not observed in the magnum, shell-gland or comb, a major metabolite, tentatively identified as pregnanedione, was found in the omental fat, skin, intestine, muscle, crop and cartilage. The conversion of progesterone to allopregnanedione was shown to occur in the magnum nuclei. At all times progesterone was the predominate steroid bound to the magnum nuclei, whereas allopregnanedione was the principal radioactive steroid bound to nuclei from the shell-gland.

Adamec et al. (6) have studied the distribution of ^{14}C-progesterone following intramuscular injection into laying hens. Eight hours after the administration, four radioactive metabolites could be detected in the droppings. Twenty-four hours after, progesterone and two other metabolites were detected in the blood whilst the number of labelled compounds in the excreta had increased to seven. The highest concentration of radioactivity after 24 hours was found in the adrenals. The liver contained a similar pattern of radioactivity as the droppings but only trace amounts of radioactivity were found in the pituitary, ovary and muscle.

Wells (292) in a more detailed study, extended these results of Adamec et al. Wells showed that there was a rapid and irreversible decrease in ^{14}C concentration of the plasma after the injection of ^{14}C-progesterone into a laying hen. The calculated plasma half-life of progesterone and its metabolites was 16-18 minutes. Analysis of a small number of tissues showed the liver to be a major site of metabolism and that the bile was the principal route for the elimination of progesterone metabolites. By contrast the kidneys contained little radioactivity. Nearly seven hours after the injection, 14-15% of the dose was recovered in the excreta as a mixture of free steroids (62%) and conjugates of sulphuric acid (38%). A Girard separation of the free steroids from the droppings revealed that 94% of these steroids were non-ketonic. By TLC it was shown that the majority of this non-ketonic material was pregnanediols (44%) and pregnanetriols (56%). One of the diols was suggested to be 5β-pregnane-3α,20β-diol but its identification was inconclusive. The triol was tentatively identified as 5β-pregnane-3α,17α-20α-ol by TLC in two solvent systems.

There have been only two reports on the study of the in vivo metabolism of radioactive progesterone in the domestic hen by examining the urine for radioactive metabolites

(40,41). After ^{14}C-progesterone was injected intramuscularly into three hens, an average of 27% of the dose was recovered in the urine. The urinary neutral steroids consisted of 65% non-ketonic and 30% ketonic material. Fifteen distinct radioactive zones were obtained from TLC of these neutral steroids, but only two steroids were positively identified. They were 5β-pregnane-3α-ol-20-one and 5β-pregnane-3α,20β-diol. Radioactive progesterone could not be detected in the urine, neither did the results provide any evidence for the presence of radioactive 5α-pregnane derivatives. In another experiment, the injection of ^{14}C-progesterone into two hens led to an average of 12% of the radioactivity of the urine being recovered as "free" steroids, 16.7% as glucuronides and 19% as sulphates.

I.5.5.2. Steroid Estrogens

The first report concerning the distribution of administered radioactive estrogen was that by Jonsson and Terenius (145) who investigated the distribution of the radioactivity in the body of week-old pullets following injection of tritiated $E_2$17β. A few hours after the injection, the oviduct had about twice the radioactivity of skeletal muscle per unit wet weight, and there was no evidence of saturation. If the birds were pretreated with non-radioactive $E_2$17β, the

radioactivity per unit wet weight in the oviduct decreased and approached that of skeletal muscle. No attempt was made to determine the chemical nature of the radioactivity in the various tissues.

Cecil (35) extended these observations when she studied the in vitro uptake of tritiated $E_2 17\beta$ by the oviduct and other body tissues. She found comparable results, with all portions of the oviduct of the immature chick having a special affinity for $E_2 17\beta$, concentrating three to six times more radioactivity than other body tissues. Furthermore, pretreatment of immature birds with non-labelled $E_2 17\beta$ reduced the ability of the oviduct to concentrate radioactivity to a level comparable to other tissues. The oviducts from non-laying hens were able to accumulate more radioactivity per unit wet weight than the oviducts from laying hens presumably due to lack of inhibition by endogenous estrogens. There was little difference in the uptake of radioactivity by the various regions of the oviduct during the different phases of the ovulatory cycle. Cecil also made no attempt to identify the radioactive compounds in the tissues.

Terenius (283) supplemented his previous work when he incubated oviduct and gizzard slices of 7- to 10-day old chicks with tritiated $E_2 17\beta$. The oviduct took up 10-20 times

more radioactivity than the gizzard on a wet weight basis and this preferential uptake was reduced by dilution with non-radioactive $E_2 17\beta$ and several non-steroidal estrogens. Progesterone or testosterone did not inhibit the uptake by the oviduct.

A more definitive study has been made by Hawkins and Taylor (113). They examined the nature of the radioactive metabolites in tissues of laying hens after the intravenous injection of tritiated $E_2 17\beta$. Of the total radioactivity taken up by the tissues, the greatest amount accountable as free steroid occurred in the follicular wall, and the greatest proportion of conjugated radioactivity was found in the bile. The blood plasma contained approximately equal amounts of free and conjugated radioactivity. The free estrogen fraction of nearly all the tissues studied contained more than one compound, the composition varying from 80% $E_2 17\beta$ in the liver to virtually none in the follicular wall. An unidentified compound accounted for 83% of the free radioactivity in the follicle wall and it was demonstrated that this compound was neither 16,17epiE_3 nor E_3. It was suggested that it may have been 16epiE_3. The blood plasma was the only tissue studied that consistently contained a significant amount of labelled E_1. This is not surprising when one considers that this steroid has been found in hens' plasma (219,

225,235).

Hawkins et al. (114) also studied the distribution of radioactivity among various tissues following the administration of tritiated $E_2 17\beta$. The peak concentration of radioactivity in all tissues examined occurred approximately four minutes after intravenous injection. There was a marked retention in the oviduct and liver and in the cerebral tissue and the greatest uptake occurred in the pituitary gland. This pituitary uptake was found to depend on the physiological state of the bird (115). Examination of the distribution of radioactivity in the pituitary gland after a single injection of ^3H-$E_2 17\beta$ revealed significant cyclical accumulations, when calculated in terms of dpm per unit wet weight, at different stages of the ovulatory cycle. Maximum accumulation occurred in the period 12-16 hours before the expected ovulation. This cyclical uptake was absent from the hypothalamus.

The identification of radioactive metabolites in the urine or droppings after the injection of labelled estrogens has led to a greater understanding of the *in vivo* metabolism of these steroids. The initial work concerned the metabolism of $E_2 17\beta$ (171). When ^{14}C-$E_2 17\beta$ was injected intravenously into a laying hen, radioactive $E_2 17\beta$, E_1, E_3, together with

a fourth conversion product later identified as 16epiE_3 (174) were found in the gut and mixed excreta. Following the administration of ^{14}C-E_3, radioactive 16epiE_3 and 16keto$E_2$17β were also detected in the faeces of laying hens (173). Ainsworth and Common (9) subsequently injected ^{14}C-16-keto$E_2$17β intravenously into a non-laying hen and demonstrated the presence of 16epiE_3, but not E_3, in the droppings. In a further study, the intravenous injection of ^{14}C-E_1 led to the formation of the following metabolites in the urine: $E_2$17β, E_3, 16epiE_3, 17epiE_3, 16keto$E_2$17β and also an acid labile compound thought to be 16ketoE_1 (7). Later work, however, proved that at least 90 per cent of the radioactivity of this compound was attributable to $E_2$17α (198).

Ainsworth and Common (9) then injected ^{14}C-16-keto-$E_2$17β intravenously into non-laying hens and identified radioactive 16-keto$E_2$17β and 16epiE_3 in the urine. Injection of radioactive 16-epiE_3 led to the detection of 16epiE_3 and 16keto$E_2$17β as the major radioactive conversion products in the urine (10). Hertlendy and Common (121) reported briefly the identification of E_3, 16epiE_3, 17epiE_3, and 16,17epiE_3 as urinary conversion products of ^{14}C-$E_2$17β injected intravenously into a laying hen. The conversion of ^{14}C-$E_2$17β and ^{14}C-E_1 to radioactive $E_2$17α was demonstrated by Mulay and Common (197), and the conversion of tritiated $E_2$17α to E_1

has been also demonstrated (196).

Recently Mathur and Common (182) detected radioactive 16epiE_3, E_1, $E_2$17α and 16keto$E_2$17β in the urine after the injection of a mixture of $E_2$17β-4-^{14}C and $E_2$17β-17α-^{3}H. They subsequently detected radioactive $E_2$17α, $E_2$17β, all four estriol epimers, and 16keto$E_2$17β in the urine after the injection of a mixture of $E_2$17α-4-^{14}C and $E_2$17α-17β-^{3}H (183). By comparing the ratio dpm ^{3}H:dpm ^{14}C, they determined that (a) 16epiE_3 and 16keto$E_2$17β were derived from $E_2$17β and E_1 was not an intermediate in this conversion; (b) that $E_2$17α was derived from E_1; and (c) that 17epiE_3 and 16,17epiE_3 were derived directly from $E_2$17α and that E_1 was not an intermediate in this conversion.

A study has been made of the distribution patterns of the <u>in vivo</u> conversion products of injected radioactive E_1 and $E_2$17β in the urines of laying and non-laying hens (51). Irrespective of whether ^{14}C-E_1 or ^{14}C-$E_2$17β were injected, the major radioactive phenolic steroids in the urine were $E_2$17β, E_1 and $E_2$17α, in that order, when the hen was not in lay, and E_1 and $E_2$17α or $E_2$17β, in that order, when the hen was in lay.

Work by Mulay <u>et al</u>. (195) indicated that a considerable amount of the phenolic steroids in the urine of the hen

were present as conjugates which were not completely cleaved by β-glucuronidase and that these conjugates included doubly-conjugated material. Subsequent work on the injection of a mixture of potassium-E_1-^{3}H-SO_4 and sodium-E_1-$^{35}SO_4$ into a laying hen, revealed that the E_1-SO_4 was metabolized to estradiol monosulphate without prior hydrolysis of the sulphate group (184). Mathur et al. (186) then injected a massive dose of ^{14}C-$E_2$17β (50 mg, specific activity 0.2 μCi/mg) into each of four similar hens. Analysis of the urine revealed (a) the virtual absence of glucuronides, (b) the presence of a high proportion of diconjugates as well as monoconjugates of the phenolic steroids and (c) the presence of a high proportion of sulphates in both conjugate fractions. When tritiated $E_2$17β-disulphate was subsequently injected intramuscularly into a mature non-laying hen, the only radioactive material identified in the urine was $E_2$17β-17-SO_4 (187).

I.6. A SYNOPSIS OF OVARIAN STEROID BIOSYNTHESIS AND METABOLISM IN THE NON-MAMMALIAN TETRAPODA

I.6.1. Biosynthesis

A primary pattern of biosynthesis seems to underlie the formation of steroids in mammalian endocrine tissues

(257). The biosynthetic pathways involved have been extensively reviewed (257); and only the salient features concerning the ovary will be mentioned here. The two carbon atoms of acetate are readily converted to steroids by the ovary. Cholesterol is an intermediate in all cases. The biosynthesis of steroids from cholesterol seems to involve either pregnenolone or 17α-hydroxypregnenolone as an intermediate. The formation of estrogens from pregnenolone may follow two alternate pathways depending upon the type of ovarian tissue. One, the Δ^5 pathway, includes 17α-hydroxypregnenolone, dehydroepiandrosterone, Δ^5-androstenediol androstenedione and testosterone. The other, the Δ^4 pathway, involves progesterone, 17α-hydroxyprogesterone, androstenedione and testosterone. The 19-hydroxylated derivative of either androstenedione or testosterone is an obligatory intermediate in the formation of estrogens from androgens. Figure 1 summarises these biosynthetic pathways.

The data on the amphibian, reptilian and avian ovarian steroid biosynthesis may now be examined in relation to this well-documented mammalian pattern. The available information is summarized in Figures 2, 3 and 4. These diagrams indicate that the biosynthesis of ovarian steroids is basically similar in amphibia, reptiles and birds. These <u>in vitro</u> studies

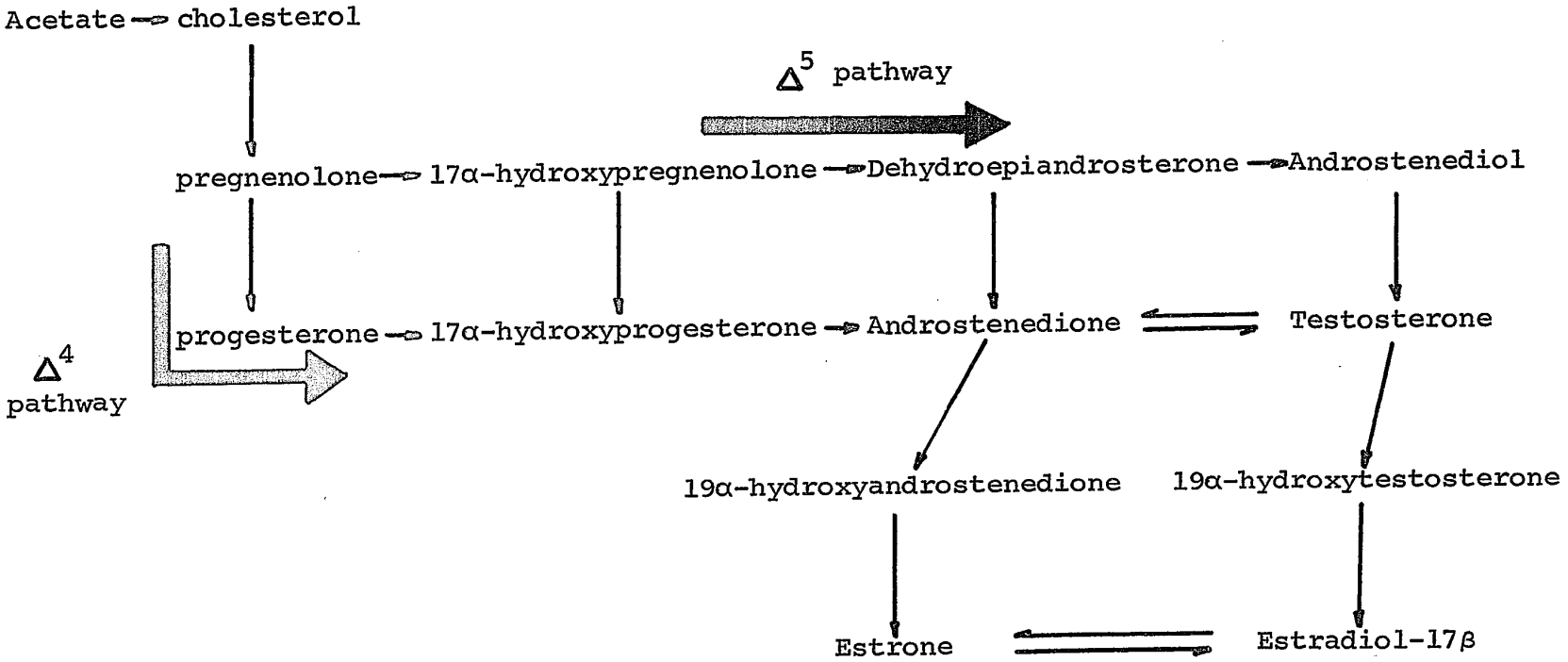
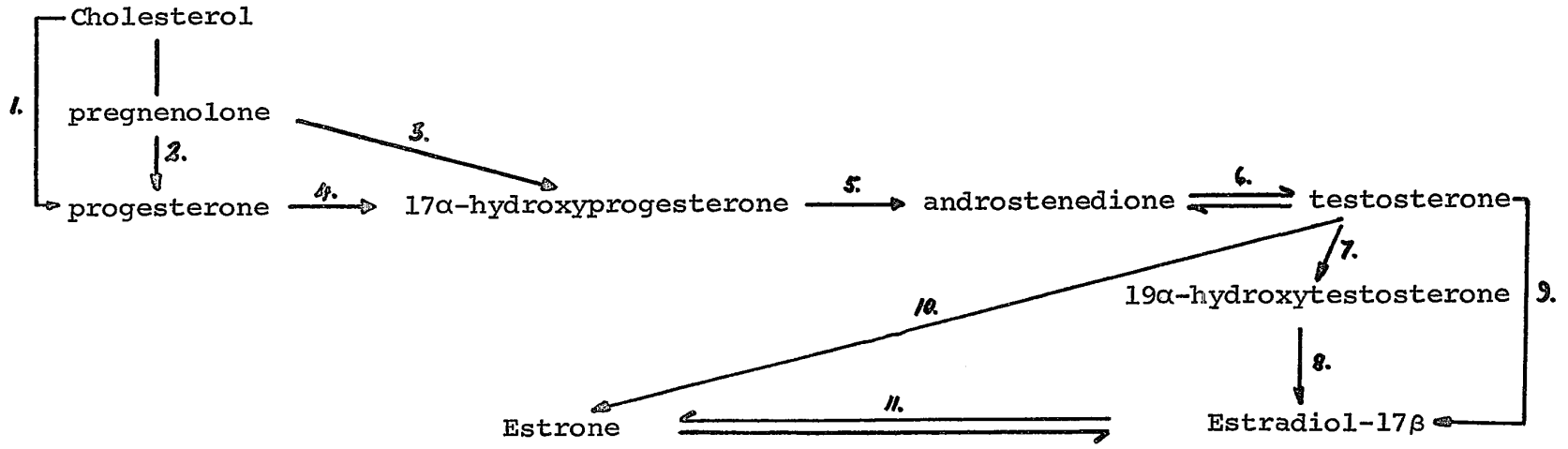
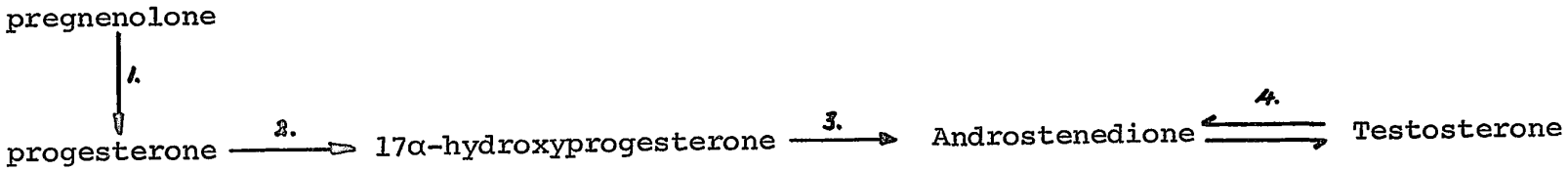


Figure 1. Biosynthetic pathways of androgen and steroid estrogen formation in mammals. (After Samuels and Eik-Nes (257)).



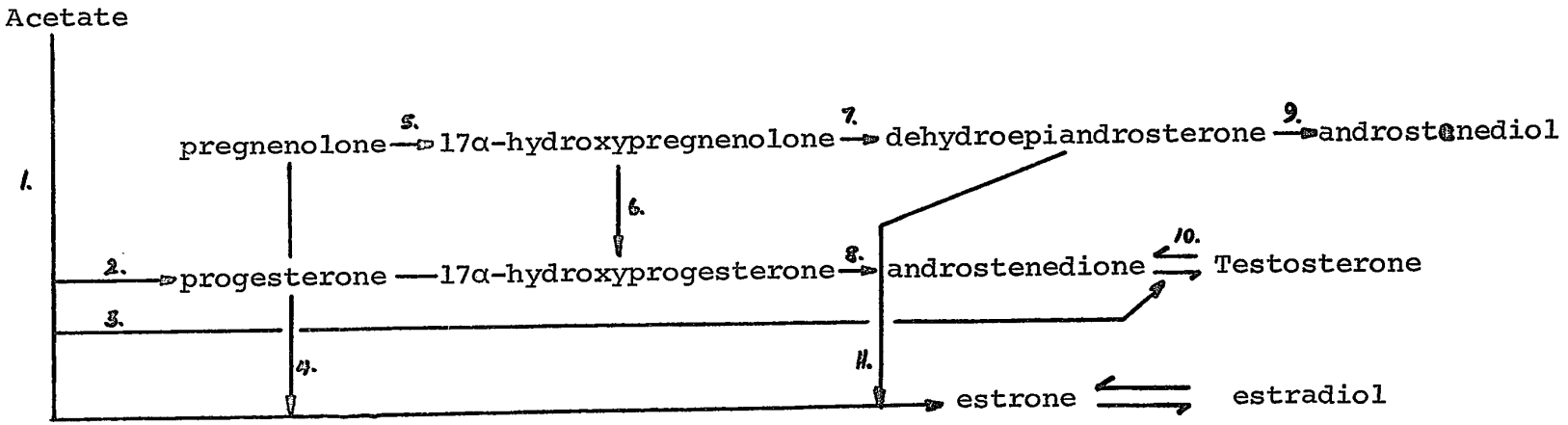
Conversion	References
1	238
2	300
3	33
4	33,228,238,246,300
5	33,228,238,243,246,300
6	33,224,228,238,246,300
7	228
8	ibid.
9	246
10	228,246
11	238

Figure 2. A summary of the in vitro studies on ovarian steroidogenesis in amphibia.



Conversion	References
1	30,31
2	30
3	ibid.
4	ibid.

Figure 3. Ovarian steroidogenesis in reptiles.



Conversion	References
1	23,112
2	23
3	ibid.
4	112
5	81
6	ibid.
7	ibid.
8	ibid.
9	112
10	81
11	112

Figure 4. Pathways of ovarian steroid formation in birds.

suggest that the Δ^4 pathway is favoured in the amphibia and reptiles whereas both the Δ^4 and Δ^5 routes are implied in birds. Even if one has regard to the relatively sparse data for the 'lower' vertebrates, it does seem likely that a basic pattern of ovarian steroid biosynthesis exists in all vertebrates.

I.6.2. Metabolism

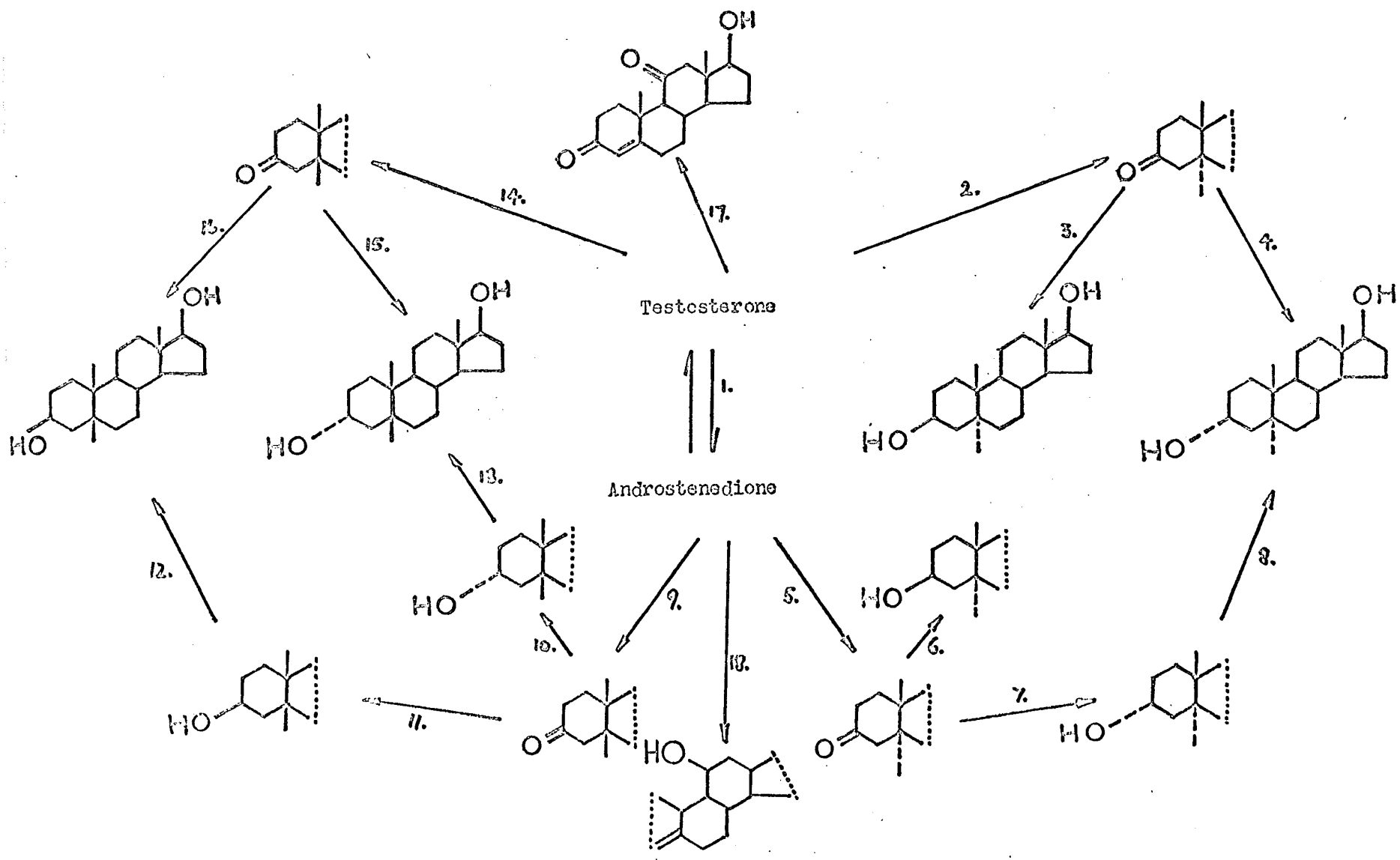
In most mammalian species, the major portion of the steroid hormones secreted appears in the urine or faeces as relatively inactive compounds reduced in ring A and conjugated with either glucuronic or sulphuric acid. The liver appears to be the major site of these changes. Some species excrete a major proportion of their steroid metabolites via the bile and gut whereas others excrete a major part by way of the urine. The metabolic pathways of the various classes of steroids have been reviewed recently (257).

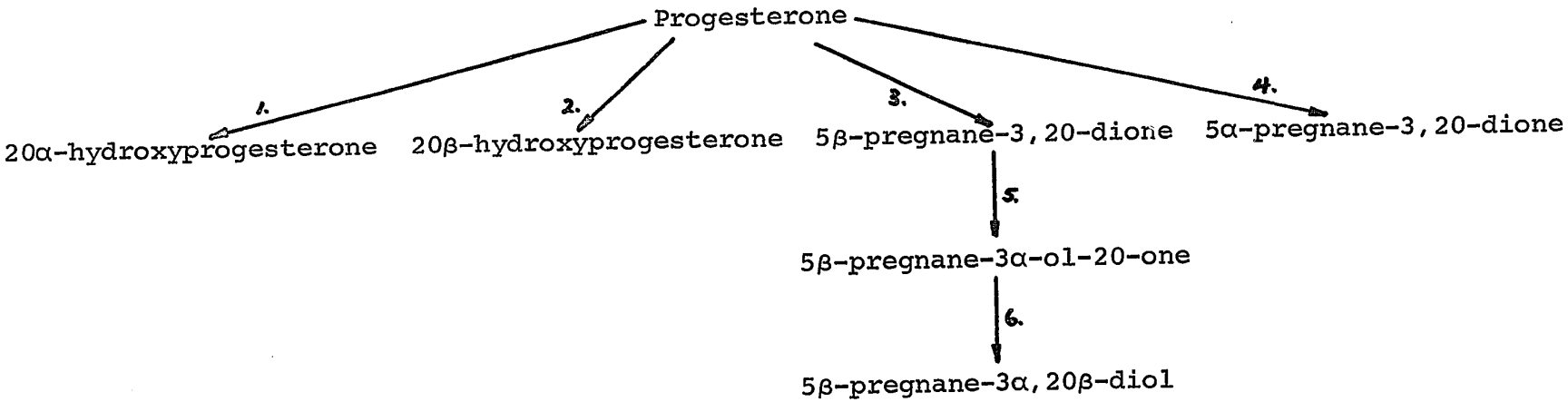
The pertinent data on the metabolism of progestins, androgens and estrogens are summarised in Figures 5, 6 and 7. These diagrams indicate that the metabolism of the ovarian steroids in the non-mammalian tetrapoda closely resembles the pattern observed in the mammals.

The same pattern of steroid biosynthesis and metabolism probably exists in all chordates and possibly in other

Figure 5. Androgen metabolism in non-mammalian Tetrapoda.

Conversion	Reference
1	30,33,81,224,228,238,246,300
2	26,33,224,246
3	26
4	26
5	26,33,224,246
6	26
7	26,33
8	26,31,33
9	26,33
10	26
11	26
12	26,31
13	25,26,31
14	25,26
15	25,26
16	33
17	238
18	238


Testosterone
Androstenedione
1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
13.
14.
15.
16.
17.
OH
HO
O



Conversion	Reference
1	208
2	208,228
3	40,199,292
4	194
5	40,199,292
6	40,199,292

Figure 6. Progesterone metabolism in the non-mammalian Tetrapoda.

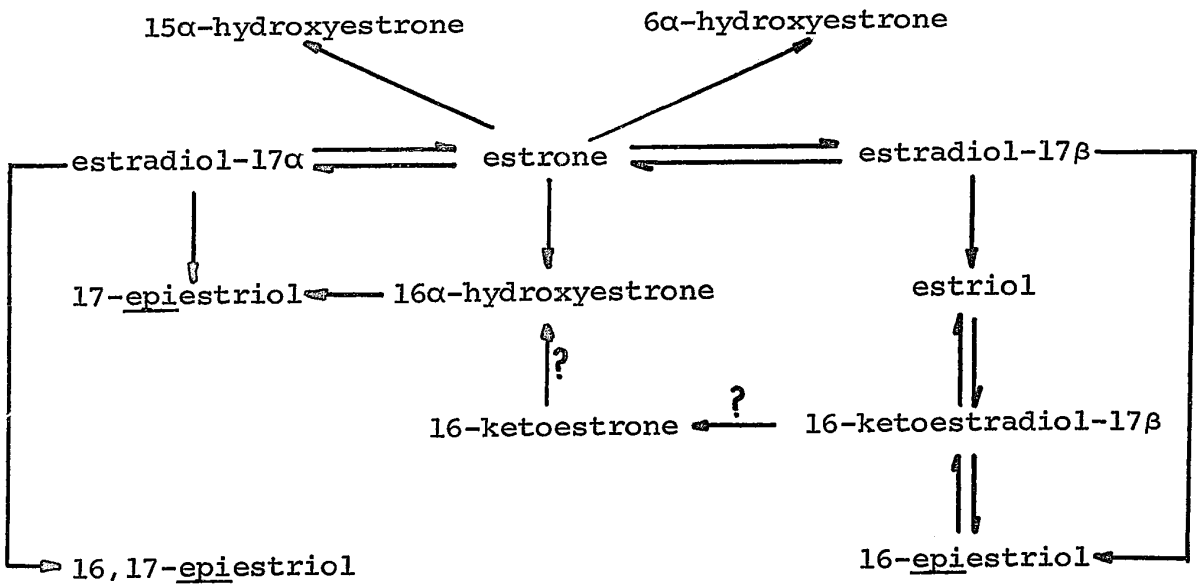


Figure 7. Estrogen metabolism in the non-mammalian Tetrapoda. (After Mathur and Common (183), Ozon and Breuer (226), Rao et al. (242) and Raud and Hobkirk (244)).

animals and even also in plants. The similarity throughout the vertebrates in the synthesis of steroid hormones may be related to the biochemical potentiality of the embryonic coelomic epithelium in chordates. The endocrine components of the gonads of both sexes and the adrenocortical tissue of mammals and its homologues in lower forms are known to be related embryologically in so far as all develop from the coelomic epithelium of the embryo (17). It is not surprising, therefore, to find the same general pattern of steroidogenesis in the vertebrate gonads and adrenals. The similarity may be regarded as reflecting an example of biochemical homology.

CHAPTER II

THE HORMONAL INFLUENCES ON REPRODUCTION AND OVULATION IN THE NON-MAMMALIAN TETRAPODA

II.1. Introduction

The study of ovulation in the "lower" vertebrates has been hindered, until recently, by the lack of sensitive techniques to determine the hormones involved. At the same time the broad relationship between the environment and reproductive activity has been well-documented. In the majority of poikilothermic animals, reproduction is rhythmic and the breeding phase is restricted to a definite season of the year; this season is characteristic of the species. Much of the information on the relationship between ovarian steroids and ovulation has been obtained from *in vitro* studies on whole or excised portions of the amphibian ovary.

II.2. Amphibia

The general relationship between environmental factors, such as light and daylength, temperature and the availability of food, and the breeding seasons of amphibia have been reviewed recently (109) and only the *in vivo* and *in vitro* studies on ovulation itself will be considered here.

II.2.1. Steroid Administration In Vivo

Barr (16) has reviewed the effect of steroid administration in vivo on amphibian ovulation and this aspect will be discussed only briefly in this review.

The experimental conditions used by different workers have differed greatly from worker to worker and it is doubtful whether much significance can be attached to the results. Two other points contribute to this doubt. The administered steroids are normally dissolved in arachis oil, or some similar vehicle, and it has been observed that this material may block the peritoneal funnels or interfere with the lymph hearts and may cause high mortality. Secondly, relatively very large doses of steroids have been used, i.e., usually greater than 1 mg per animal, and hence the results most likely reflect a pharmacological rather than a physiological effect (16).

Bearing these reservations in mind, one may perceive two general patterns in the results to date. The more obvious is the consistent failure of the steroid estrogens to produce ovulation in any of the six species studied. Secondly, some experiments have indicated that when steroids were administered simultaneously with pituitary extracts, all of them, whether androgenic, estrogenic or progestogenic induced ovulation, in contrast to the negative results

obtained by injecting the steroids by themselves.

II.2.2. Steroid Administration In Vitro

The effect of exposing whole ovaries or fragments of ovaries in vitro to steroids has been reviewed by Barr (16) and only recent developments in this field will be considered.

One subject that calls for clarification is that of oocyte maturation and oocyte ovulation. In most vertebrates completion of meiotic maturation of the oocytes normally occurs in association with the gonadotrophin-induced event of ovulation (262). These two processes had been generally considered directly related, until recent in vitro experiments on amphibia demonstrated that oocyte maturation can be induced independently of either the pituitary hormones or the ovarian tissues normally involved in ovulation (263).

Ovulation has likewise been shown to be induced by steroid hormones. In vitro maturation is stimulated by progesterone and certain other steroids (260). In many cases this occurs without a simultaneous induction of ovulation. Furthermore, partial or complete removal of the follicular tissues surrounding the oocyte does not interfere with this steroid-induced maturation (260,261). Ovulation and oocyte maturation appear, therefore, to be separate

events and can occur independently of each other. Both can be induced by a single gonadotrophic hormone and it appears that the presence of follicular cells is required for the initiation of maturation by gonadotrophic hormones.

It has been suggested that these two processes are the result of the synthesis or release of an ovarian intermediate, presumably a steroid resembling progesterone, in response to gonadotrophic stimulation of the ovarian follicle (261,296). It would appear that the state of differentiation, or potentiality of the tissues involved in ovulation and maturation is as important as the presence or secretion of a steroid hormone. The maturation and ovulation activities of gonadotrophins are probably inseparable. It is the intrafollicular differentiation due to differing cellular responsiveness which determines which process will occur. This differentiation is most likely a seasonal process and, if so, then this could explain the different effects of hormones on ovulation and maturation observed throughout the year (263).

It has long been known that steroids play a role in amphibian ovulation. Progestational, androgenic and certain adrenocortical steroids are effective stimulants of ovulation, whereas estrogenic steroids have been consistently ineffective in stimulating ovulation under similar <u>in vitro</u>

conditions (296). Recent evidence indicates that progesterone acts on the follicle wall to initiate ovulation (260, 261) and that the inhibitory effects of the estrogens on gonadotrophin-induced ovulation are the result of interference by the steroid with some initial action of the gonadotrophin on the follicle wall (263).

Wright (297) has provided additional evidence that gonadotrophins effect ovulation in amphibia by stimulating the synthesis of an ovulatory steroid. He observed that lymph-sac injection of cyanoketone (2α-cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one), a specific inhibitor of 3β-HSDH, 12 to 16 hours prior to excision of the ovary, results in the total inhibition of gonadotrophin-induced ovulation *in vitro*, but does not affect the ovulation induced by progesterone or by combinations of progesterone and pituitary extract. Pregnenolone induced ovulation *in vitro* in normal frog ovaries, though not in ovaries from cyano-ketone-treated animals. These results suggest that the capacity of frog ovarian tissue to form 3-keto-Δ^4 steroids is a requisite for gonadotrophin-induced ovulation. It may be noted that $E_2$17β in some cases causes a stoichiometric pattern of 3β-HSDH inhibition similar to that produced by cyanoketone (27), a fact which may explain the

inhibitory role of estrogens on gonadotrophin-induced ovulation.

Although Wright's results demonstrate that a 3-keto-Δ^4 steroid is necessary for ovulation, they do not indicate which steroid is immediately involved. Indeed, it may be that several steroids are equally effective as long as they incorporate the required active molecular structure. There are, however, two reports that implicate progestins as the steroids most likely to be involved. Thronton (286), using a bioassay, detected a progesterone-like factor in the plasma of B. bufo injected with a dose of human chorionic gonadotrophin sufficient to induce ovulation. This factor, considered to be of ovarian origin, was first detected four hours after the injection of gonadotrophin, reached a peak after eight to nine hours and fell to undetectable levels before ovulation began. Xavier and Ozon (300) obtained evidence from preovulatory females of Nectophrynoides occidentalis that pregnenolone was converted primarily to progestins and that neither androgens nor corticoids were found. Furthermore, progesterone is the most potent steroid inducer of in vitro oocyte maturation (260,261,269). Since the two processes of maturation and ovulation are closely related, it is possible that the same type of steroid is involved in both.

The initial action of gonadotrophin on the follicular wall leading to ovulation is as yet unknown. It does seem to involve protein synthesis, for the gonadotrophin-induced ovulation is inhibited by actinomycin-D and puromycin (13,264). This protein may be collagenase (13) or possibly a polypeptide associated in some way with the enzyme 3β-HSDH. The initial action of gonadotrophin may not be so simple, however, since a recent report suggests that the gonadotrophin stimulating maturation does not induce a macromolecular synthesis necessary for steroid release but rather simply induces the release of existing steroid from the follicular epithelial cells (189).

II.3. Reptiles

The available data on the relationship between ovarian steroids and ovulation in reptiles are, in general, exiguous. Nevertheless, the hypophysis-gonadal axis is generally well-documented (288). There are some data on the effects of injecting and also of implanting progesterone and $E_2$17β into various areas of the brain. Progesterone injections appear to inhibit follicular development, to induce some follicular atresia and thereby to retard ovarian growth (34). Implants of progesterone into the hypothalamus were

very effective in preventing further ovarian development, an action that mimicked that of hypophysectomy (34). Subcutaneous implants of $E_2 17\beta$ seem to have no affect on ovulation, whereas ovulation is blocked if $E_2 17\beta$ is implanted into the median eminence and anterior hypothalamus (34). Oviducal regression occurs in lizards with intrahypothalamic estrogen implants (34), and this provides some evidence for the existence of a negative feedback for the control of estrogen secretion by the ovary and also indicates that hypothalamic implants of estrogen interferes with gonadotrophin release.

II.4. Hormones and Reproduction in Wild Birds

The roles of hypophyseal and gonadal hormones in the reproduction of wild birds have been the subject of many excellent reviews. These include the reproductive endocrinology of birds (287), endocrinological aspects of behaviour (29,77,302,304), incubation patch development (143) and the control of avian reproductive cycles (80,130). It would be beyond the scope of this review to give any but a brief summary of the relationship of hormones to the reproduction of wild birds.

The hormones most directly implicated in the reproduction are the gonadotrophins, prolactin and the gonadal

hormones. At the end of their breeding season, most birds are reproductively quiescent, with regressed gonads and with a pituitary refractory to stimulation. The onset of the next breeding season begins with stimulation of pituitary activity by environmental changes, including photoperiod. This stimulation results in the secretion of gonadotrophins, which in turn stimulate the gonads to secrete gonadal steroids. These, together with the appropriate environmental conditions, stimulate the first stage of reproduction, including song, fighting, delineation of territory and courtship.

Incubation begins either with the arrival, or just after, the laying of the clutch. The physiological factors that control the serial ovulation and also whether the clutch is determinate or indeterminate are largely unknown, although prolactin has been implied in the control of clutch size. In most species the female takes over incubation, although in some species this devolves upon the male. It is not fully understood how the formation of brood patches influences the incubation behaviour. There is also some controversy as to which hormones are actually involved in this behaviour, although there is agreement that prolactin and progesterone are predominant (250).

The conflict arises as to whether prolactin acts

directly on the higher brain centres, or via the gonad, leading to the secretion of progesterone.

II.5. Ovulation in the Domestic Hen

The laying cycle of the domestic hen comprises a series of eggs, called a sequence, laid on successive days, successive sequences being interrupted by a pause of one or more day's duration. Whilst the time of ovulation cannot be easily recorded in the hen, it can be estimated rather closely from observation of oviposition. Early data indicated that ovulation normally followed oviposition by an average of 30 minutes (291). Although this is true for birds laying sequences of four to eight eggs, ovulation and oviposition seem coincident in long sequences.

Under optimal lighting conditions (usually a photoperiod of 12 to 16 hours) a hen typically lays the first egg of a sequence in the early hours of the morning and succeeding ovipositions occur progressively later in the following days until the sequence ends with an egg laid late in the afternoon. The difference in hours between the times of day of two successive ovipositions is called the lag (90). The greatest value of lag is some two to three hours and this occurs at the beginning and at the end of a sequence.

Lag approaches zero in the middle of long sequences.

II.5.1. Gonadotrophins of the Domestic Hen

The presence of gonadotrophic activity in the avian pituitary, first demonstrated in the pigeon, has been known for many years (249,287). It is only comparatively recently that the gonadotrophins have been partially characterised and that levels of gonadotrophic activity in the pituitary and blood have been related to ovulation.

Fraps et al. (87) reported the first fractionation of chicken anterior pituitary extracts by ammonium sulphate precipitation. They showed that the presumed LH fraction, but not the presumed FSH fraction, was active in inducing premature ovulation in the hen.

Chicken FSH and LH have been separated recently and also partially purified (274), and the two hormone fractions, when measured by accepted bioassays, gave valid parallel line assays when compared with standard preparations of human and ovine gonadotrophins (97). These results indicate that the anterior pituitary of the domestic hen contains both follicle-stimulating and luteinizing hormones.

It is currently accepted that the secretion of gonadotrophins by the anterior pituitary of vertebrates is controlled

by hormonal substances secreted by the hypothalamus and carried to the pituitary by the hypothalamic-hypophyseal portal vascular system (71). It is advocated that for each trophic hormone there is manufactured in the hypothalamus, a substance which induces the synthesis and release of the trophic hormones from the pituitary. These hypothalamic substances are known as releasing factors. Present evidence indicates that these releasing factors are short-chain polypeptides.

Recent in vitro and in vivo studies (83,134,137,151, 222,278-280) have demonstrated that both FSH releasing factor (FSH-RF) and LH releasing factor (LH-RF) are present in the hypothalamus of the domestic hen and of the Japanese quail. The in vitro results for LH-RF have to be accepted with caution, however, as they are based on the subsequent assay for the LH released using the ovarian ascorbic acid depletion (OAAD) assay. This assay is now known to be non-specific for LH in the cockerel (135). These results do suggest that, in the domestic hen, the secretion of gonadotrophin from the anterior pituitary is controlled by the hypothalamus and higher brain centres. This view is confirmed by the effect of lesions in the paraventricular nucleus, the median eminance and the pars tuberalis, because such lesions result in

interruption of ovulation for long periods. Lesions elsewhere in the hypothalamus had little lasting influence (106).

II.5.2. The Hormonal Control of Ovulation in the Domestic Hen

The role of pituitary hormones, especially LH and the role of the ovarian steroids, in the ovulatory cycle of the hen and the neuroendocrine mechanisms controlling them have been the subject of extensive reviews (91,102,104,106). Essentially, the two major events to be incorporated into any theory are the phenomena of daily lag and the missed ovulation which causes interruption of the sequence. Another consistent feature that must be included is that the release of LH from the pituitary must occur some six to eight hours before ovulation (91,92). Despite the lack of basic information, several attempts have been made to account for the events in the ovulation cycle of the domestic hen in neuroendocrine terms (19,90,209,287). Of these, Fraps' (90) hypothesis displays the greatest degree of accord with available experimental evidence.

According to Fraps' theory, follicular growth and maturation are dependent on FSH secreted by the pituitary. The theory holds that there is a relatively steady output of FSH plus some LH in the normally laying hen, but that release of extra LH from the pituitary at intervals triggers

ovulation. The release of this extra LH from the pituitary is considered to be stimulated by an hypothetical 'excitation hormone' which acts via a neural pathway. The threshold for the action of the 'excitation hormone' is held to be subject to a diurnal rhythm. Fraps' theory involves the idea that the excitation hormone level starts at a set time in relation to preceeding ovulation and increases until it reaches the threshold level for release of LH and consequent ovulation. This hypothetical rhythm in threshold level provides an explanation for the circumstance that an ovulation due to occur in late afternoon does not occur, but is delayed until early the next day, that is, a day on which no egg is laid, thus leading to a pause in the laying sequence. Fraps' explanation is that the rising level of 'excitation hormone' misses the time of low threshold on those days when ovulation does not occur.

Nelson et al. (213) used the OAAD assay to measure plasma LH activity at intervals throughout the cycle and reported peak values at 8, 13 and 21 hours before ovulation. Bullock (28), using the same method, confirmed the observation of three peaks of LH activity and also observed that whenever peaks of LH activity did not occur, there was no ovulation and, consequently, a pause in egg-laying. Heald

et al. (116) used the OAAD assay to measure the LH content of the pituitary. They reported that pituitary LH activity decreased shortly after an egg had been laid and remained low for several hours, after which it rose to a high value at four to eight hours before the next ovulation and then decreased rapidly. They interpreted this decrease as corresponding to the release of ovulatory LH. In a later paper (117) the corresponding data for the terminal egg of a sequence were presented. The pituitary LH activity remained constant for 12-14 hours after ovulation of the last egg of the sequence, then rose to a peak 12 hours before ovulation and decreased to lower levels at four to eight hours before ovulation.

The results of Tanaka and Yoshioka, which show peaks of pituitary LH activity at 20 and at 8 hours prior to ovulation (279), have further confused the picture. Recently, Constantine (ex 55) demonstrated a peak in pituitary LH activity seven hours prior to ovulation. Five hours before ovulation there was a significant decrease in pituitary LH activity and a marked increase in such activity in the plasma. The foregoing results are open to criticism in view of the reported evidence that the OAAD assay is not specific for LH in the adenohypophysis of the cockerel (135). The discrepancies between the results shown in Table 5 are not

unexpected, therefore, and it must be concluded that the OAAD assay has not led to an unequivocal resolution of the time relationship between LH release and ovulation.

Table 5. Time of peak levels of LH and FSH in the pituitaries and plasma of laying hens.

	Time of peak levels of gonadotrophin (hours before ovulation		Reference
	Pituitary	Plasma	
LH (OAAD assay)	14,21	8,14,21	213
	-	8,14,20	28
	0, 8	-	116
	8,20	-	279
	0, 7	5	58
		11	150
FSH	22,14,6	11,25	129

More recently Cunningham and Furr (55), using a radioimmunoassay for avian LH (84), have reported that plasma LH concentration shows a two- to three-fold increase from basal levels to reach a maximum value four to seven hours before ovulation. No such peak was seen in birds which failed to ovulate during the experimental period. An elevated level of LH was detected some 20 to 23 hours before ovulation in five out of the twenty birds studied, but the small number of observations precludes any definite conclusion as to the

physiological reality of this early LH peak.

Kamiyoshi and Tanaka (150) performed bioassays of FSH activity of pituitaries from laying hens. They found that pituitary FSH content fluctuated during the ovulatory cycle and concluded that a peak release of FSH from the pituitary occurred some eleven hours before ovulation. Imai and Nalbandov (129), using the same bioassay as Kamiyoshi and Tanaka, supplemented these results when they demonstrated changes in FSH activity of the anterior pituitary and of blood plasma during the laying cycle. They reported two peaks of FSH activity in the plasma and three in the pituitary. The highest plasma FSH activity occurred some eleven hours prior to ovulation, with a second minor peak about 25 hours before ovulation. The first pituitary peak was relatively small as compared with the other two peaks. The second and third pituitary FSH peaks were found at points in time preceeding the two plasma peaks (Table 5).

The available evidence indicates that all three classes of ovarian steroids - progestins, androgens and estrogens - may affect the ovulatory cycle of the domestic hen. Progesterone, however, is especially effective in this regard (106,287,288).

Administration of progesterone enhances premature ovulation of the hen's follicle (86) by activating certain

regions of the hypothalamus, but not the pituitary (240,241). The injection of large doses of progesterone suppresses ovulation (148,265), although the significance of this is doubtful, in so far as the effect may be pharmacological rather than physiological. Four recent papers have indicated that there is a definite fluctuation in the concentration of plasma progesterone in relation to ovulation (14,55, 154,234). All four papers report a significant peak of plasma progesterone at some four to seven hours prior to ovulation. There is an abrupt decline before ovulation begins. This peak of progesterone was not detected in the plasma when ovulation did not occur.

There is some evidence indicating that estrogens may be concerned in ovulation. It has been mentioned previously that there are fluctuations in urinary estrogen excretion during the ovulatory cycle, oviposition, and by inference, ovulation, always being associated with falling estrogen excretion (37,185). Fraps (90) observed that injection of estradiol benzoate 28 to 11 hours before an ovulation suppressed that ovulation in 55% of the treated hens. There is also evidence for significant cyclic accumulations of radioactivity in the pituitary of the laying hen after the injection of tritiated $E_2 17\beta$, the greatest accumulation

being in the period 16 to 12 hours before the expected ovulation.

Van Tienhoven (287) has stated that the injection of testosterone is about half as effective as the injection of progesterone in causing premature ovulation in the domestic hen. This testosterone-induced ovulation also seems to be mediated by a neural mechanism.

Although these results suggest an intimate association between progesterone and estrogen and the release of LH by the pituitary, the lack of comparable data for androgens precludes any conclusions as to the nature of the 'excitation hormone' proposed by Fraps. The exact causal relationships between the ovarian steroid hormones and gonadotrophin release in the domestic hen also remain to be elucidated.

PART II

EXPERIMENTAL

CHAPTER I

THE EXPERIMENTAL METHOD

The relatively new experimental technique used in this thesis has revolutionized the whole field of endocrinology and is applicable to the assay of both polypeptide and steroid hormones. The method exploits, on the one hand, the extreme delicacy of radioactive measurement and, on the other, the almost unique chemical specificity which characterizes many biochemical reactions of the proteins. The term saturation analysis may be used to describe the general nature of the principle involved, relying on progressive saturation of a specific reagent by the test compound. This method found its initial practical application in two assays published in 1960, one for serum thyroxine and one for serum insulin (78).

Since 1960 variations of the original concept have been applied and have given rise to a number of analytical methods each with a different name. Figure 8 attempts to relate these terms together. The two most common terms, competitive protein-binding (CPB) and radioimmunoassay (RIA), merely reflect the origin of the protein used in the assay. Both CPB and RIA have been the subject of many extensive

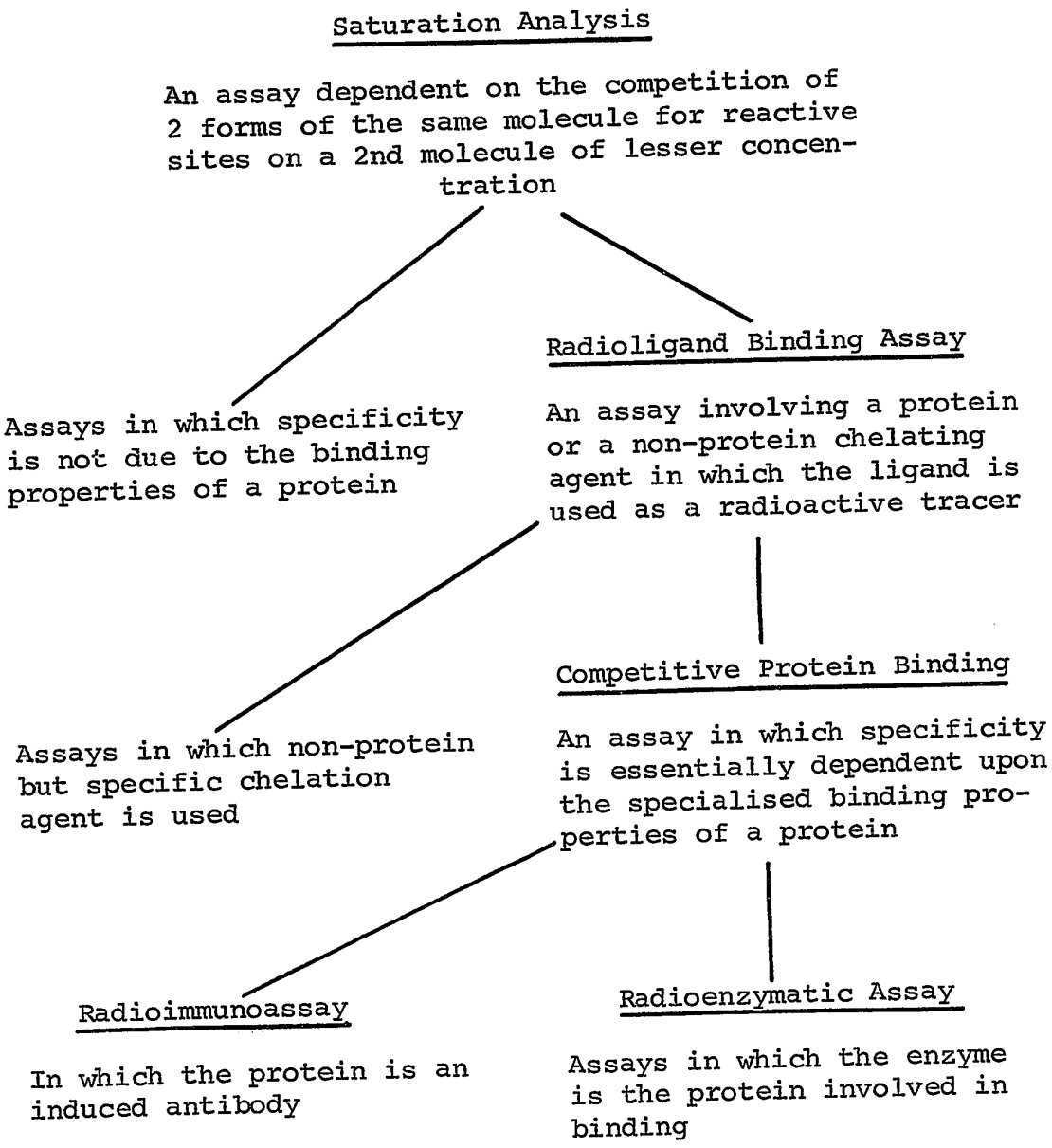


Figure 8. An interpretation of the current nomenclature of saturation analyses. (After Murphy (203,205)).

and excellent reviews (70,200,216,233) and the theoretical and practical considerations will be discussed only briefly.

CPB is defined as that form of saturation analysis in which one reacting molecule is a protein with a high affinity and high specificity for the other, which is thus a ligand. RIA methods depend on the highly specific reactions between either polypeptide or steroid hormones and their antibodies. The difference between CPB and RIA lies in the fact that the protein in the former assay is already present in the donor animal, whether circulating in the plasma or present in the target tissue, whereas in the latter assay it is induced in the serum by immunization.

Briefly the assay depends on the competition between radioactive hormone and non-radioactive hormone for specific binding sites on a protein. These interactions obey the law of mass action, so that if the amount of protein and radioactive hormone are kept constant, then as more and more non-radioactive hormone is added to the system it will displace progressively more radioactivity from the protein and the amount of radioactivity bound to the protein will fall. The standard curve of the assay is obtained by plotting the amount of bound radioactivity against the amount of non-radioactive hormone added. The requirements for such an

assay are, therefore, a suitable binding protein and an isotopic form of the substance to be measured.

When one applies the general principle to the assay of steroids in biological fluids, four main problems present themselves, and these are (a) choice of binding protein, (b) removal, if present, of binding proteins from the test samples, (c) separation of competing analogues from the system and (d) separation of the protein-bound radioactivity from the non-protein-bound radioactivity.

The first decision is the choice of an assay protein, and this choice resolves into considering plasma proteins, tissue proteins or antibodies. While the plasma proteins are certainly the most readily available, they are perhaps the least specific. It is important that there should not be high concentrations of proteins with a high capacity and low affinity, such as albumin. This can be accomplished by simply diluting the plasma. Sometimes preliminary purification of the plasma proteins, for example separating the actual binding globulins, results in improvements in the assay. Tissue proteins are highly specific for their corresponding active hormone. Lack of stability has been a problem, but this is usually due to the choice of donor animal. Sheep uterine cytosol for example, is stable for over a year

at -10°C, whereas rabbit uterine cytosol is only stable for three months and has to be stored in liquid nitrogen. Antibodies are difficult to prepare and one can never be sure of obtaining precisely the same antibody composition twice in a row. Once a suitable batch of high titre is obtained, however, it will last for a considerable time. Thus, the time spent to immunize the donor animal initially is extremely worthwhile. Many excellent reviews on the methods of preparing antibodies to steroid and polypeptide hormones are available (70,149,233).

The second problem is the removal or destruction of interfering endogenous binding proteins. This is most simply done either by heat or organic solvent extraction of the sample. In many instances adequate removal and subsequent specificity of the steroid in question can be achieved by judicious use of a simple separation procedure. For example, progesterone can be readily assayed by prior extraction into petroleum ether (201).

The third problem is the separation of competing analogues. This is the single, and in fact, the only troublesome aspect of CPB and RIA. With satisfactory separation systems, specificity would cease to be a problem entirely. The difficulty about most separatory systems, especially TLC,

is that they give rise to high blank values. Recently, column chromatography on Sephadex LH-20 seems to have overcome this problem (205,206).

Lastly, there is the problem of separating the bound from non-bound radioactivity. This can be done in many ways and the choice is usually a matter of convenience and personal preference. The common method used is adsorption, either by Florisil or by dextran-coated charcoal. Figure 9 summarises the general procedures involved in saturation analyses.

Step	Purpose
1. Sample preparation	
a. Deproteinization	To destroy or remove any binding protein from the sample.
b. Purification	Separation of any competing analogues.
2. Quantitation	
a. Equilibriation with tracer and assay protein	Permit the sample ligand and tracer ligand to react with the assay protein in proportion to their concentrations.
b. Separation of the protein-bound and unbound radioactivity	Determine the distribution of the radioactivity.
c. Count the radioactivity	

Figure 9. Some methodological considerations involved in saturation analyses. (After Murphy (203,205)).

CHAPTER II

PROGESTERONE CONCENTRATION IN PERIPHERAL PLASMA OF LAYING HENS AS DETERMINED BY COMPETITIVE PROTEIN-BINDING ASSAY

II.1. Introduction

Recent reviews have discussed in great detail many aspects of the occurrence and estimation of progesterone in the plasma (144,193). The rapid development of new techniques for progesterone estimation, notably those making use of CPB or RIA, have made possible the application of progesterone assays on a much larger scale.

The CPB assay of Murphy (201), based on the competition between progesterone and tritiated corticosterone for binding sites on the corticosterone-binding globulin (CBG) of dog plasma was the first to be reported. Corticosterone-^{3}H of high specific activity, is bound to CBG and subsequently displaced by the addition of unlabelled progesterone to the system. Most of the subsequent CPB assays of progesterone have been based on modifications of this original method.

Murphy (201) suggested that the specificity of the method could be improved by extracting the plasma with a particular petroleum ether that extracted essentially only

progesterone. This idea was further advanced when the specific properties of one particular lot of petroleum ether were utilized to get a semiquantitative assay for progesterone (139,140,141). Several investigators have since reported improvements of this method (46,68,76,169).

The specificity of the method can be improved by introducing various separation steps that remove the interfering compounds from the initial extraction. Neill et al. (212) were the first to include a thin-layer chromatographic separation of the initial plasma extract. Many subsequent workers have also included a TLC step, or steps (21,125,131, 247,248,256,266,273,301), whereas others have included either a paper (178) or a celite-column chromatographic step (275).

Recently radioimmunoassays, usually including celite-microcolumn chromatography, have been developed and have given greater sensitivity to the assay of progesterone in the blood (4,100,272).

II.2. Materials and Methods

II.2.1. General Materials and Apparatus

(a) Reference and radioactive steroids:- Crystalline progesterone, M.P. 128-129.5°C, lot No. U3676, was obtained from Mann Research Laboratories, New York. Progesterone-4-^{14}C (21.7 mCi/mMole) and corticosterone-1,2-^{3}H (41.5 Ci/mMole),

were purchased from Amersham-Searle Corp., Don Mills, Ontario.

(b) Chemicals:- PPO (2,5-diphenyloxazole) and POPOP (1,4-bis-2,5-phenyloxazole benzene) were obtained from Packard Instrument Cmpy., Downer's Grove, Illinois. 2',7'-Dichlorofluorescein was purchased from Eastman-Kodak, Rochester, New York. Florisil (100-200 Mesh) was obtained from Fisher Scientific Co., Montreal.

(c) Reagents:- Petroleum ether, 30°-60°C, lot No. 7106P (Anachemia, Montreal) was redistilled between 30° and 60° and stored in amber bottles in the dark. The solvent properties of the petroleum ether are critical for the method, and therefore sufficient was prepared in one operation for all assays included in the present study. Benzene ('spectranalyzed'), methanol ('spectranalyzed') and toluene (certified ACS, lot T-324) were obtained from Fisher Scientific Co., Montreal. The scintillation cocktail consisted of 0.30 g PPO and 0.01 g POPOP dissolved in 1.0 litre of toluene. An 0.2 per cent solution of 2',7'-dichlorofluorescein in absolute ethanol (Consolidated Alcohols Ltd., Toronto) was used to visualize the thin-layer chromatograms.

(d) Standard solutions:- The primary standard solution of crystalline progesterone was prepared in 'spectranalyzed' methanol at a dilution of 1.0 mg/ml. The working

solution was prepared from this primary standard at a final dilution of 0.02 μg/ml. Both standard solutions were stored at 4°C.

(e) Protein-binding solution:- The protein-binding solution was prepared by dissolving 2.5 ml of dog plasma (obtained from the MacIntyre Animal Centre, McGill University) and 0.4 ml of a solution of tritiated corticosterone (10 μCi/ml in ethanol), in 97.1 ml of glass-distilled water. Care was taken to add the plasma after adding most of the water to avoid precipitating the protein with ethanol.

(f) Apparatus:- The Shandon outfit for the TLC was purchased from Consolidated Laboratories (Canada) Ltd., Toronto. Glass plates (20 x 20 cm) were coated with a 0.25 mm thick layer of Silica Gel G (Merck). The plates were air-dried for 30 minutes and then were run once in absolute methanol-concentrated HCl (9:1 v/v) and twice in Lisboa's (168) solvent system N (benzene:ethanol, 95:5). The plates were air-dried between each step. After the final solvent run the plates were activated at 100-110°C for one hour and stored in a desiccator over anhydrous Silica Gel. Detection of progesterone on the thin-layer chromatograms was performed by spraying the plates with the dichlorofluorescein solution and then viewing the plate under ultra-violet light.

Radioactivities were measured by liquid scintillation spectrometry (Packard Model 3003). The counting efficiencies for ^{3}H and ^{14}C, determined from appropriate quenching curves, were 26.5% and 75%, respectively.

II.2.2. Procedure

The laying hens (Rhode Island Red males x S.C. White Leghorn females) were housed singly in cages of a laying battery fitted with an automatic timing device.

Blood samples (about 2 ml) were withdrawn from the wing by means of a heparinized syringe fitted with a No. 23 needle. It was quite feasible to obtain successive samples at three to five hour intervals over a 24 to 30 hour period. The sample was centrifuged immediately and the plasma stored at -10°C pending analysis. Samples that displayed any signs of haemolysis were discarded.

The assay method was a slight modification of that first described by Murphy (201). Plasma (0.5 ml), in duplicate, was extracted with redistilled petroleum ether (2 x 4 ml) and the separation after each extraction was facilitated by centrifugation. The petroleum ether was evaporated in a current of nitrogen. The rest of the assay was carried out as described by Murphy (201) except that 74 mg of Florisil was used to separate the bound and unbound corticosterone.

II.3. Results and Discussion

II.3.1. Specificity

The specificity of the method has been previously discussed (pp97-98) and is a function of the properties of the petroleum ether used and of the binding protein. To assess the specificity of the method as applied to laying hens' blood, the progesterone in petroleum ether extracts of plasmas, and in similar extracts of the same plasmas to which known amounts of progesterone had been added before extraction, was isolated by TLC in Lisboa's system N (168). This system was found efficient for the separation of progesterone from the 20-hydroxy epimers of progesterone and from 5β-androstanedione, steroids which are present in hens' plasma (98). Elution of the steroids from appropriate areas of the plate was done with absolute methanol (2 x 3 ml followed by 1 x 1 ml). The solvent was evaporated from the eluates in a current of nitrogen and the progesterone in the residues measured by the protein-binding assay.

Progesterone in successive areas scraped from plates was measured by the assay. The only appreciable amounts found corresponded in mobility with authentic progesterone. The overall recovery, measured by the recovery of ^{14}C-progesterone applied in separate lanes, was 65±3.2%(SD). When

correction for this recovery was applied to the values, measured by CPB, for progesterone in the petroleum ether extracts and the extracts plus known amounts of progesterone, the values obtained accounted for not less than 90% of the values obtained for the same plasmas when the TLC was not included (Table 6).

The foregoing observations showed that a TLC step was not essential for the assay of progesterone at the concentrations which occur in the peripheral blood plasma of laying hens. This finding was not unexpected. Although 5β-androstanedione is present in such plasma (98) in concentrations comparable with that of progesterone, it does not compete with tritiated corticosterone for binding sites on the CBG. The only steroid present which does compete with progesterone in the assay is 20α-hydroxyprogesterone (284), but this is the less abundant epimer in the plasma (98) and it competes but weakly.

It was concluded that the specificity of the assay was at least reasonably adequate over the ranges of concentration found in plasmas from laying hens and that a TLC step could be omitted. Similar conclusions on the assay of progesterone in other vertebrates have also been reached (140,251).

Table 6. A comparison of the assay with and without a thin-layer chromatographic step.

Amount progesterone added (ng)	Amount progesterone measured without TLC		Amount progesterone measured with TLC		Amount progesterone measured with TLC corrected for 65% recovery from the plate		Percentage of progesterone measured with TLC as opposed to that measured without	
2.0 ng	1.80	1.70	1.05	1.01	1.60	1.55	90	92
	1.50	1.60	0.88	0.97	1.35	1.49	91	94
	1.60	1.70	0.99	1.02	1.52	1.56	95	91.5
	1.80	1.80	1.08	1.07	1.66	1.64	92	91.5
	1.50	1.60	0.88	0.94	1.35	1.44	90	90.0
6.0 ng	5.00	5.00	2.99	2.96	4.60	4.55	92	91.5
	5.60	5.80	3.46	3.54	5.32	5.44	95	94
	4.90	4.90	2.90	2.90	4.46	4.46	91	91
	4.60	4.70	2.70	2.70	4.15	4.15	90.5	90
	5.00	5.10	2.94	2.98	4.52	4.58	90.5	90
10.0 ng	9.60	9.20	5.86	5.50	9.02	8.46	94	92
	8.80	8.90	5.15	5.50	7.92	8.46	90	95
	9.50	9.40	5.79	5.68	8.91	8.74	94	93
	8.00	8.00	4.84	4.84	7.45	7.45	93	93
	8.60	8.40	5.09	5.00	7.83	7.69	91	91.5

II.3.2. Accuracy

The accuracy of the method was examined by recovery experiments. Amounts of progesterone of 2 ng, 6 ng and 10 ng were added to duplicate laying-hen plasma samples (0.5 ml), which were then assayed together with corresponding duplicate blank samples. The recoveries are presented in Table 7.

Table 7. Recoveries of progesterone added to 0.5 ml-samples of laying-hen plasma.

Amount Added ng	No. Duplicates N	Recovery ng ± S.E.	%
2.0	11	1.7 ± 0.03[a]	85
6.0	7	5.1 ± 0.12[b]	85
10.0	9	9.1 ± 0.14[b]	91

[a]Variance significantly different ($P < 0.01$) from variances of recoveries bearing superscript b.

II.3.3. Precision

This term denotes the reproducibility of the results and is usually expressed in terms of the standard deviation, computed from the expression $Sx = \sqrt{(\Sigma d^2/2N)}$ (271), where (d) is the difference between the two individual determinations in a duplicate analysis and (N) is the number of such duplicate analyses, that is, the number of pair analyses.

An overall average value for the precision was calculated from 27 duplicate analyses covering the range 2.0 to 10.0 ng/ml by applying the above expression. The calculated value was $S\bar{x} = \pm 0.29$ ng.

II.3.4. Sensitivity

In the present context, sensitivity is measured by the least amount of the substance that can be distinguished with a given degree of probability from zero amount, and as used in this sense, it is really an extension of the term precision. Thus, if the value of $S\bar{x}$ has been established for a series of samples of low concentration, such that any variation of $S\bar{x}$ with the value of x may be neglected, then sensitivity may be computed from the relation,

$$\text{sensitivity} = t \times Sx/\sqrt{n}$$

where (t) is the tabulated value of Student's "t" for the number of degrees of freedom available for the calculation of Sx at the desired probability level; (n) is the number of analyses in the array, usually single, duplicate or triplicate.

In the present assay, the least amount distinguishable from zero amount was calculated from the data for recovery of amounts of 2.0 ng, because Sx for recovery was not independent of concentration. The calculated value for

duplicate determinations was 0.27 ng ($P < 0.05$).

II.3.5. Occurrence of an Ovulatory Cycle of Blood Progesterone Level

Times of oviposition were known within ±10 minutes and hence the values for blood progesterone concentration could be related to times of oviposition. Ovulation of an egg laid on the following day may be assumed to have occurred within half an hour of the oviposition on the preceeding day, although the actual time of ovulation is unknown. If no egg was laid on the succeeding day, it could be presumed that no ovulation had accompanied the oviposition. This presumption may not always hold because in some cases "internal" laying occurs, that is the ovum fails to be engulfed by the infundibulum, and is slowly resorbed from the body cavity. With these reservations, plasma progesterone levels could be related in time to times of oviposition accompanied by ovulation, or in the case where no ovulation occurred, that is, in the case of the last egg, (CT), of a sequence, they could be related to oviposition unaccompanied by ovulation. Results were obtained for nine ovulations within a laying sequence and for five CT eggs, that is, for five days of no ovulation. These observations were made on different hens.

The average levels varied considerably from bird to bird. The time intervals from oviposition also differed. To compare the data, therefore, the values for each ovulatory cycle were calculated as percentages of the highest value observed for the cycle in question and these percentages were then plotted against times before or after oviposition. The data for other than CT eggs are presented in Figures 10 and 11. The data for CT eggs are presented in Figure 12.

Figures 10 and 11 show that each of the nine hens for which preovulatory values were observed displayed a peak level of blood progesterone at four to six hours before ovulation, assuming ovulation to occur within half an hour of oviposition. Corresponding plots (Figure 12) of the data for the five hens for which oviposition was not succeeded by ovulation displayed a completely different picture. The values showed a general decline in progesterone concentrations from the time of oviposition.

While the major result of this study relates to the occurrence, in a given bird, of the peak level of plasma progesterone at four to six hours before ovulation, the actual levels measured are of interest and are presented in Table 8. The observed values range from 0.5 ng/ml to 12.5 ng/ml and are in close agreement with those previously reported (Table 3).

Figure 10. Plasma progesterone values plotted against hours before (-) or after (+) oviposition accompanied by ovulation. The values for each individual hen are expressed as percentages of the maximal value observed during the ovulatory cycle in question.

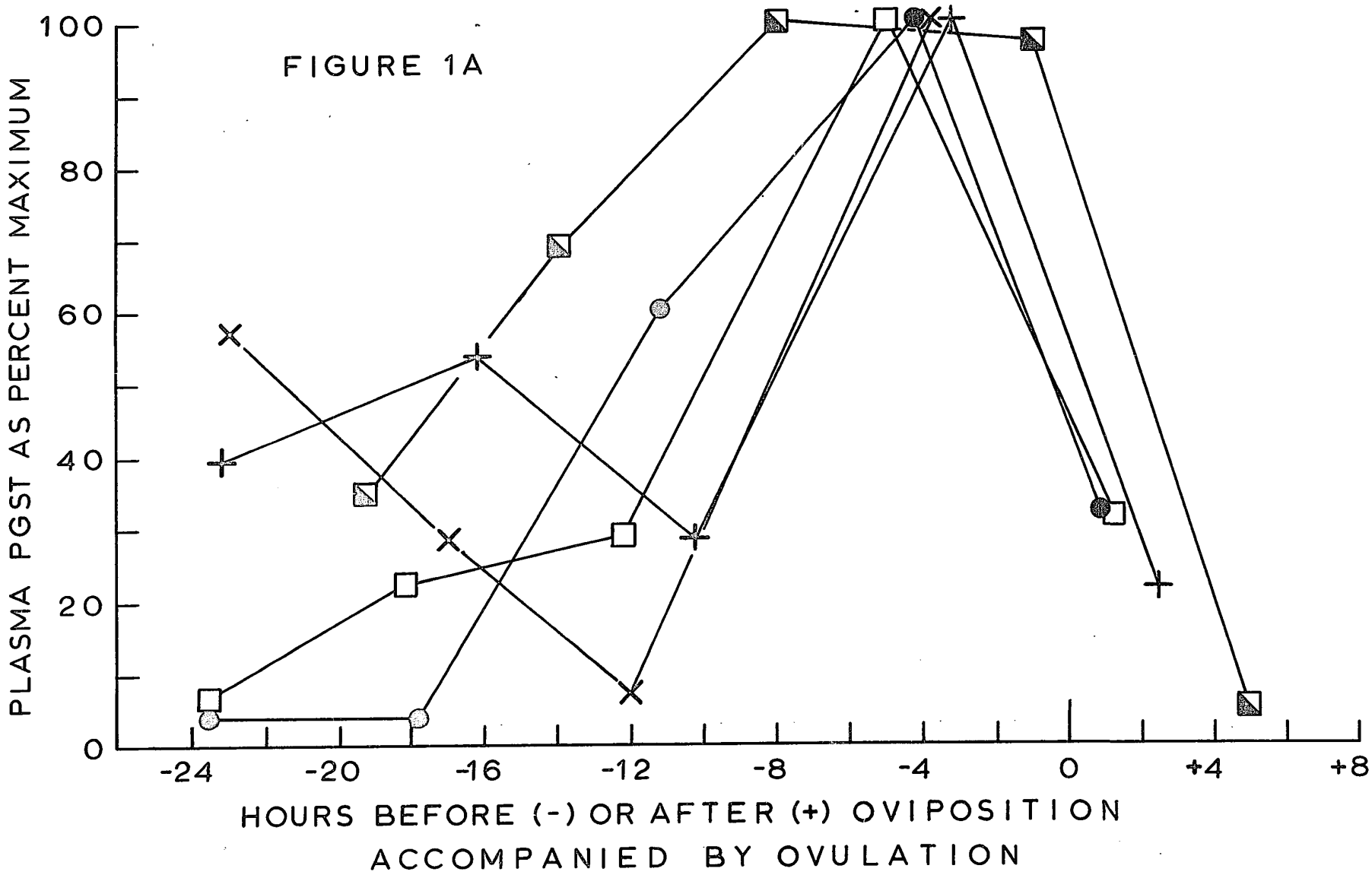


FIGURE 1A
PLASMA PGST AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 11. Plasma progesterone values plotted against hours before (-) or after (+) oviposition accompanied by ovulation. The values for each individual hen are expressed as percentages of the maximal value observed during the ovulatory cycle in question.

FIGURE 1B

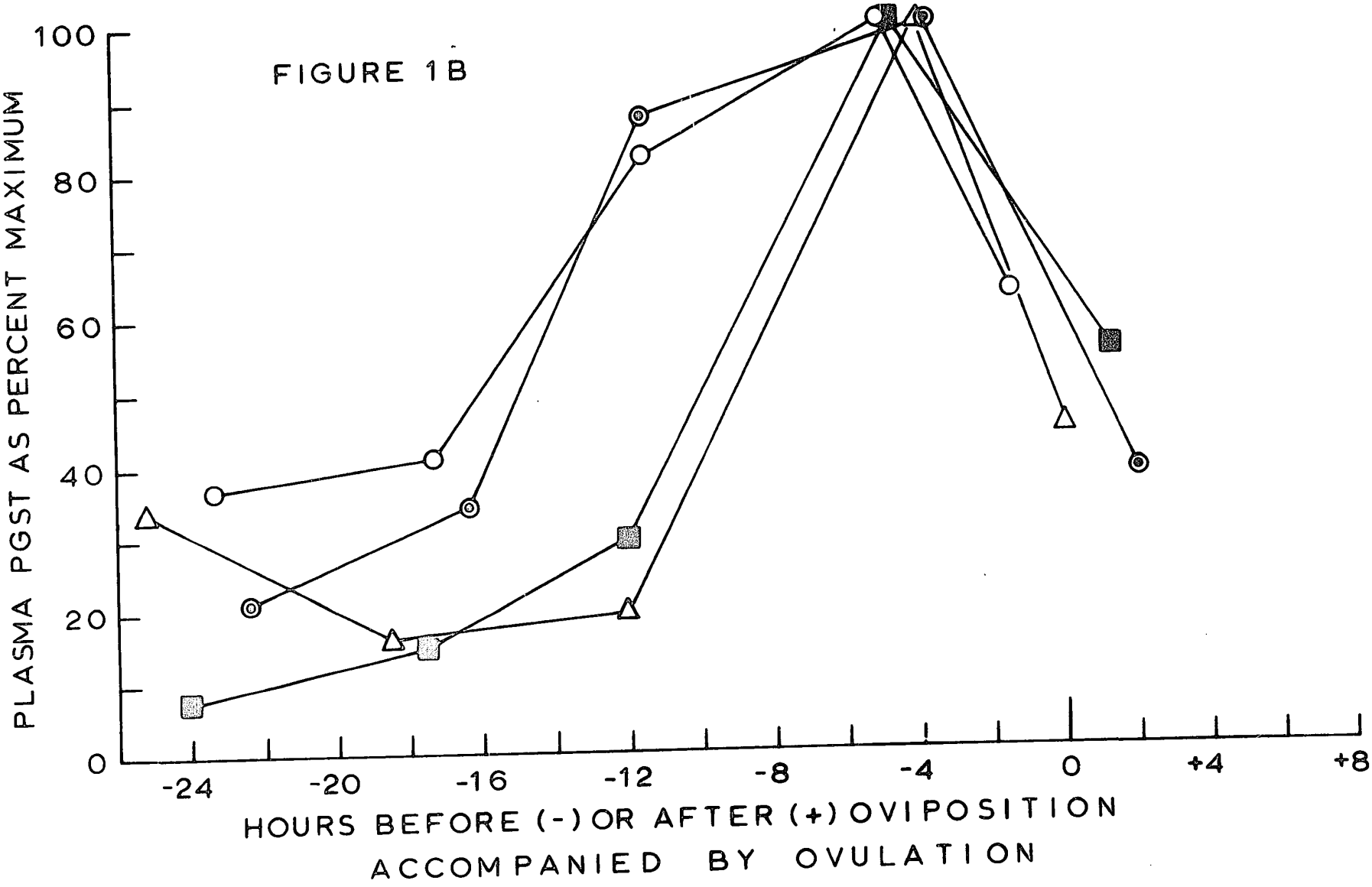

PLASMA PGST AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 12. Plasma progesterone values plotted against time before (-) or after (+) an oviposition unaccompanied by an ovulation. Values for each individual hen are expressed as percentages of the maximal value observed during the period for the bird in question.

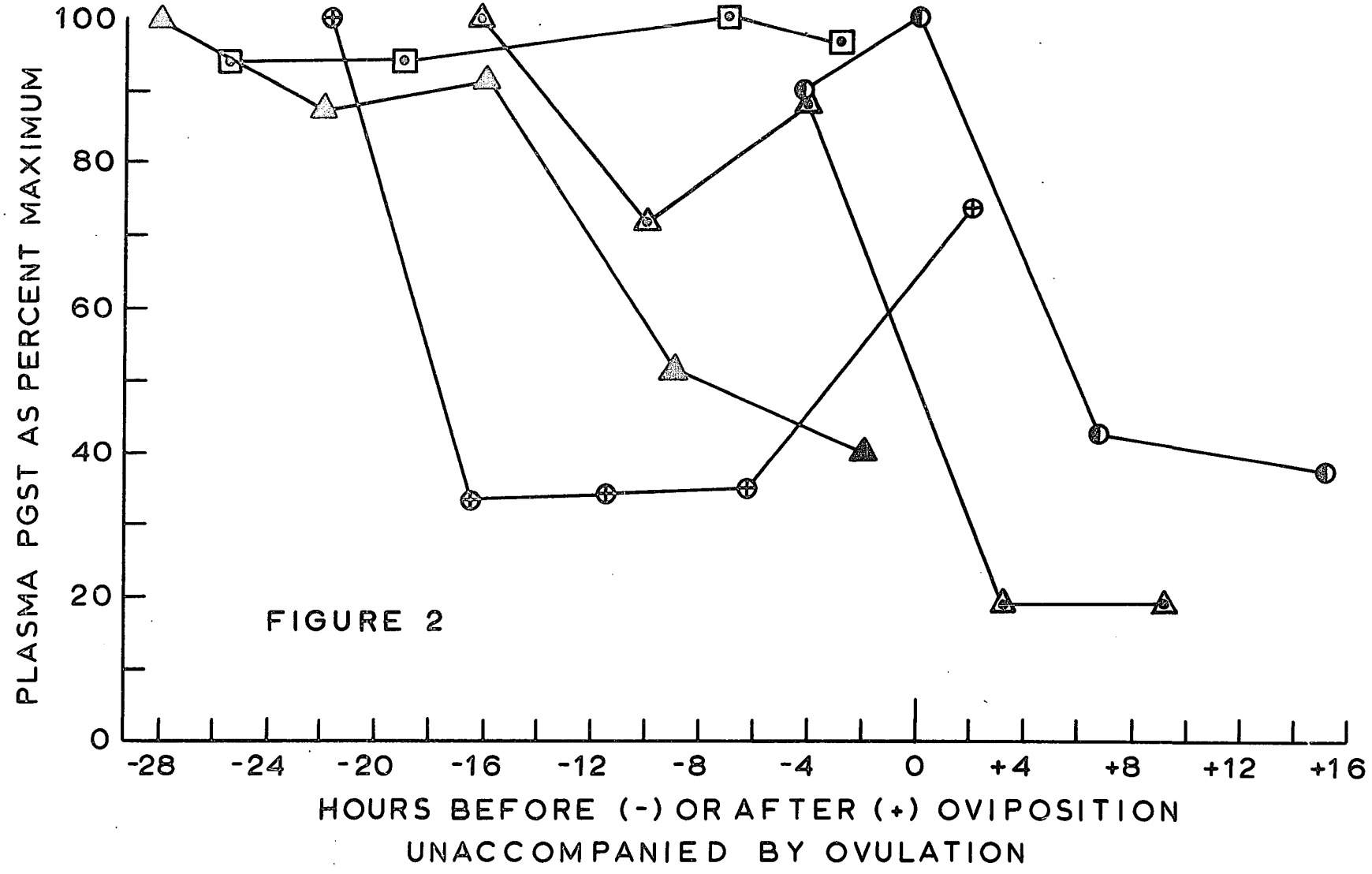

PLASMA PGST AS PERCENT MAXIMUM
100
80
60
40
20
0
-28
-24
-20
-16
-12
-8
-4
0
+4
+8
+12
+16
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
UNACCOMPANIED BY OVULATION

FIGURE 2

Table 8. Progesterone levels (ng/ml) in blood samples from individual hens for single-ovulatory cycles and for the periods of ovulatory pause (values not corrected for procedural losses, which ranged from 9 to 15%, Table 7).

Bird No.	No. Samples Drawn During Cycle	Progesterone, ng/ml		
		Min.	Max.	Average
	Preceding an Ovulation			
1	5	0.5	3.0	1.39
2	5	1.0	4.8	2.70
3	5	0.2	4.3	2.65
4	5	1.2	5.6	2.72
5	5	0.5	3.1	1.18
6	5	0.4	5.4	2.24
7	5	3.2	8.8	5.68
8	5	0.5	5.0	2.00
9	4	0.5	2.8	1.35
	Preceding a Day of No Ovulation			
2	4	4.6	12.5	8.40
5	4	2.6	2.8	2.70
7	5	1.0	2.6	1.94
10	5	0.5	1.2	0.65
11	5	1.0	3.0	1.96

CHAPTER III

SATURATION ANALYSIS OF THE ESTRONE AND ESTRADIOL CONCENTRATIONS IN THE PERIPHERAL PLASMA OF LAYING HENS

III.1. Introduction

The reported levels in the blood of laying hens, ranging from 2.0 to 31.2 ng/ml, would seem to allow the measurement of estrone and estradiol by the technique of Roy (255). This method, based on a Kober-Ittrich fluorometric technique was found capable of measuring as little as 10 ng of estrone in 10 ml of whole blood from pregnant cows (253). When this method was applied to whole blood or plasma from laying hens, consistently negative results were obtained even though the volume of blood assayed was increased to 50 ml (252). It was concluded from this study that either the method was inapplicable to hens' blood, or that the levels in hens' blood lie far below those reported.

This problem of assaying the extremely low amounts of steroid estrogen in the peripheral blood of various animals has been largely resolved by the use of protein-binding and radioimmunoassays. The CPB assay was developed first and was initially based on the use of sheep (267) or rabbit

(53,54,158,159,190,191,207,290) uterine cytosol. Subsequently, following the discovery of a sex hormone binding globulin (SHBG) that bound testesterone and estradiol-17β in the plasma (202,204), some workers developed a CPB assay using the SHBG in late pregnancy plasma (72,157,188,277). More recently the use of RIA has led to improved specificity and sensitivity in the measurement of plasma estrogens (1,2,79,138,298).

III.2. Materials and Methods

III.2.1. General Materials and Apparatus

(a) Kober-Ittrich study:- the materials and methods were identical to those reported elsewhere (252).

(b) Reference and radioactive steroids:- crystalline estrone, estradiol-17α and estradiol-17β were obtained from Steraloids Inc., Pawling, N.Y. Estradiol-17β-2,4,6,7-^{3}H (100 Ci/mMole) was purchased from Amersham-Searle Corp., Don Mills, Ontario.

(c) Chemicals:- charcoal (Norite A), sodium azide, THAM (Tris(hydroxymethyl)aminomethane) and sucrose, were obtained from Fisher Scientific Co., Montreal. Gelatin (granular), NaCl, $Na_2HPO_4 \cdot 7H_2O$, $NaH_2PO_4 \cdot 4H_2O$ and EDTA (disodiumethylene-diaminetetraacetate) were purchased from

Anachemia, Montreal. Sephadex LH-20 was purchased from Pharmacia, Montreal, and Dextran (Grade D) from Schwarz-Mann, New York, N.Y.

(d) Reagents:- benzene and methanol (both 'Spectranalyzed' grade) were obtained from Fisher Scientific Co., Montreal, and were used as received. Diethyl ether (reagent) was purchased from Fisher Scientific Co., Montreal, and was freed from peroxides and redistilled immediately before use. The scintillation fluid for the radioactivity measurements was Aquasol, purchased from New England Nuclear, Montreal.

(e) Buffer solutions:- The phosphate buffer A was prepared by dissolving 5.38 g $NaH_2PO_4 \cdot 7H_2O$, 16.35 g $Na_2HPO_4 \cdot 4H_2O$, 9.00 g NaCl, 1.00 g Na azide and 1.00 g gelatin in 1000 ml of glass distilled water (3). The Tris buffer A, pH 8, was prepared by mixing 25 ml of 0.2 M THAM, 13.4 ml of 0.2 N HCl and 61.6 ml of glass distilled water. Sucrose was added to a final concentration of 0.25 M. The Tris buffer B, pH 8, was prepared in a similar way as the Tris buffer A except EDTA was added to a final concentration of 0.01 M instead of sucrose. All buffers were stored at $4^{o}C$.

The dextran-coated charcoal suspension was prepared by dispersing 250 mg of Norite A and 25 mg Dextran D in 100 ml of the phosphate buffer A.

(f) Standard solutions:- The primary standard solutions

for estrone, estradiol-17α and estradiol-17β were prepared in a methanol solution at a concentration of 10 μg/ml. The working solutions were prepared from these primary standard solutions at a final concentration of 1 ng/ml. All standard solutions were kept at 4°C.

(g) Tritiated estradiol-17β solution:- the stock solution was prepared in methanol at an activity of 10 μCi/ml. One ml of this solution was dried down under nitrogen in a glass-stoppered 125 ml Erlenmeyer flask, 100 ml of phosphate buffer A added, and then the solution shaken well. The flask was stored at 4°C and a small quantity was poured out each day sufficient for the assay. Care was taken not to warm this final stock solution every day.

(h) Sheep Uterine Cytosol:- a mature ewe was ovariectomized and two weeks later the uterus was removed. The excess blood was washed from the uterus, and the uterus generally cleaned-up, with ice-cold Tris buffer A. The uterus was weighed approximately and then homogenized in a Waring blendor using four 30-second pulses, with three volumes of cold Tris buffer A per gram of uterus. The resultant suspension was spun at 4000 g to remove the bulk of the debris and the supernatant was then spun at 105,000 g at 4°C for 1½ hours. The resultant supernatant, which was the required cytosol fraction, was then pooled and 5 ml

lots frozen individually at -10°C.

To determine the final dilution at which cytosol was to be used in the assay, 0.5 ml of serial dilutions of cytosol in cold Tris buffer B were incubated overnight with approximately 10 nCi of tritiated estradiol-17β. After separating the bound and unbound radioactivity with the dextran-coated charcoal solution, the radioactivity bound in the supernatant was measured and plotted against the dilution of cytosol. Determinations at all dilutions were done in quadruplicate. It is arbitrarily taken that a dilution resulting in 50% binding is the optimal concentration of assay protein (188) and, according to this criterion, a dilution of 1/15 of the original cytosol was the most appropriate. Therefore, in the CPB assay, 0.5 ml of a 1/15 solution of cytosol in Tris buffer B was used as the binding protein.

(j) Radioimmumoassay:- the estradiol antibody used in this assay was a gift from Dr. Burton Caldwell, Department of Obstetrics and Gynaecology, Yale University, and had been prepared by immunizing a sheep against an estradiol-17β-bovine-serum-albumin conjugate (233). The serum was supplied undiluted and a dilution curve was prepared in a similar manner to that already described for the CPB assay, using,

however, the phosphate buffer A to dilute the antiserum. The optimal dilution range was from 1:30,000 to 1:50,000, and the assay was set up using a dilution of 1:40,000. The original antiserum was frozen in 0.1 ml lots at -10°C and the 1:40,000 solution was made up each day from a 1:500 stock solution which was kept at 4°C. This stock solution was still viable after five months.

(k) Apparatus:- disposable culture tubes (13 mm x 100 mm; Becton, Dickinson and Co., Montreal) were used throughout. London Luer hypodermic syringes (2-ml capacity) were obtained from Canadian Laboratories, Montreal. Disposable Pasteur pipettes were obtained from Fisher Scientific Co., Montreal. The tritiated estradiol solution and the antiserum were dispensed using a 2-ml repipette ("Repette", Jencons, Hertfordshire, England) that was calibrated to dispense approximately 0.1 ml. The actual cpm of dispensed tritium was $17,784 \pm 31.7$ (SD, N=20). Radioactivities were measured by liquid scintillation spectrometry (Packard Model 3003). The counting efficiency for tritium was 21.5%.

III.2.2. Procedure

(a) Kober-Ittrich study:- the method was identical to that described elsewhere (252).

(b) Saturation Analyses:- the experimental hens and

method of blood collection were essentially the same as previously described (II.2.2.) except that a No. 26 gauge needle was used for the withdrawal of blood samples in this study. To reduce the possibility of any interconversion of the estrogens the plasma samples were extracted as soon as possible after collection. Samples that displayed any signs of haemolysis were discarded.

Plasma (0.5 ml) was extracted in duplicate with diethyl ether (2 x 5 ml), the ether being separated by a disposable Pasteur pipette after centrifugation. The ether extract was evaporated to dryness in a current of nitrogen, dryness being ensured by continuing passage of nitrogen for ten minutes after removal of visible liquid. If the extracts were not thoroughly dry they would not dissolve in the solvent used for column chromatography, the insoluble material most likely being hydrated phospholipids.

Microcolumns of Sephadex LH-20 were prepared in glass hypodermic syringes (2-ml capacity). The syringes were plugged with a disc of glass filter-fibre and Sephadex (0.4 g) was slurried with the eluting solvent (benzene:methanol, 85:15) and transferred, with two rinses, to the syringe. A second glass filter-fibre disc was placed on top of the Sephadex and each column was washed with 30 ml of eluting

solvent before use. The columns were then allowed to run dry.

The dry estrogen extract was dissolved in 0.1 ml of the eluting solvent and the solution transferred to the column by means of a Pasteur pipette. The tube was then rinsed by a further addition of 0.1 ml of eluting solvent which was similarly transferred to the column. Eluting solvent (1.1 ml) was then added and the eluate discarded. More solvent (0.6 ml) was added and the eluate (estrone) was collected. The column was then rinsed with 0.2 ml of solvent and the eluate discarded. Further solvent (1.0 ml) was added and the eluate (the estradiols) collected. It should be noted that the column was allowed to run 'dry' after each elution. Two additional columns were packed and used as blanks. High and inconsistent blank values were encountered if the columns were used more than once, even when 30 ml of the eluting solvent was used to wash each column before re-use. Accordingly, columns were prepared afresh for each assay.

CPB assay:- the appropriate column eluate was evaporated to dryness under nitrogen and 0.5 ml of the diluted cytosol added (to each tube and the tubes were shaken for two minutes). Approximately 0.1 μCuries of estradiol-17β-2,4,6,7-^{3}H was then added to each tube and the mixture shaken for two minutes. All tubes were covered and incubated

at 4°C overnight. Dextran-coated charcoal suspension (0.5 ml) was then added and the mixture was shaken for two minutes. The tubes were then spun at 4000 g for 15 minutes and the resultant supernatant carefully decanted into counting vials containing 10 ml of Aquasol. The vials were shaken thoroughly and placed in the liquid scintillation spectrometer at least two hours before counting began. The appropriate series of standards were prepared in duplicate in a similar way with each batch of assays. To assess the reproducibility of the standard curves, six standard curves for estrone and estradiol-17β were taken at random from those accumulated in the course of the work. The two sets of data were averaged and plotted as in Figure 13. This figure indicates that the standard curves were reasonably reproducible.

RIA:- the procedure followed was almost the same as that described for the CPB assay, excepting that 0.1 ml of diluted antisera was used for the incubation and 1.0 ml of the charcoal suspension was used to separate the bound from the unbound radioactivity. To assess the reproducibility of the standard curves for the RIA, nine standard curves for each steroid, estrone, estradiol-17α and estradiol-17β, were taken from those accumulated in the course of the work. The three sets of data were averaged and the results plotted as in Figure 14. This figure indicates the standard curves for

Figure 13. Standard curves for estrone (closed circles) and estradiol-17β (circled dots), obtained by the CPB assay. Crossed lines indicate standard errors (n=6).

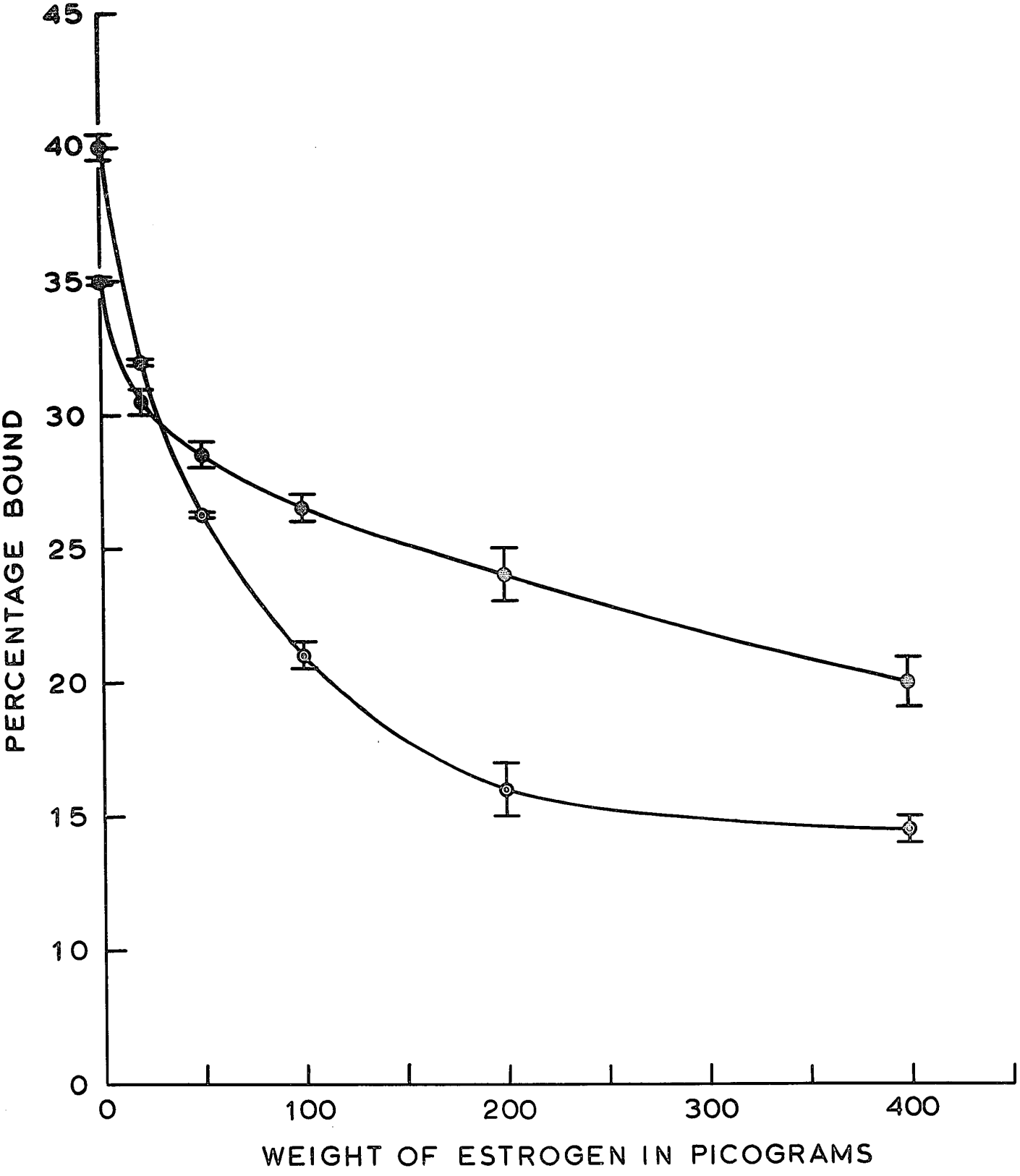
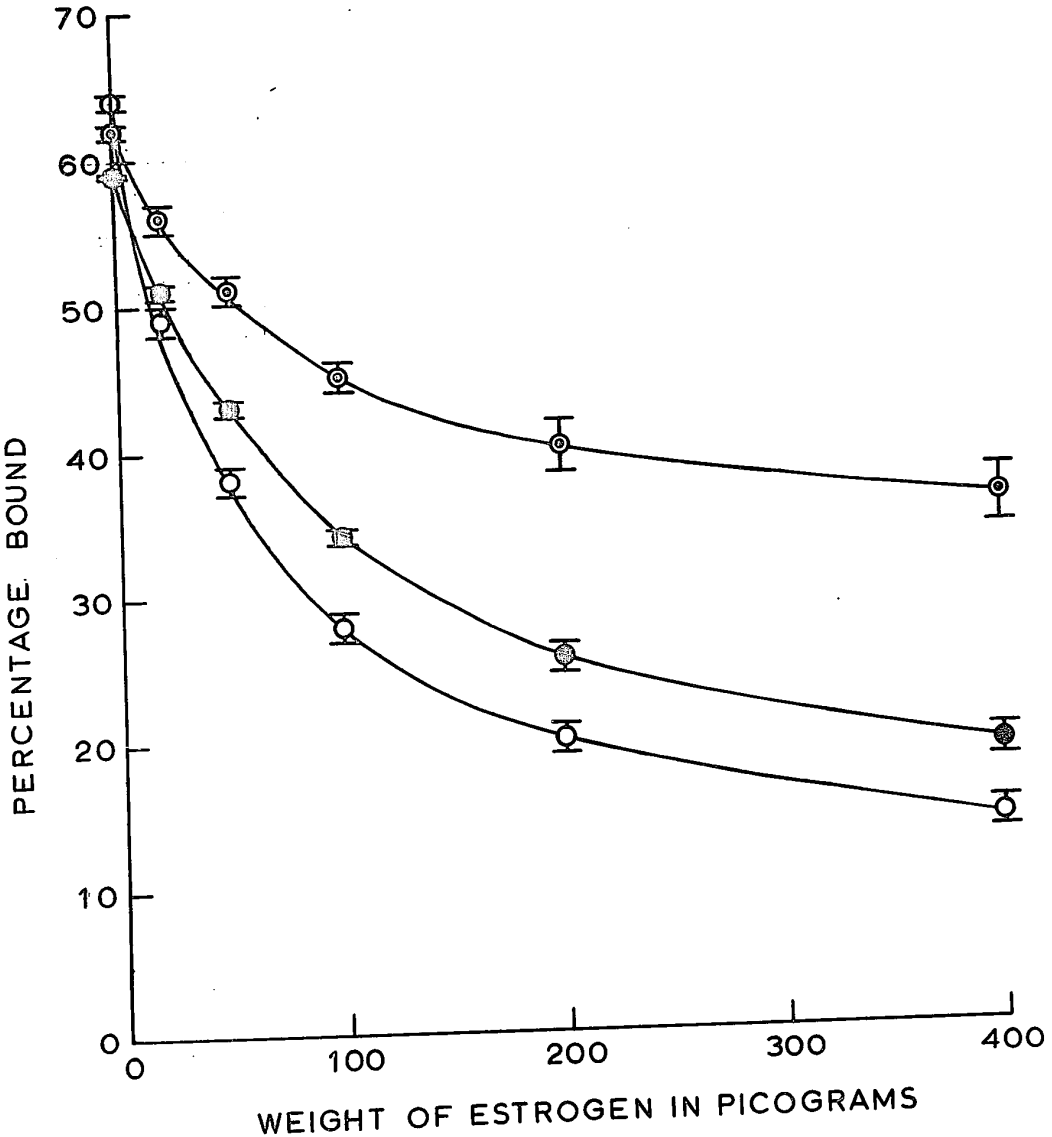

45
40
35
30
25
20
15
10
0
PERCENTAGE BOUND
0
100
200
300
400
WEIGHT OF ESTROGEN IN PICOGRAMS

Figure 14. Standard curves for estradiol-17α (circled dots), estrone (solid circles), and estradiol-17β (open circles) obtained by RIA. Crossed lines indicate standard errors (n=9).


70
60
50
40
30
20
10
0
PERCENTAGE BOUND
0
100
200
300
400
WEIGHT OF ESTROGEN IN PICOGRAMS

the RIA, as for the CPB assay, were reasonably reproducible.

III.3. Results and Discussion

III.3.1. The Kober-Ittrich Study

Previous work in this laboratory had shown that the Kober-Ittrich method of Roy (255) was not suitable for the measurement of total estrone in the blood of laying hens either because of interfering substances or because the concentrations were actually below the limit of detection of the method (252). Shortly after this work had finished, there was published a report in which the free estrone, estradiol-17β and estriol were measured in laying turkey-hen plasma by alumina chromatography and a subsequent Kober reaction (15). Since in this report, the estrogens were extracted from whole blood without prior acid hydrolysis and since hydrolyses had always been performed in this laboratory, either on whole blood or plasma, it was of interest to determine whether the extraction of laying-hens' plasma without prior acid hydrolysis would lead to the detection of estrone and estradiol-17β.

Accordingly the extraction procedure of Bajpayee and Brown (15) was modified slightly in that plasma instead of whole blood was used. The residue obtained after defatting was methylated and the procedure of Robinson et al. (253)

used from then on. Initially ten ml of plasma was extracted in duplicate, but neither estrone nor estradiol-17β could be detected. The volume of plasma was then increased to 50 ml and the remaining red blood cells washed with 50 ml of avian isotonic saline (239) and the plasma and saline wash combined. When this solution was processed, neither steroid estrogen could be detected. In the final experiment, 50 ml of plasma and 50 ml of the saline wash of the red blood cells were pooled and divided into two equal lots. These plasma solutions were then acid-hydrolysed for 30 minutes and the hydrolyseate processed by the same method as for the first two experiments. Again neither estrone nor estradiol could be detected.

The reported sensitivity of the method for estrone was 0.95 ng (252) and it should have been able to detect the amounts of estrone that had been reported as being present in laying-hens' blood (Table 2). This suggests strongly that the concentration of total estrone in the peripheral plasma of laying hens is in the sub-nanogram range. This suggestion was verified from the report that the free estrone concentrations in whole blood of laying hens and laying turkey hens were 0.9 ng/ml and 0.13-0.5 ng/ml, respectively (15).

III.3.2. Competitive Protein-Binding Assay

During the course of this study it was found that the supply of cytosol became rapidly depleted and that if the circulating levels of estrone and estradiol-17β were to be measured on a great number of birds then a constant supply of cytosol would have to be consistently on hand. Because of the considerable inconvenience of obtaining ewes, especially ovariectomized ewes, from which the uterine cytosol could be prepared, the bulk of the experimental work was done using the donated anti-serum. Enough data were accumulated, however, on the relative binding of the steroid estrogens to enable a comparison of the standard curves of the two methods. The levels of estrone and estradiol were also determined on five laying hens throughout the ovulatory cycle using the CPB assay. The results from these studies are shown in Figures 15 and 16.

An average value for the precision of the method was estimated by taking the 25 duplicate analyses for both estrone and estradiol from the CPB assay of the estrogens in the five ovulatory birds. These values ranged from 75-220 pg/ml for E_1 and from 105-365 pg/ml for E_2. The standard deviation, calculated from $S\bar{x}=\sqrt{(\Sigma d^2/2N)}$, was $\pm$13.2 pg/ml for estrone and $\pm$14.6 pg/ml for estradiol. On this basis

Figure 15. Plasma estradiol values (determined by CPB), plotted against time before (-) or after (+) an oviposition accompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

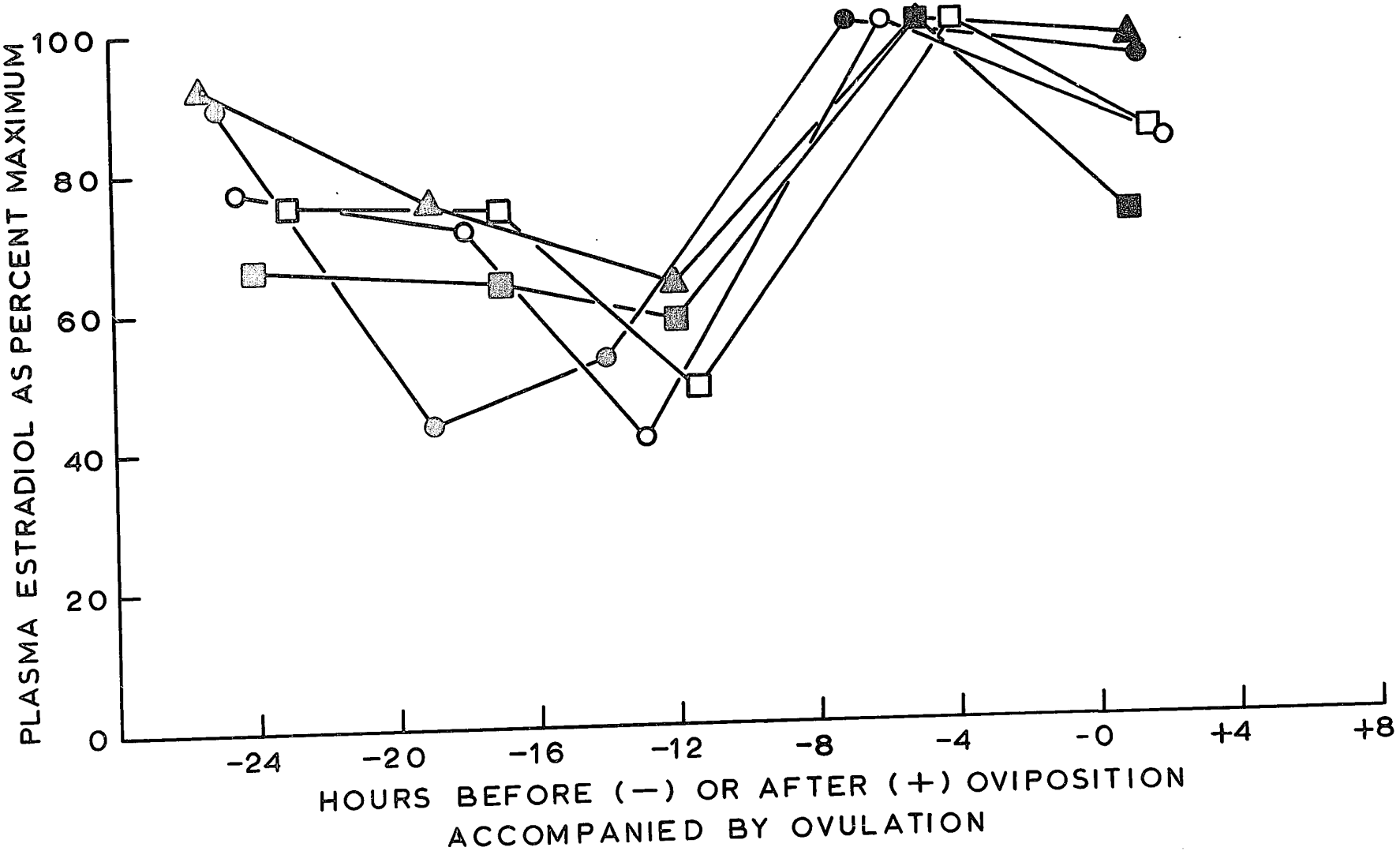
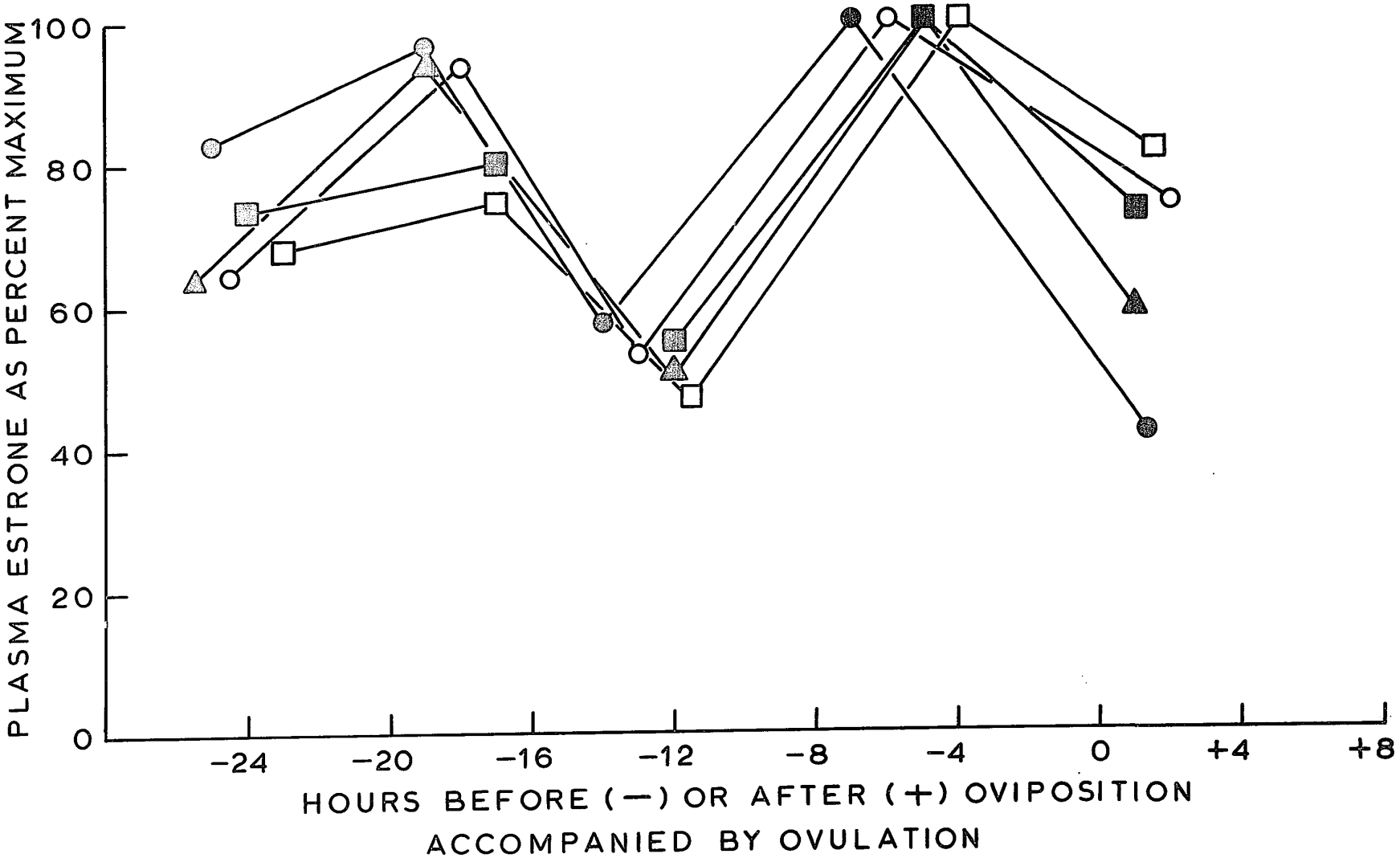

PLASMA ESTRADIOL AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
-0
+4
+8
HOURS BEFORE (—) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 16. Plasma estrone values (determined by CPB), plotted against time before (-) or after (+) an oviposition accompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.


PLASMA ESTRONE AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (—) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

the least amount distinguishable from zero amount ($P = 0.01$ and calculated for duplicate determination) was 25.8 pg/ml for estrone and 28.6 pg/ml for estradiol.

III.3.3. Radioimmunoassay

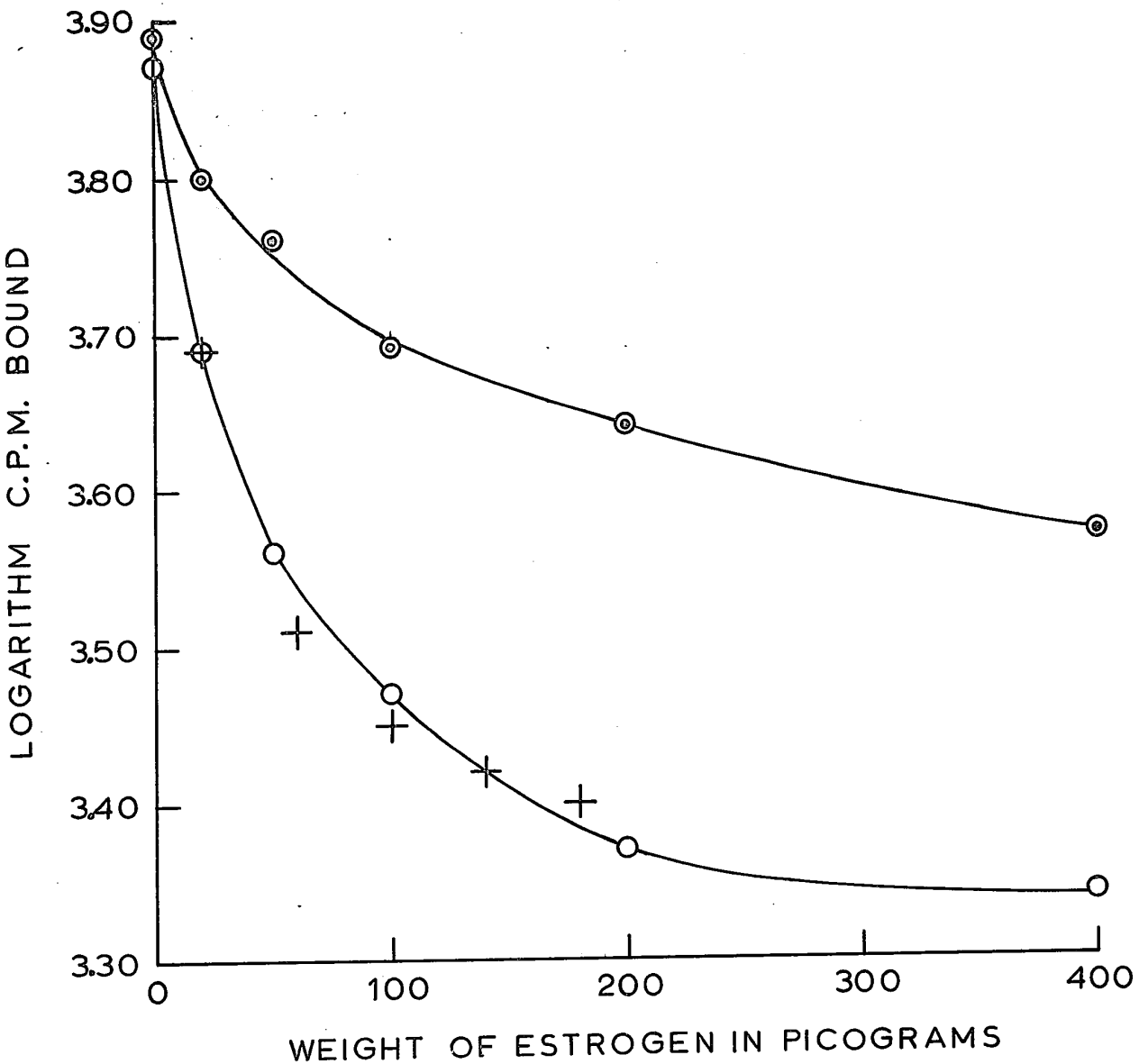
(a) Specificity of the Method

The chromatographic system adequately separated estrone from the estradiols, but did not separate the two diols (298). To examine the effect of an increasing concentration of estradiol-17α in a mixture of the two diols, a standard curve for the same total amount of estradiol (200 pg) but with a decreasing proportion of estradiol-17α was constructed in quadruplicate. As shown in Figure 17, the contribution of the 17α epimer to the total estradiol binding was negligibly small. It was concluded that the results for estradiol reported in this thesis reflect mainly the 17β epimer. Wu and Lundy (298) have shown that a similar estradiol antibody was relatively specific for estradiol-17β among a considerable number of steroid estrogens tested.

(b) Accuracy

Amounts of estrone and estradiol-17β of 50,100 and 200 pg were added in duplicate to separate 0.5 ml portions of the same laying hens' plasma and were then assayed together with corresponding duplicate blank samples. The recoveries

Figure 17. Effect of estradiol-17α on measurement of estradiol-17β. Estradiol-17α (circled dots) and estradiol-17β (open circles). The crosses indicate values for estradiol-17β in presence of estradiol-17α (see text).


3.90
3.80
3.70
3.60
3.50
3.40
3.30
LOGARITHM C.P.M. BOUND
0
100
200
300
400
WEIGHT OF ESTROGEN IN PICOGRAMS

are presented in Table 9 and may be considered satisfactory for an assay of this kind.

Table 9. Recoveries of estrogen added to 0.5-ml samples of laying-hen plasma.

Amount Added pg	No. Duplicates, N		Recovery, % ± S.E.	
	E_1	$E_2 17\beta$	E_1	$E_2 17\beta$
50	8	6	76.0 ± 10.0	68.3 ± 7.0
100	15	10	77.2 ± 7.8	73.8 ± 8.3
200	8	6	75.0 ± 10.4	66.7 ± 8.1

(c) Precision

An average value for the precision of the method was estimated by picking 30 duplicate analyses for both estrone and estradiol-17β at random from all available data for ovulating and non-ovulating hens. These values ranged from 10 to 162 pg/ml for estrone and 42 to 210 pg/ml for estradiol. The standard deviation, calculated from $Sx=\sqrt{(\Sigma d^2/2N}$, was ±7.2 pg/ml for estrone and ±6.8 pg/ml for estradiol. The corresponding confidence limits (degrees of freedom = 29, P=0.01) were ±3.6 pg/ml and ±3.4 pg/ml for estrone and estradiol, respectively.

(d) Sensitivity

The least amount distinguishable from zero amount (P=0.01 and calculated for duplicate determinations) was 14.0 pg for estrone and 13.1 pg for estradiol.

(e) Blank Values

The column blanks in the RIA were very low, 2.64 ± 0.82 (SD,N=13) pg for estrone and 3.72 ± 0.93 (SD,N=13) pg for estradiol. Ideally the blank values are produced by samples which differ from normal unknowns only in that they are known not to contain the compound being estimated. In saturation analyses the only situation where the blank is subtractable is where a contaminant is reacting with the binding protein with an equilibrium constant identical to that of the compound one is setting out to measure, that is, the contaminent and the steroid to be measured have the same affinity for the binding protein. This can rarely, if ever, happen. One must know, however, what order of magnitude the blank reading is as compared to the sensitivity of the method. If the blank is consistently far below the calculated sensitivity of the method, then it is causing no interference in the assay. This was the situation in the present study. Even if the detection limit is calculated from the apparent blank concentration by adding two standard deviations to the mean of the blank values, the results of 4.28 pg for

estrone and 5.58 pg for estradiol still lie below the calculated sensitivity.

(f) Occurrence of an Ovulatory Cycle of Blood Estrogen Level

The procedure for relating times of blood sampling to time of oviposition was identical to that already described in Section II.3.4. Data were obtained for 13 ovulations within a laying sequence and for 12 comparable intervals where ovulation did not occur. In order to better compare the data for different birds, the estrogen values for each ovulatory cycle were recalculated as percentages of the highest value observed for the individual cycle in question and these percentages were then plotted against times before or after oviposition. The data for estrogen concentration from ovulating birds are presented in Figures 18 to 21. The data for non-ovulating birds are presented in Figures 22 to 23.

The average concentrations ($\pm S_{\bar{x}}$) of estrogen, uncorrected for procedural losses, during four-hour intervals of the ovulatory cycle are shown in Figure 24. The corresponding data for non-ovulating birds are presented in Figure 25. Paired "t" tests were used to determine the degree of significance for differences between the average concentrations for each four-hour interval during the ovulation cycle. The

Figure 18. Plasma estradiol values, determined by RIA, plotted against time before (-) or after (+) an oviposition accompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

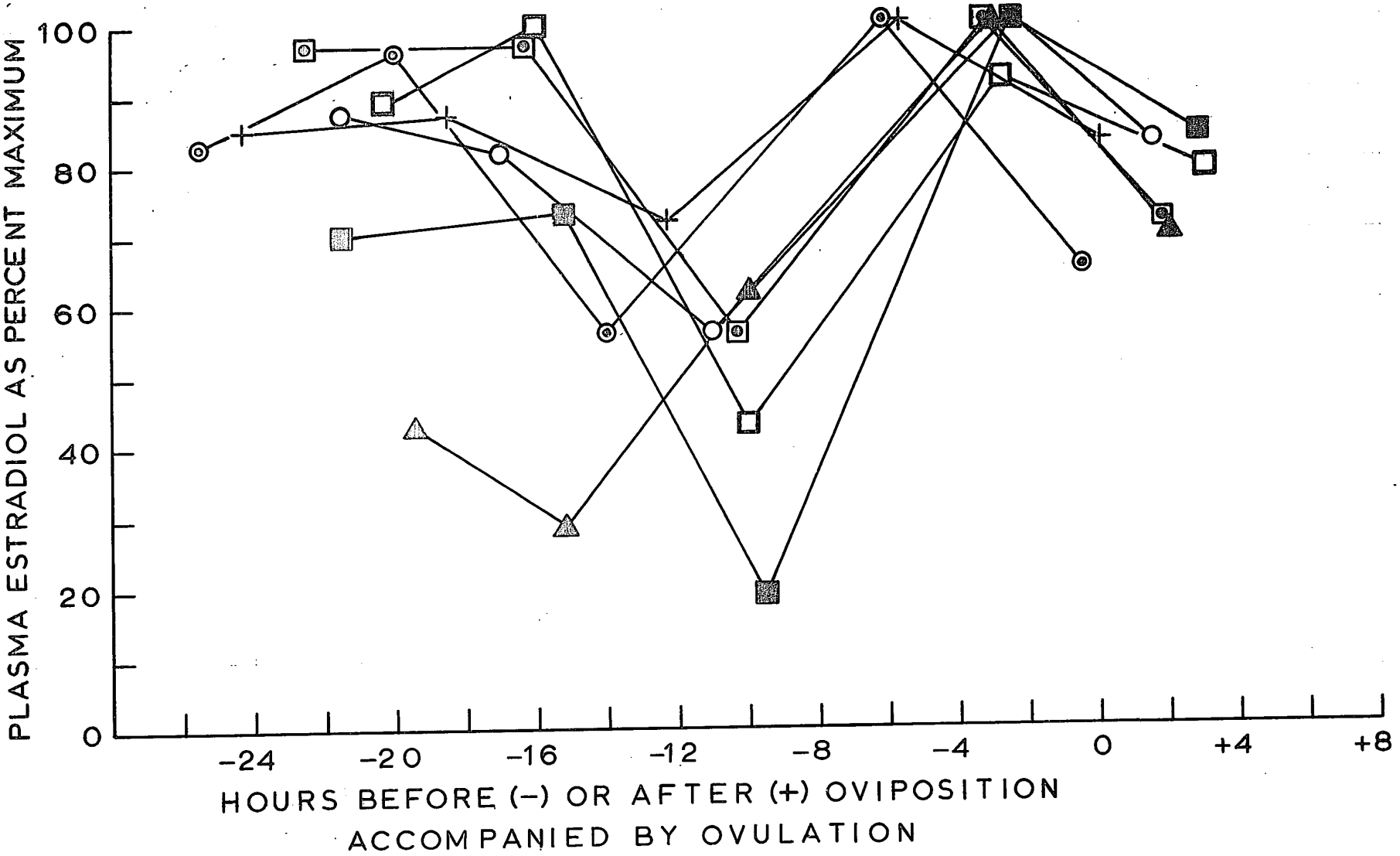

PLASMA ESTRADIOL AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 19. Plasma estradiol values, determined by RIA, plotted against time before (-) or after (+) an oviposition accompanied by ovulation. Values for each individual hen expressed as percentaces of the maximal value observed during the period for that bird.

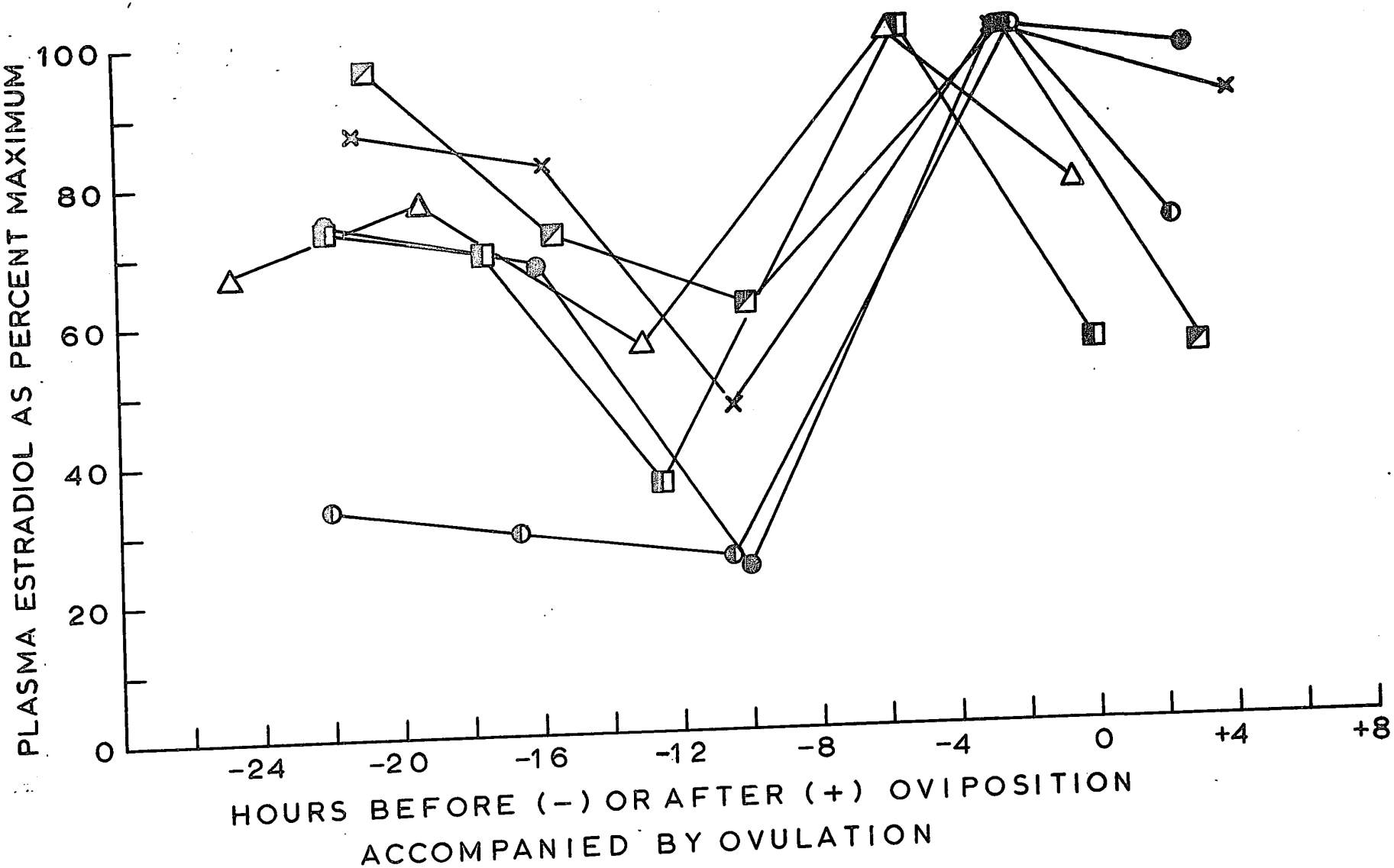

PLASMA ESTRADIOL AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 20. Plasma estrone values (determined by RIA) plotted against time before (-) or after (+) an oviposition accompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

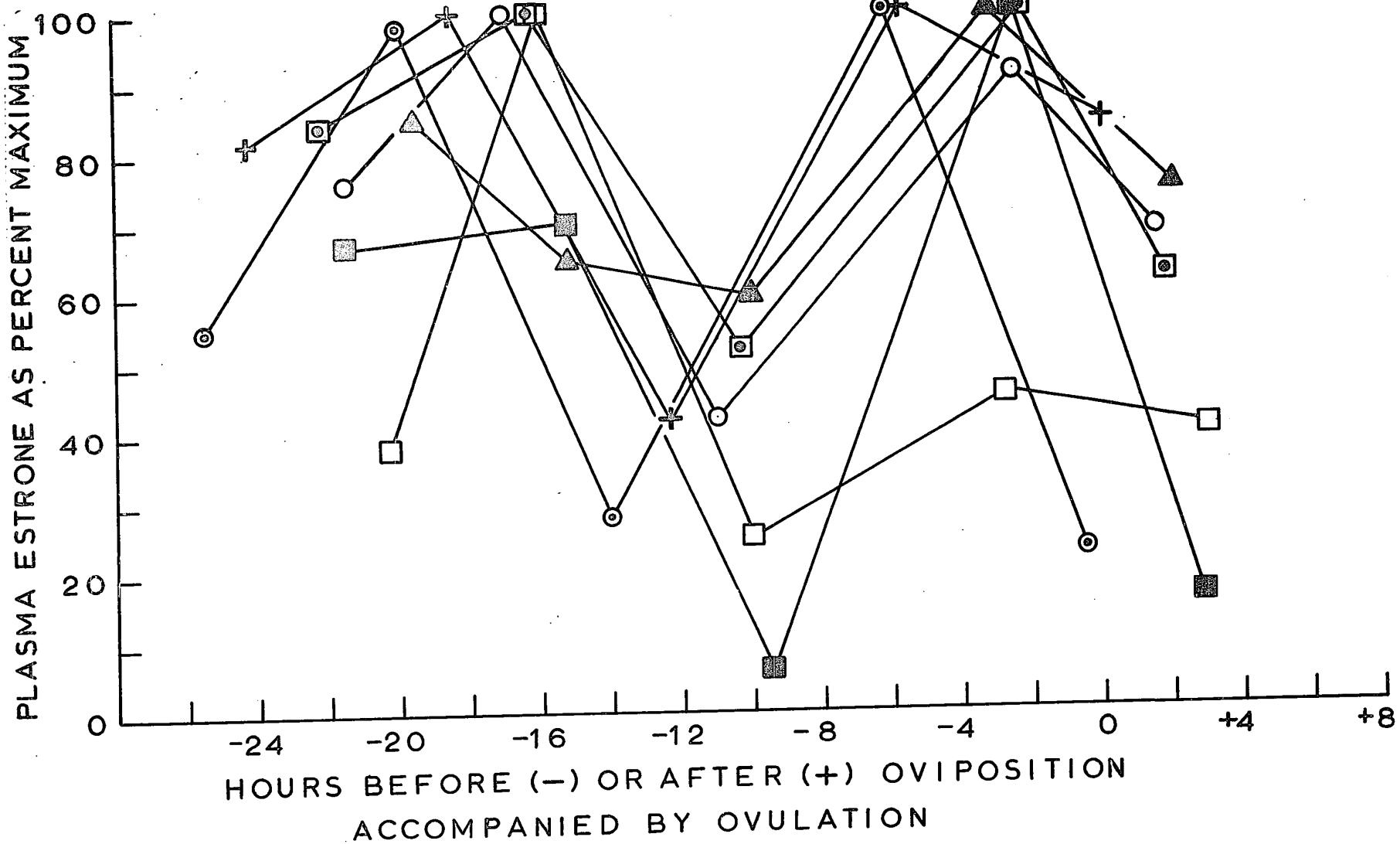

PLASMA ESTRONE AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (−) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 21. Plasma estrone values (determined by RIA) plotted against time before (-) or after (+) an oviposition accompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

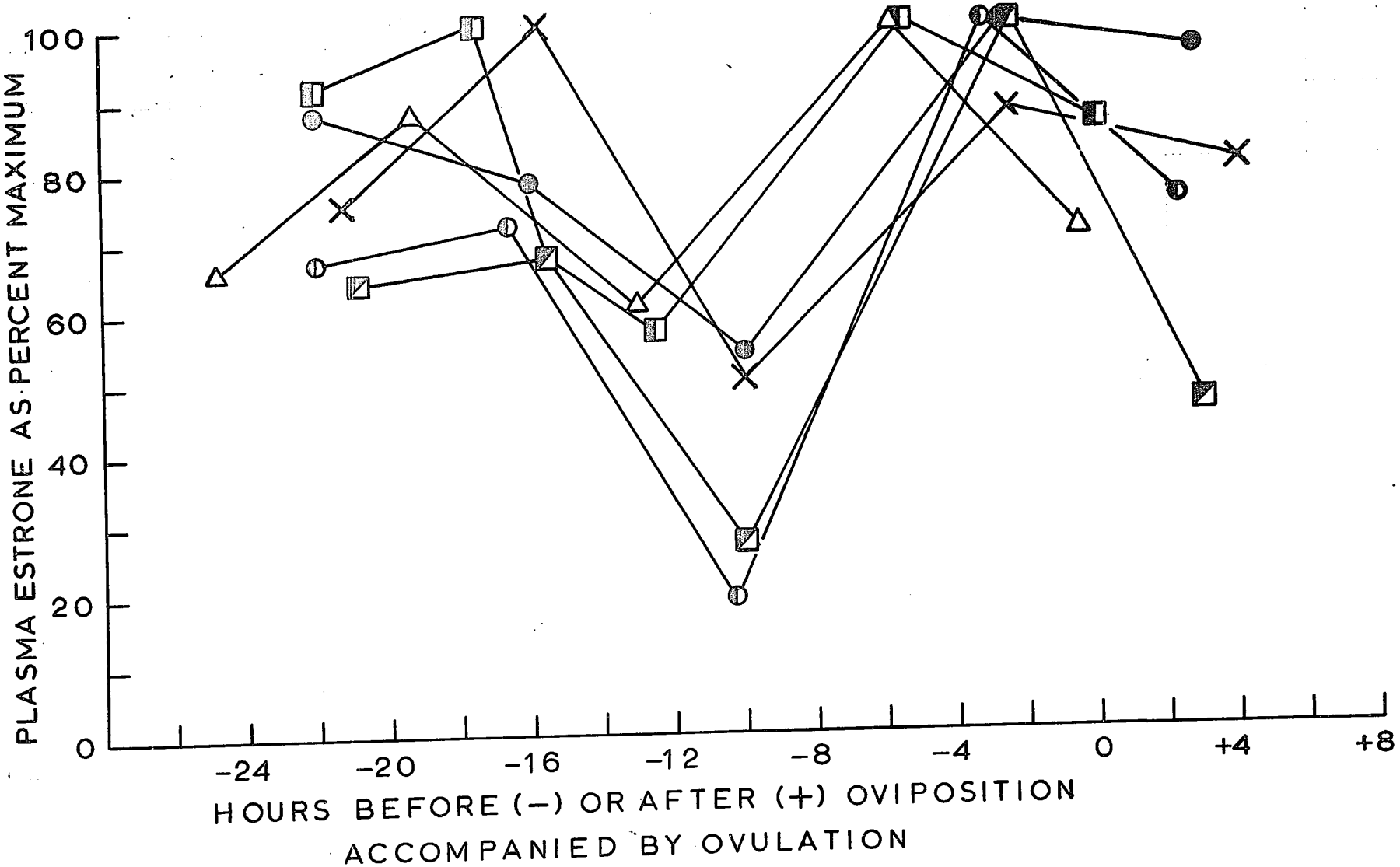

PLASMA ESTRONE AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 22. Plasma estradiol values, determined by RIA, plotted against time before (-) or after (+) an oviposition unaccompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

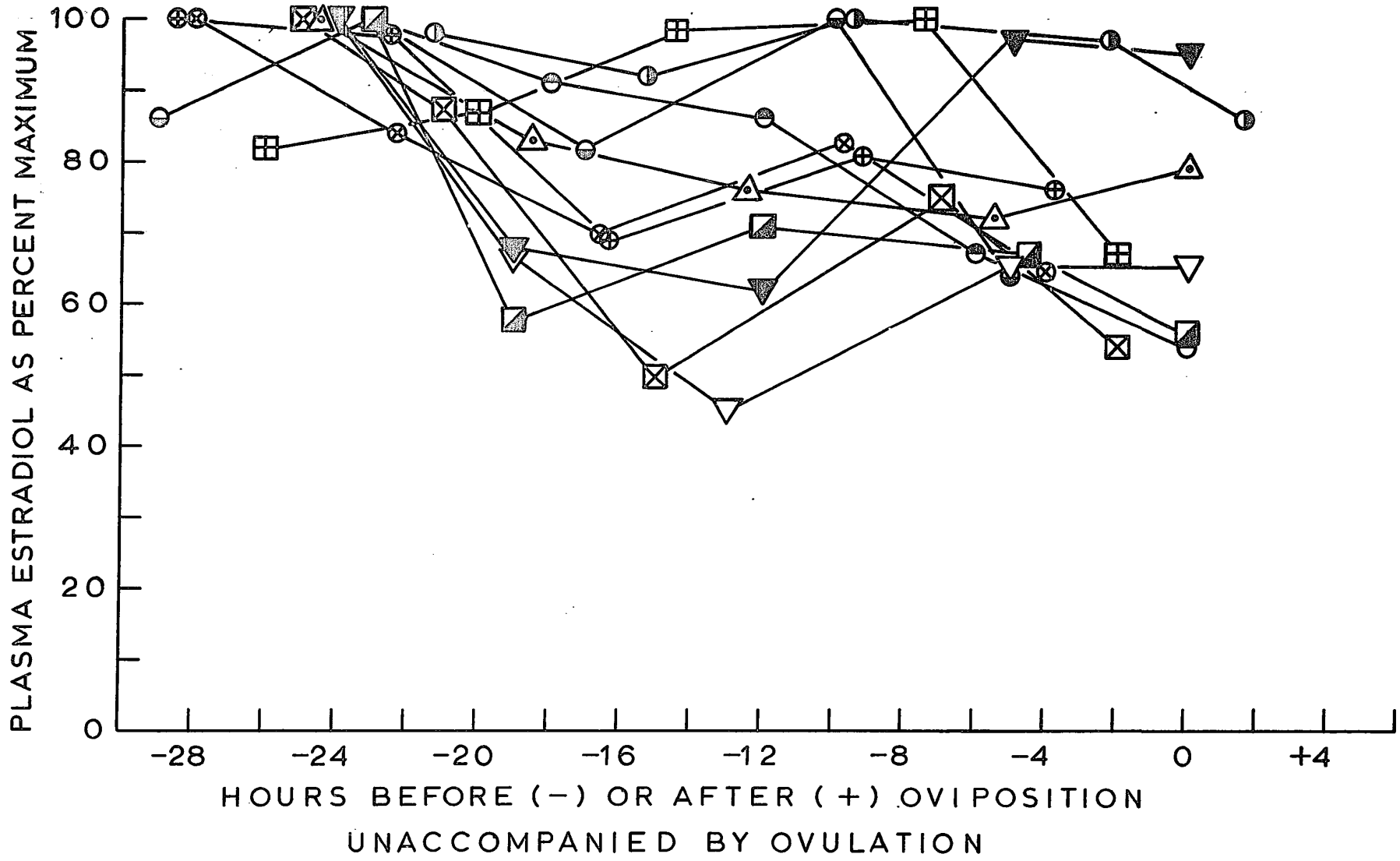

PLASMA ESTRADIOL AS PERCENT MAXIMUM
100
80
60
40
20
0
-28
-24
-20
-16
-12
-8
-4
0
+4
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
UNACCOMPANIED BY OVULATION

Figure 23. Plasma estrone values, determined by RIA, plotted against time before (-) or after (+) an oviposition unaccompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

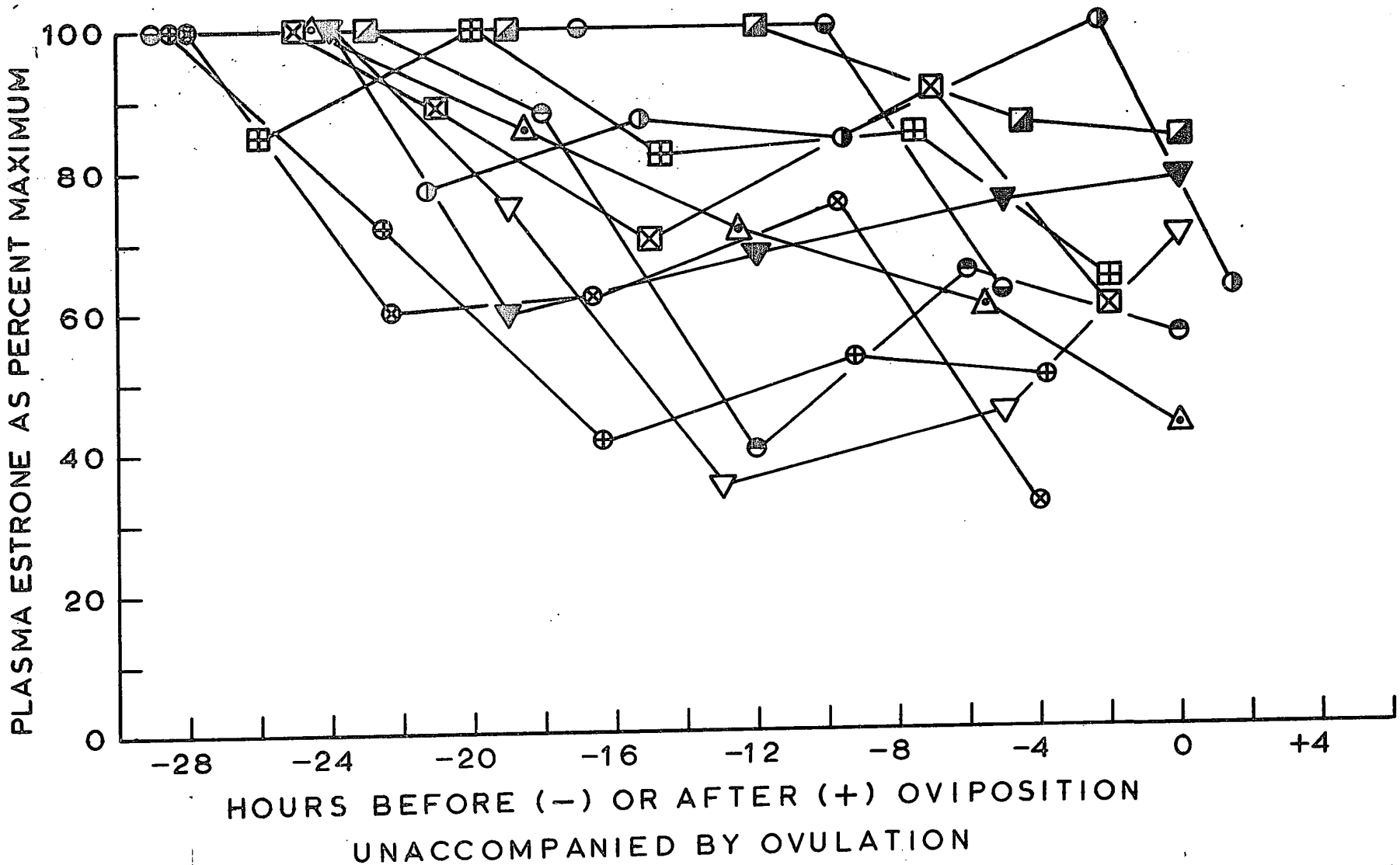

PLASMA ESTRONE AS PERCENT MAXIMUM
100
80
60
40
20
0
-28
-24
-20
-16
-12
-8
-4
0
+4
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
UNACCOMPANIED BY OVULATION

Figure 24. Average estradiol values (full line) and estrone values (broken line), determined by RIA, plotted against corresponding intervals before (-) or after (+) oviposition accompanied by ovulation. Crossed lines indicate standard errors.

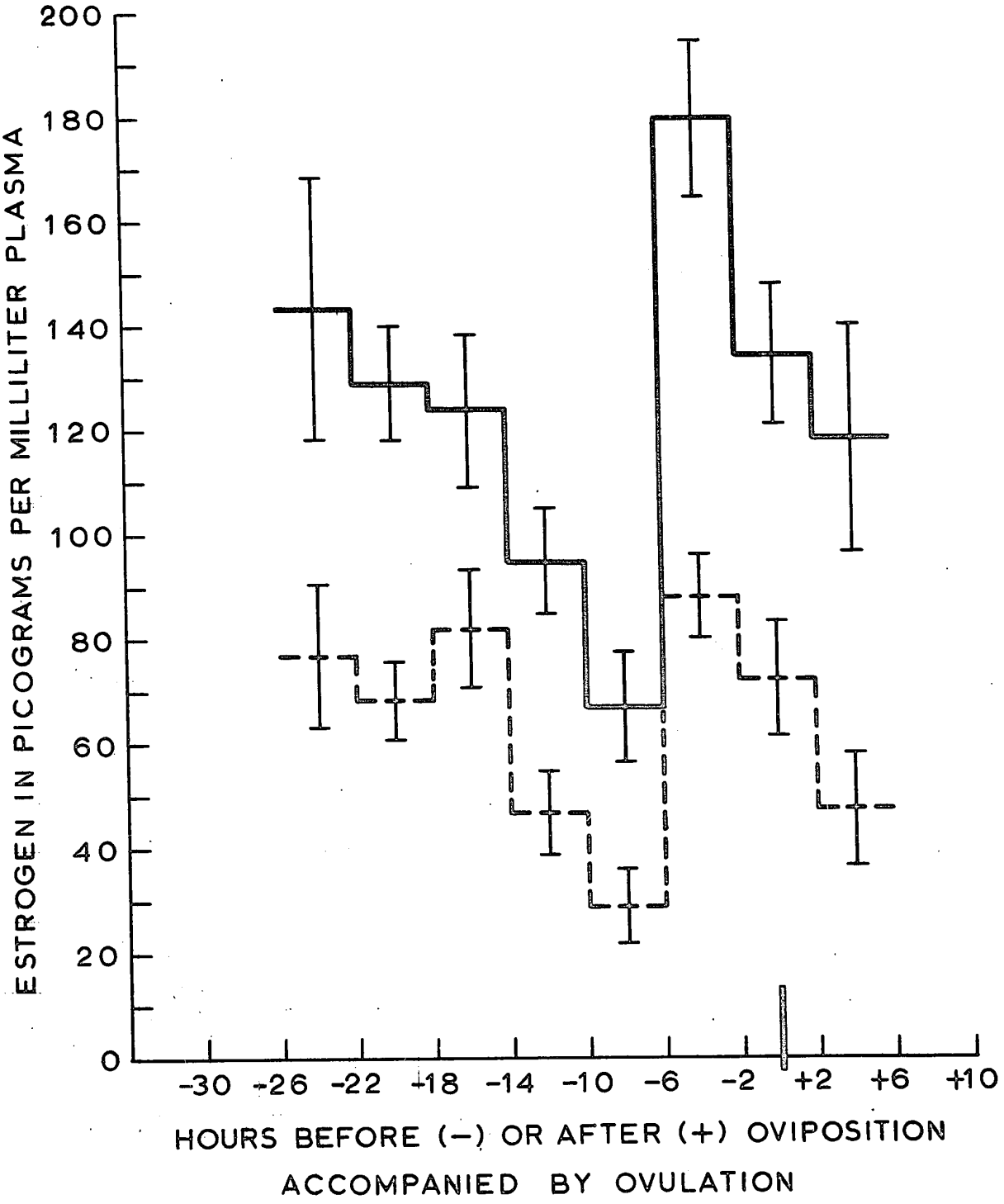
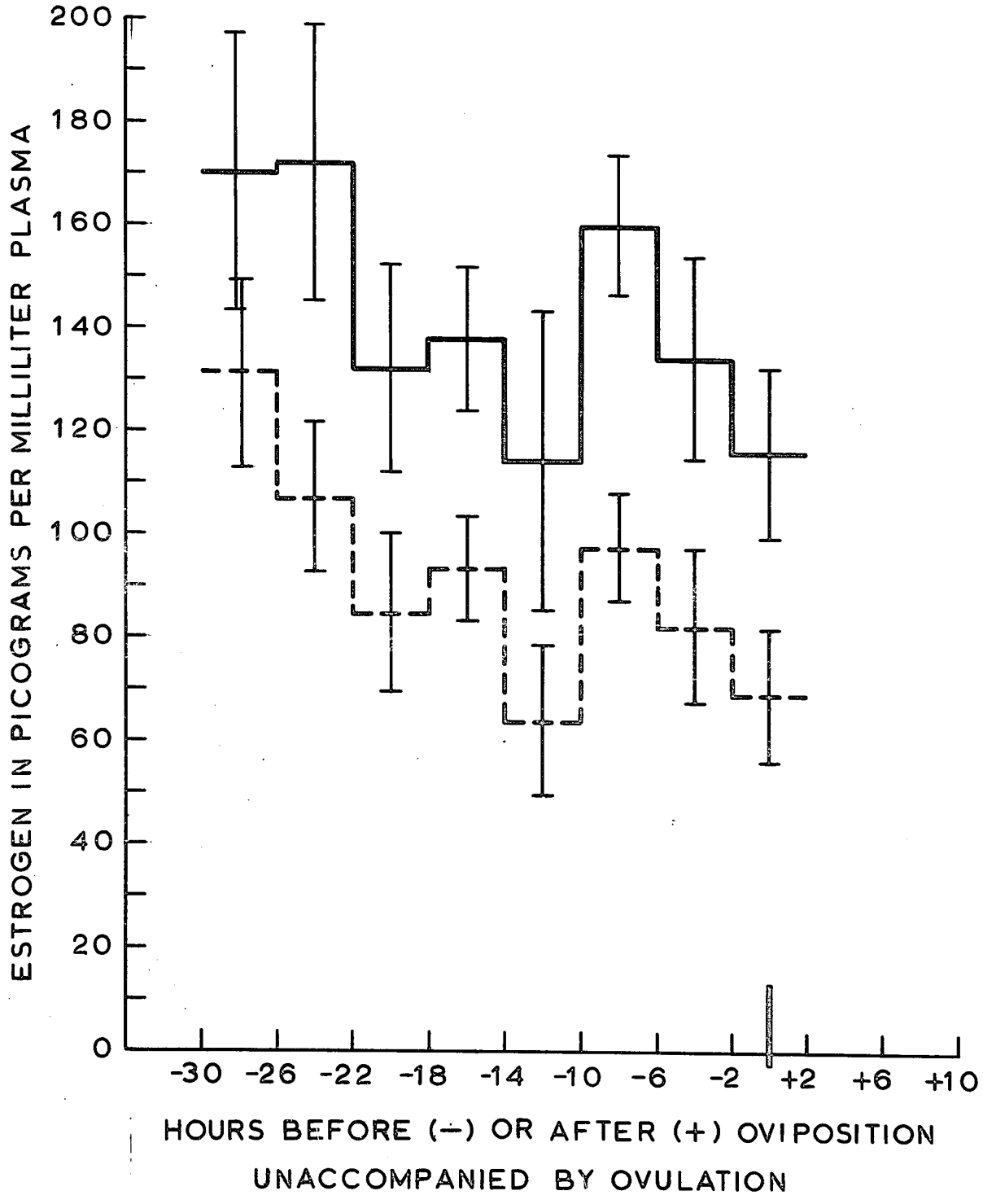

ESTROGEN IN PICOGRAMS PER MILLILITER PLASMA
200
180
160
140
120
100
80
60
40
20
0
-30 -26 -22 -18 -14 -10 -6 -2 +2 +6 +10
HOURS BEFORE (−) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 25. Average estradiol values (full line) and estrone values (broken line), determined by RIA, plotted against corresponding intervals before (-) or after (+) oviposition unaccompanied by ovulation. Crossed lines indicate standard errors.


ESTROGEN IN PICOGRAMS PER MILLILITER PLASMA
200
180
160
140
120
100
80
60
40
20
0
-30 -26 -22 -18 -14 -10 -6 -2 +2 +6 +10
HOURS BEFORE (−) OR AFTER (+) OVIPOSITION
UNACCOMPANIED BY OVULATION

results of these tests are shown in Table 10.

Table 10. Results of paired 't' tests between average four-hour concentrations of estrogen before or after oviposition accompanied by ovulation.

Hours Before (-) or After (+)	Estrone Average (pg/ml)*	Estradiol Average (pg/ml)*
-26 to -22	77^{bcdef}	144^{cdefg}
-22 to -18	69^{bcd}	129^{cde}
-18 to -14	82^{defg}	124^{bcd}
-14 to -10	47^{ab}	95^{ab}
-10 to -6	29^{a}	66^{a}
-6 to -2	88^{defg}	180^{fg}
-2 to +2	72^{bcde}	134^{cdef}
+2 to +6	47^{abc}	117^{abc}

*Means carrying the same superscript letter are not significantly different (P=0.05).

Concentrations of estradiol for birds with ovipositions accompanied by ovulation displayed a marked drop over the interval 18 hour to 10 hour before oviposition, as shown in Figures 18 and 19. This drop led to minimal concentrations at about 14 hours to 8 hours before oviposition. The concentration then rose sharply to peak values at six to two hours before oviposition. The data also suggest the

existence of a second, though much less prominent peak around 22 to 18 hours before oviposition. The corresponding estrone concentrations displayed a similar pattern (Figures 20 and 21) but with indications that the earlier maximum and succeeding minimum may have lagged behind those for estradiol by about two to four hours. The corresponding plots of the data for periods where ovulation did not occur displayed a different pattern, as shown in Figures 22 and 23.

Statistical examination of the average results for four-hour intervals over the ovulation cycle showed that the drops to the minimum average value for ten to six hours before oviposition were significant for both estrone and estradiol, as were also the subsequent rises to the peak at six to two hours before oviposition (see Table 10 and Figure 24). Oviposition and, by inference, ovulation were associated with falling plasma estrogen concentrations. A similar statistical examination of the average for four-hour intervals for periods where ovulation did not accompany oviposition showed that the fluctuations did not attain significance at $P=0.05$. Figure 25 suggests that there was a general decline in plasma estrogen, with some slight indication that low levels may tend to occur around the middle hours of the night.

(g) A Comparison of the CPB Assay and the RIA Used in this Study

A series of experiments was performed to compare the standard curves obtained by CPB and RIA for estrone and estradiol-17β. Sets of standards for either estrone or estradiol were assayed simultaneously in duplicate by both CPB and RIA. This enabled the standard curves to be compared under identical experimental conditions. The results are shown in Figures 26 and 27. The most obvious difference is that the antibody binds consistently more radioactivity. The almost identical slope between the curves obtained by both assays for estradiol-17β indicates that both are effective in measuring the low amounts of estradiol. RIA is more effective, however, in assaying estrone. This demonstrates the ease with which one can set up a CPB assay to measure steroid estrogens in the picogram range provided that a consistent supply of binding protein is available. It also demonstrates that the RIA will most likely supersede the CPB assay in future because of the extremely high titre of binding protein that can be obtained from an immunized animal.

The RIA proved the more precise assay and displayed an average standard deviation of about one half that for the CPB assay. The actual values for the precision of the two methods may lie more closely together than this because

Figure 26. Comparison of the standard curves for estradiol-17β determined by CPB (closed circles) and RIA (open circles). Crossed lines indicate standard errors (n=5).

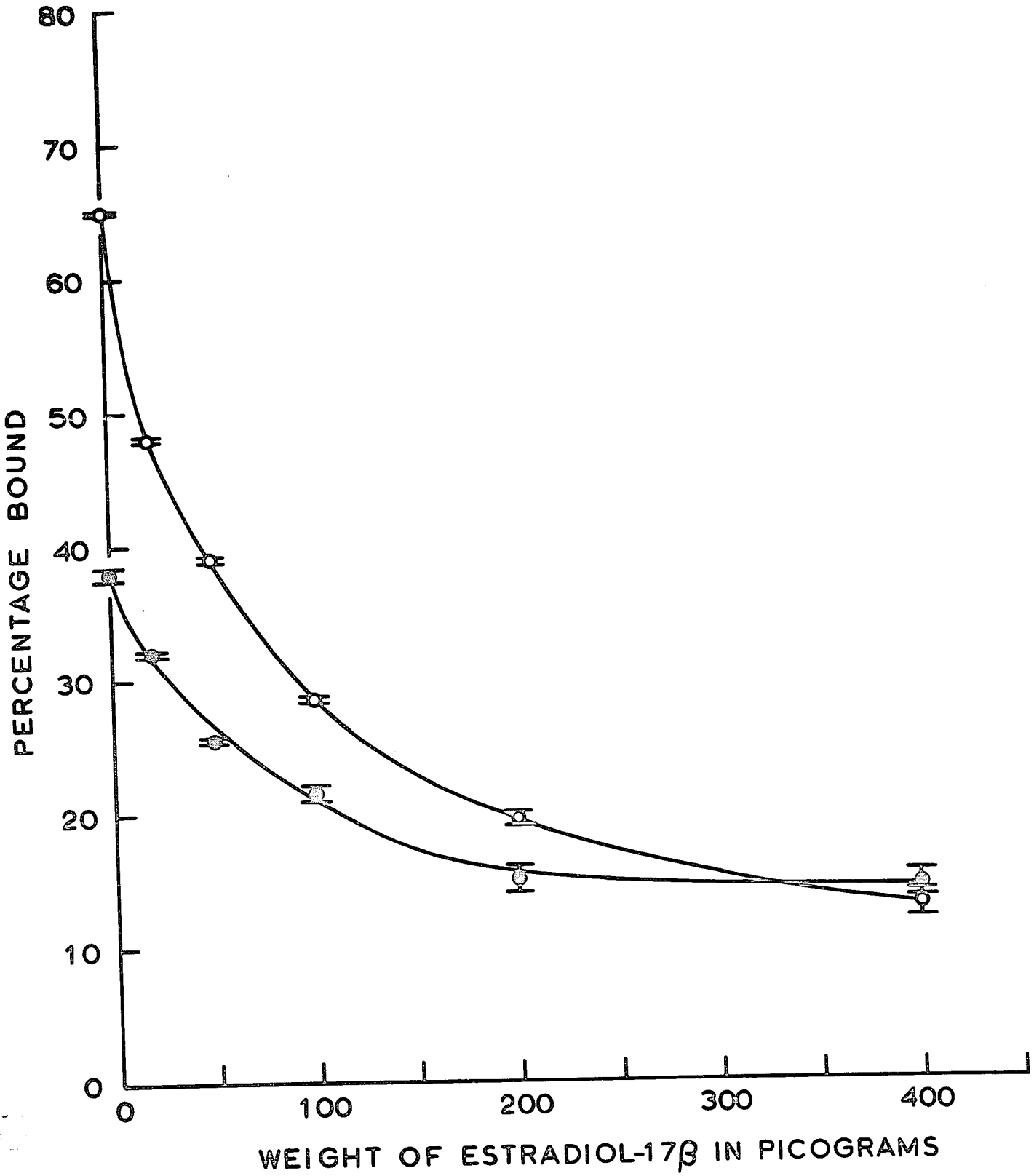
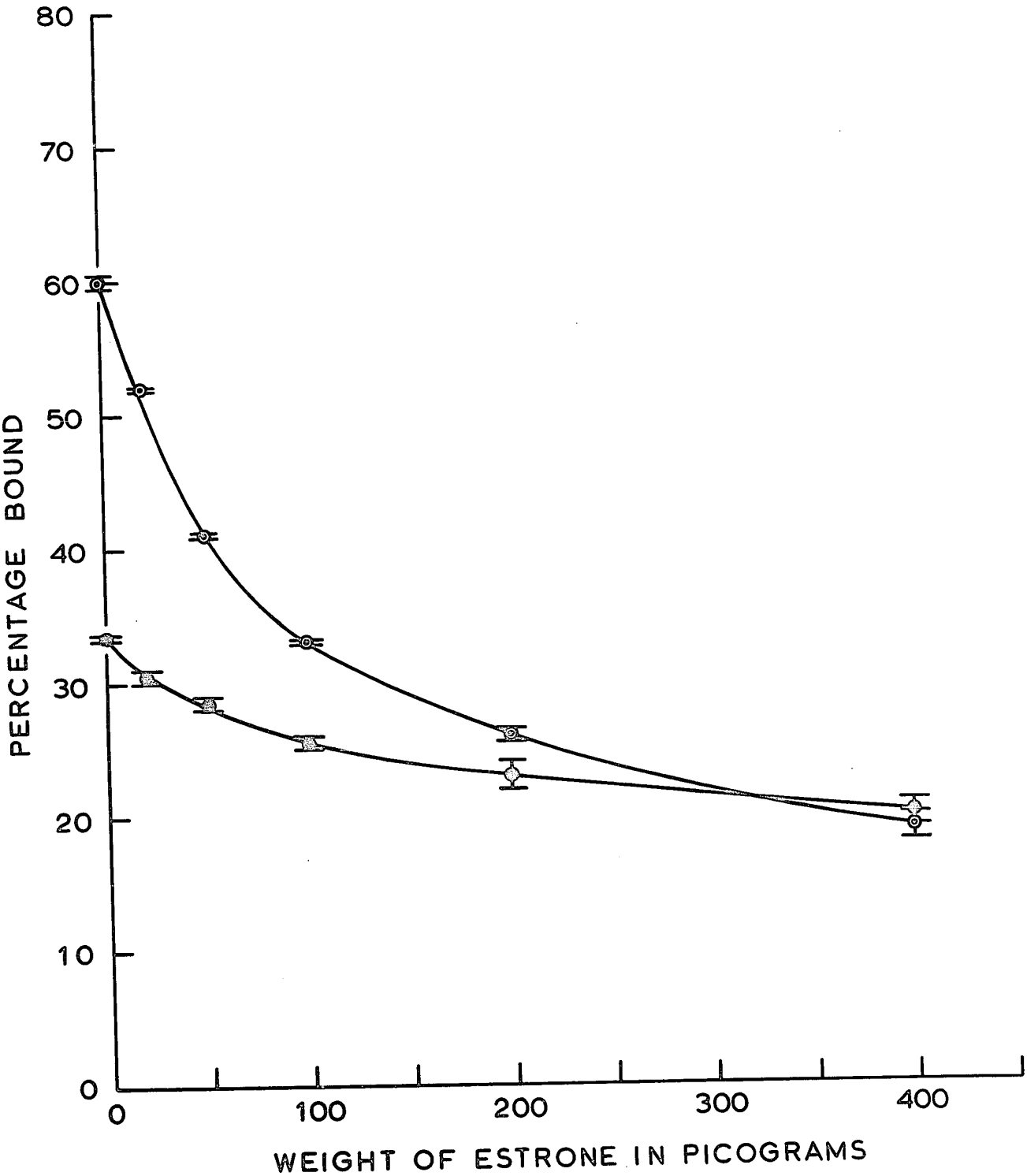

80
70
60
50
40
30
20
10
0
PERCENTAGE BOUND
0
100
200
300
400
WEIGHT OF ESTRADIOL-17β IN PICOGRAMS

Figure 27. Comparison of the standard curves for estrone determined by CPB (closed circles) and RIA (open circles). Crossed lines indicate standard errors (n=5).


80
70
60
50
40
30
20
10
0
PERCENTAGE BOUND
0
100
200
300
400
WEIGHT OF ESTRONE IN PICOGRAMS

the precision for the CPB assay was calculated from only five birds, and these were the first five birds that the author studied by saturation analysis. The improvement in reproducibility with the RIA may, therefore, reflect an improvement of experimental technique. This possibility receives some support from the circumstance that the experimental parameters for both CPB assay and RIA reported by various authors are very similar (1,207).

CHAPTER IV

LEVELS OF ANDROGENS IN THE PERIPHERAL PLASMA OF LAYING HENS IN RELATION TO OVULATION

IV.1. Introduction

The term androgen signifies a substance, usually a naturally occurring C_{19} steroid, which acts in animals to produce and maintain the secondary sex characteristics of the male. The role of androgens in normal female reproduction is not completely understood. It has been observed that, in adult women, the highest plasma levels of testosterone occur at the time of ovulation and during the luteal phase of the cycle. During menstruation the levels fall to that seen in oophorectomized females (75). The physiological significance of this increased androgen concentration during ovulation is not known.

The advent of saturation analysis has enabled more routine measurement of androgens in the plasma of animals to be undertaken, but research has been concentrated to date on the male. Females have been studied mostly in relation to the etiology of the various virilizing syndromes. The original saturation analyses involved CPB with use of the sex hormone binding globulin (SHBG) present in female plasma.

High concentrations of SHBG occur during pregnancy and the most common source for the SHBG has been human third trimester pregnancy plasma (69, 155). The reliability of the published CPB assays for plasma testosterone and the comparison between this method and GLPC determinations have been the subject of careful investigation (20, 69, 215). More recently, RIA has been used to measure testosterone, antibodies being induced by using either testosteron-17-bovine serum albumin or testosterone-3-bovine serum albumin (39, 47, 73, 99, 133, 214).

The reported levels of testosterone in the plasma of laying and non-laying hens range from around 200 to 122 pg/ml (96, 220, 259), and androstenedione has been measured at a concentration of 980 pg/ml (220). There is some experimental evidence to support the view that androgen plays an important role in relation to calcium balance in birds (48, 146) and some more recent evidence that this effect of testosterone is mediated through its affect on calcitonin secretion (56). This evidence suggests in turn the possibility of a relationship between circulating androgen and ovulation in the domestic hen, but no information on this point has been hitherto available. The subject of this chapter is an experimental study of this relationship.

IV.2. General Materials and Apparatus

(a) Reference Steroids:- crystalline testosterone, androstenedione, dehydroepiandrosterone, 5β-androstanedione and androsterone were obtained from Steraloids Inc., Pawling, N.Y.

(b) Radioactive Steroids:- testosterone-1,2,6,7-^{3}H (91 Ci/mMole), testosterone-1,2-^{3}H (45 Ci/mMole), androsterone-1,2-^{3}H (53 Ci/mMole), androstenedione-1,2-^{3}H (48 Ci/mMole), dehydroepiandrosterone-7-^{3}H (21 Ci/mMole) and dihydrotestosterone-1,2-^{3}H (39.4 Ci/mMole) were purchased from New England Nuclear, Montreal. Each, excepting testosterone-1,2,6,7-^{3}H, was diluted in 10 ml of methanol to provide a final concentration of 10 μc/ml and stored at 4°C.

(c) Chemicals:- charcoal (Norite A) and sodium azide were obtained from Fisher Scientific Co., Montreal. Gelatin (granular), NaCl, $Na_2HPO_4 \cdot 7H_2O$ and $NaH_2PO_4 \cdot 4H_2O$ were purchased from Anachemia, Montreal. KH_2PO_4 was obtained from Allied Chemicals, New York, N.Y. Sephadex LH-20 was bought from Pharmacia, Montreal and Dextran (Grade D) from Schwarz-Mann, New York, N.Y.

(d) Reagents:- benzene and chloroform (both "Spectranalyzed" grade) were obtained from Fisher Scientific Co.,

Montreal and were used as received. Normal heptane (toxigraphic grade) was purchased from Anachemia, Montreal, and was also used as received. Diethyl-ether (reagent) was obtained from Fisher Scientific Co., Montreal, and was freed from peroxides and redistilled immediately before use. Absolute ethanol was obtained from Consolidated Alcohols Ltd., Toronto and was redistilled twice before use. The eluting solvent for the column chromatography consisted of n-heptane:chloroform:ethanol, 50:50:1. The scintillation fluid was identical to that described in the progesterone assay.

(e) Buffer Solutions:- the phosphate buffer A used in the RIA was identical to that used for the estrogen assay. The phosphate buffer B used in the CPB assay consisted of a mixture of 87.7 ml of 1/15 M KH_2PO_4 and 12.3 ml of 1/15 M $Na_2HPO_4 \cdot 7H_2O$, the resultant reaction of this buffer being pH 6.0. The dextran-coated charcoal suspension for the RIA was prepared identically as described for the estrogen assay. The suspension for the CPB assay was prepared by dispersing 250 mg of Norite A and 25 mg of Dextran D in 100 ml of phosphate buffer B. All buffer solutions were stored at 4°C.

(f) Standard Solutions:- the primary standard solutions of the androgens were prepared in methanol at a

concentration of 10 μg/ml. The working standard solutions were prepared from the primary standards to give a final concentration of 10 μg/ml. All standard solutions were kept at 4°C.

(g) Tritiated Testosterone Solution for RIA:- the stock solution was prepared by drying-down 0.1 ml of the original testosterone-1,2,6,7-^{3}H in a glass-stoppered 10 ml volumetric flask. Exactly 10 ml of phosphate buffer A was then added and the flask was shaken well. The final working solution was prepared by taking 1.0 ml of the stock solution and diluting it to 100 ml with phosphate buffer A in a glass-stoppered volumetric flask. The resultant concentration of activity was 10 nC/ml. The flask was stored at 4°C and a sufficient quantity for the assay was poured out each day.

(h) Protein-tracer Solution for the CPB Assay:- term pregnancy plasma (60 ml) was obtained from the Lakeshore General Hospital, Pointe Claire, Quebec (by courtesy of Dr. G. Rona), and was frozen in 2.5 ml lots at -10°C. The binding solution was prepared in a 125 ml glass-stoppered Erlenmeyer flask by dissolving 2.5 ml of term plasma and 0.2 ml of the tritiated testosterone solution (10 μc/ml in methanol) in 97.3 ml of buffer B. Care was taken to add the plasma after adding most of the buffer to avoid

precipitation of the protein by the methanol.

(j) Radioimmunoassay:- the testosterone antibody used in this study was a gift from Dr. G.D. Niswender, Department of Physiology and Biophysics, Colorado State University, and had been prepared by immunizing a sheep against testosterone-3-bovine serum albumin. The antiserum was stored in 0.1 ml lots. A stock solution was prepared by diluting 0.1 ml of the antiserum to 10 ml with phosphate buffer A. The working solution was prepared by taking 0.1 ml of the stock and diluting it to 6 ml with phosphate buffer A to provide a final dilution of the antiserum of 1:6000. The stock solution was still usable after five months in cold storage.

(k) Apparatus:- the culture tubes, Pasteur pipettes, the "Repettes" and the method of measuring the radioactivity were identical to those used for the estrogen assay. Teflon-stoppered burets were purchased from both Fisher Scientific Co., Montreal (25 ml, "Student line") and Canadian Laboratory Supplies Ltd., Montreal (50 ml).

IV.2.1. Preliminary Experiments on the Feasibility of Measuring Testosterone in Laying-Hen's Plasma by CPB Assay

(a) General Procedure for the CPB Assay of Androgens as Used in This Study.

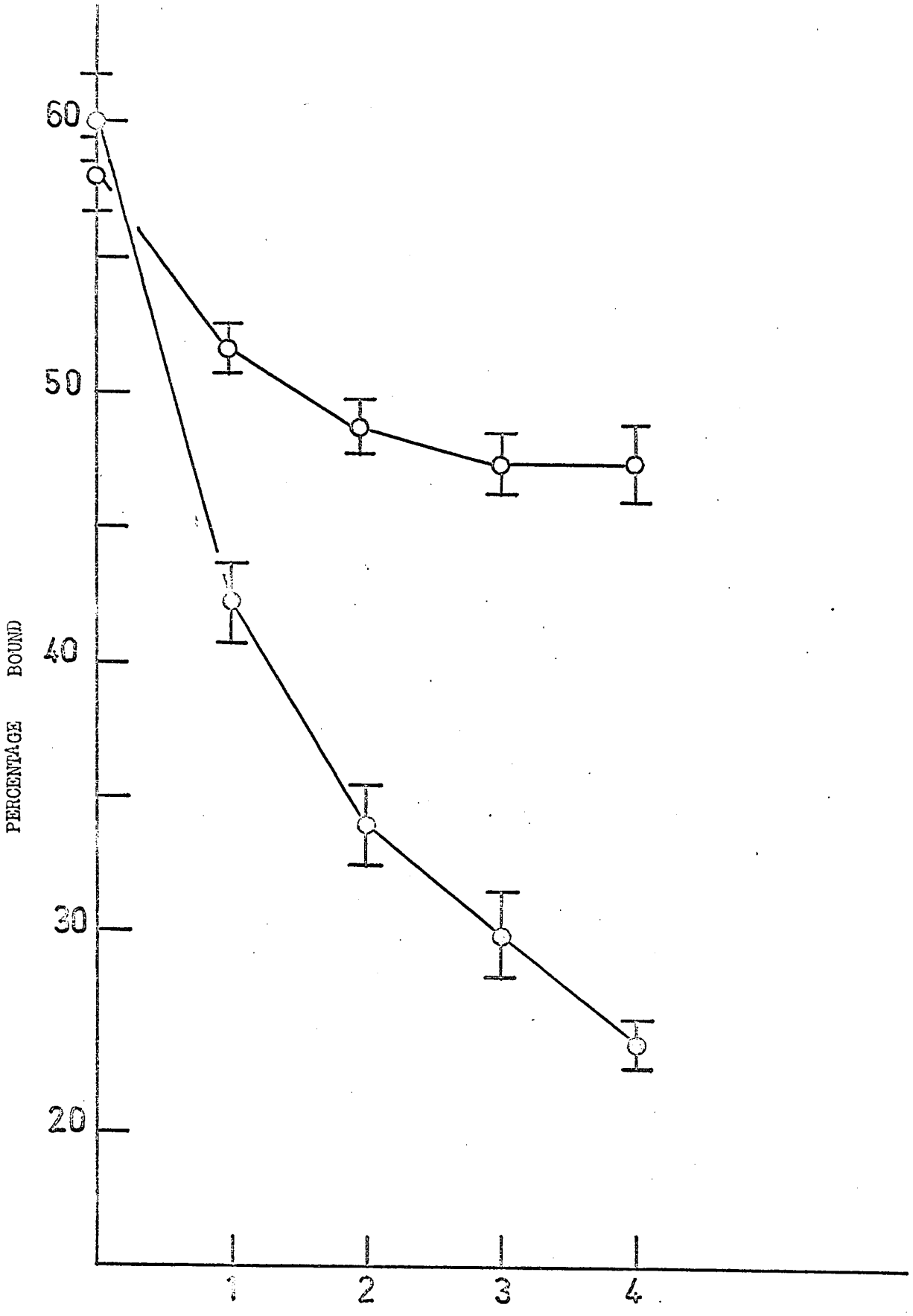
To each of the tubes containing the dried extracts to

be assayed was added 1.0 ml of the protein tracer solution and the tubes were shaken. The tubes were then incubated at 45°C for five minutes, shaken again and then incubated at 4°C for 45 minutes. Dextran-coated charcoal suspension (0.5 ml) was added and the mixture shaken for two minutes. The tubes were then spun at 4000 g and the supernatant carefully decanted into counting vials containing 12.5 ml of Aquasol. The vials were shaken vigorously and then placed in the liquid scintillation spectrometer at least two hours before counting began. The appropriate series of standards was prepared in duplicate in a similar way with each batch of assays. The standard curves obtained for the androgens are shown in Figure 28.

(b) Preliminary Experiments on the CPB Assay of the Androgens Separated by Column Chromatography.

In order to examine the feasibility of using the 31 cm Sephadex column as reported by Murphy (206), the extracts from six two-ml samples of laying-hens' plasma, obtained from different hens, were applied to separate columns. The eluates were collected in fractions each of 2.0 ml, dried down under nitrogen and assayed for androgen by CPB with testosterone as the standard. A series of columns was also run to examine the fractionation of various radioactive androgens. The eluates from these columns (also

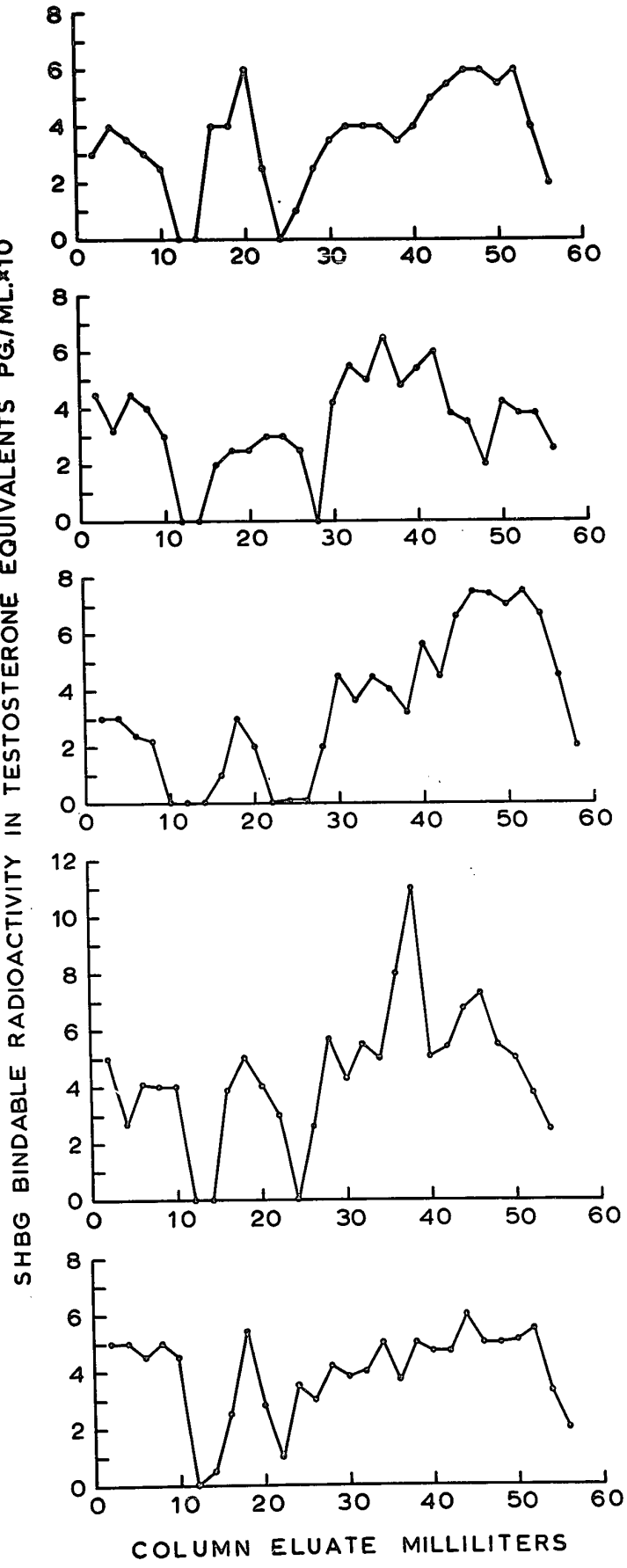
Figure 28. Standard curves for testosterone (closed circles) and androstenedione (open circles) obtained by CPB. Crossed lines indicate standard errors (n=10).

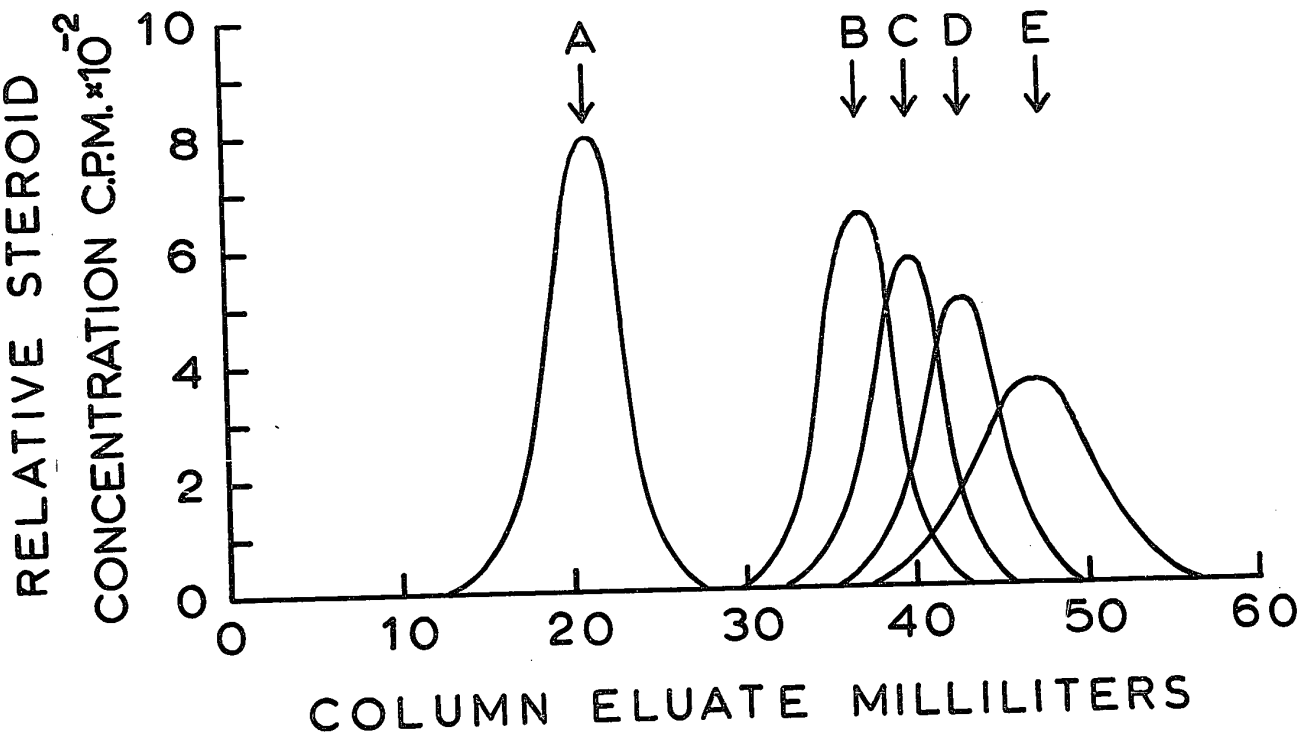

60
50
40
30
20
PERCENTAGE BOUND
1
2
3
4
WEIGHT OF ANDROGEN IN NANOGRAMS

2.0 ml fractions) were collected in counting vials. After the solvent had been evaporated 10 ml of toluene scintillation fluid was added and the radioactivity was measured. The results of these experiments are presented in Figure 29.

These results demonstrate that this column consistently separated androstenedione, but was unable to separate the four androgens, androsterone, dehydroepiandrosterone, dihydrotestosterone and testosterone. Since the SHBG in term plasma is relatively unspecific, it was felt that a separation step would be necessary if the assay was not to overestimate the testosterone concentration. As the only androgen isolated by the column was androstenedione, however, attention was focussed on the adaptation of the column for use in the CPB assay of androstenedione rather than testosterone. Accordingly, various gel heights were tried until it was found that a column length of 15 cm, corresponding to 6.5 g of Sephadex LH-20, fulfilled the requirements of a practical column. These were (a) a reasonable volume of solvent needed to prewash the column; (b) a reasonably rapid development time; and (c) maintenance of efficient and reproducible separation of androstenedione. The reproducibility of elution patterns obtained from this column closely resembled that reported by Murphy (206).

Figure 29. Fractionation patterns of androgens, extracted from laying-hens' plasma, using Sephadex LH-20 column chromatography. The androgens are measured as SHBG activity in testosterone equivalents.


SHBG BINDABLE RADIOACTIVITY IN TESTOSTERONE EQUIVALENTS PG./ML.×10⁻²
COLUMN ELUATE MILLILITERS



A. Androstenedione

B. Androsterone

C. Dihydrotestosterone

D. Dehydroepiandrosterone

E. Testosterone

Figure 28 demonstrates that the CPB assay was more specific for testosterone than for androstenedione, but with meticulous attention to experimental detail the standard curves for androstenedione were reproducible even though the decrease in percentage bound radioactivity with increasing standard concentration was far less than for testosterone. It was considered that androstenedione could reasonably be assayed by CPB over the range of 0 to 1.0 ng.

IV.2.2. Procedure in the Saturation Analysis of Plasma Androgen in the Laying Hen

(a) Blood Collection:- the experimental hens and the method of blood collection were identical to those described for the estrogen assays, except that slightly more blood, viz., approximately three ml, was taken at each sampling time. The resultant plasma was frozen at -10°C pending analysis. After thawing, the sample was divided into three sub-samples, viz., 0.2 ml in duplicate for assay by RIA, and 1.0 ml for the CPB assay.

(b) CPB Assay:- to the 1.0 ml plasma sample was added 0.2 ml of 1 N NaOH and the plasma was then extracted with diethyl ether (2 x 5 ml). The ether was evaporated to dryness under nitrogen, the same criteria for dryness being applied as for the estrogen study.

Columns were prepared by adding 6.5 g of Sephadex LH-20 dry (as received) to the 25 ml "Student line" burets, which had been filled with eluting solvent. These burets were preferred because the teflon part containing the stopcock could be completely removed from the bore of the buret, thus allowing easy removal of the Sephadex after use. The stopcocks were opened from time to time to allow the solvent to run through and to facilitate packing. The columns were washed with 50 ml of eluting solvent, left overnight and used the next day. A fresh set of columns was prepared for each batch of assays.

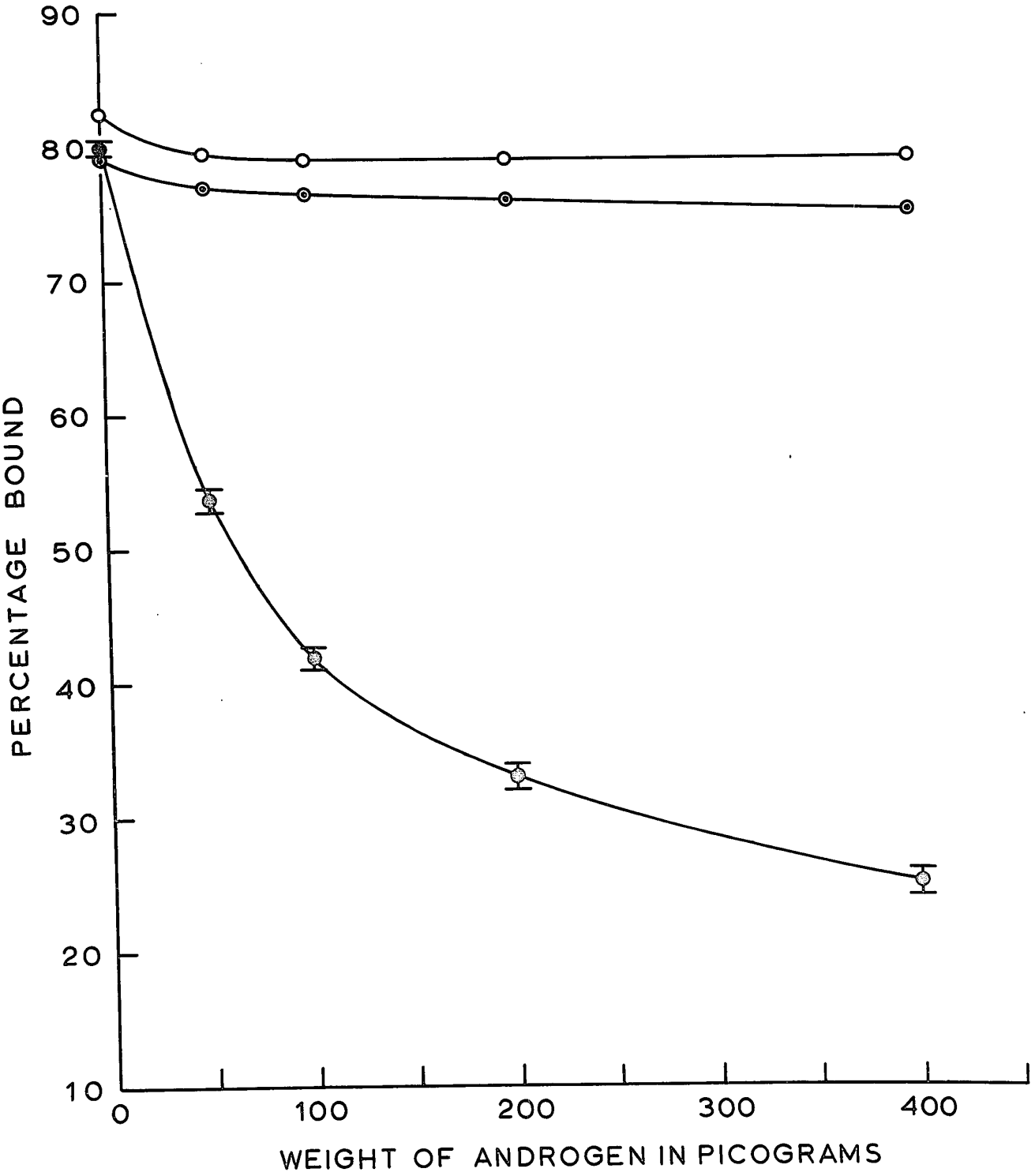
Before the sample to be assayed was placed on the column, five ml of eluting solvent was added to each column and the eluates collected and evaporated under nitrogen. These eluates were used to measure the column blanks. The dried androgen extract was dissolved in 0.1 ml of solvent and introduced onto the top of the gel by a Pasteur pipette. The tube was then rinsed with a further addition of 0.1 ml of solvent which was similarly transferred to the column. Nine ml of eluting solvent was added and the eluate discarded. Five ml of solvent was then added and the eluate, containing the androstenedione, collected. The eluate was evaporated under nitrogen and the dried extract and the corresponding

column blank were assayed by CPB by the procedure outlined above.

Because of the impracticality of taking more than three to four ml of blood from the hen and because at least one ml was needed for the column chromatography, it was impossible to perform the analyses in duplicate. To obviate problems that might result from environmental change, the elution pattern was checked every second assay by preparing an extra column that was used as a control. Tritiated androstenedione was placed on top of this column, elution of which was started 15 minutes before that of the others. The eluate was collected in one ml fractions in counting vials containing ten ml of the toluene scintillation fluid. By monitoring the radioactivity during the collection, the optimal volume for the separation of androstenedione could be determined and the appropriate volume collected from the other five columns accordingly. It was observed that, over the two months during which the assays were performed, the variation in elution of androstenedione was no more than 1.5 ml.

(c) RIA:- the anti-testosterone serum used in the RIA was more specific for testosterone than the SHBG used in the CPB (Figure 30). The RIA was performed directly

Figure 30. Standard curves for various androgens obtained by RIA. Testosterone (closed circle), androstenedione (circled dots) and dehydroepiandrosterone (open circles). The standard curves for both androsterone and 5β-androstanedione lie between those for androstenedione and dehydroepiandrosterone.


90
80
70
60
50
40
30
20
10
PERCENTAGE BOUND
0
100
200
300
400
WEIGHT OF ANDROGEN IN PICOGRAMS

on the ether extract of the plasma and no chromatographic step was included.

Plasma (0.2 ml) was extracted in suplicate with diethyl ether (2 x 2.5 ml) after the addition of 0.1 ml of 1 N NaOH. The ether was then evaporated to dryness under nitrogen. The diluted antiserum (0.1 ml) was added and the tubes were shaken for one minute. Approximately 0.1 nCi of the tritiated testosterone was then added and the tubes were shaken for another minute. The tubes were then covered and incubated overnight at 4°C. Dextran-coated charcoal (1.0 ml) was then added and the tubes were spun at 4000 g for 15 minutes. The resultant supernatants were decanted carefully into counting vials containing 12.5 ml of Aquasol and the vials placed in the counter at least two hours before counting began. The testosterone standards were prepared in duplicate in exactly the same way with each batch of assays.

IV.3. Results and Discussion

IV.3.1. CPB Assay

(a) Specificity:- the column separated androstenedione adequately from the other four androgens examined, and the assay was quite probably measuring mainly androstenedione.

(b) Accuracy:- amounts of androstenedione of 400 and 600 pg were added in duplicate to separate one-ml portions of laying hens' plasma and were then assayed together with the corresponding duplicate blank samples. The recoveries are presented in Table 11.

Table 11. Recoveries of androstenedione added to 1.0 ml samples of laying-hens' plasma.

Amount Added pg	No. Duplicates N	Recovery pg ± SE	%
400	10	260.8 ± 15.2	65.2
600	10	382.8 ± 18.4	63.8

(c) Precision:- since no duplicate analyses were performed on the samples of laying hens' plasma, no average value for the precision can be calculated from the column results. Some indication of the precision can be gained, however, by considering the results from the recovery experiments. Twenty duplicate analyses were taken and the average value for the precision, calculated from $SD = \sqrt{(\Sigma d^2/2\ N)}$ was ±19.2 pg/ml.

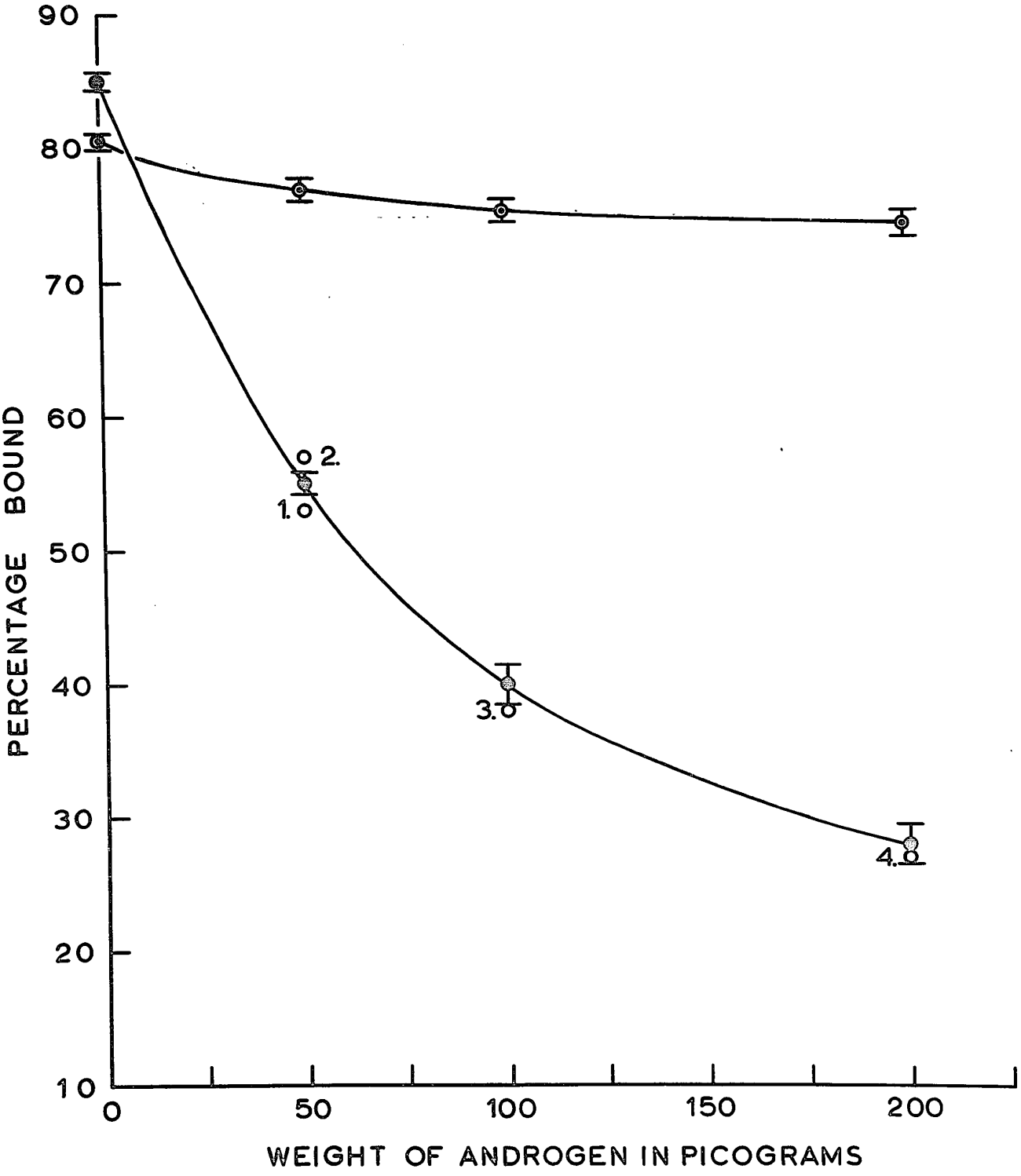
(d) Blanks:- this assay relied upon meticulous attention to detail and also on scrupulous cleanliness of the

apparatus used. By following a vigorous cleaning procedure for the columns and also by using 'Spectranalyzed' grade solvents, the elution blanks of the five ml aliquots were kept low. The average blank value for 20 determinations was 32.0 ± 12.1 (SD) pg. The 'detection limit' (78) of this assay, calculated by adding two standard deviations to the mean of the blank values, was ±66.2 pg.

IV.3.2. Radioimmunoassay

(a) Specificity:- the antiserum showed major cross-reactions only with dihydrotestosterone (77%) and 5α-androstan-3α,17β-diol (26%) (133). As dihydrotestosterone was difficult to separate from testosterone on the column used, the plasma was extracted and assayed without prior separation of the androgens. The effect of androstenedione on the RIA was observed in a similar way to that described for the estradiol-17α and -17β interaction. These results are shown in Figure 31, which shows that even a ratio of androstenedione to testosterone of 4:1 did not change the standard curve. As dihydrotestosterone has not been found in the blood of the domestic hen, and as androstenedione, known to be present in the blood, does not interfere with the assay for testosterone, the results of the RIA probably reflect mainly the levels of testosterone in the plasma.

Figure 31. Effect of androstenedione on measurement of testosterone by RIA. Androstenedione only (circled dots) and testosterone (closed circles). The open circles indicate values for testosterone in presence of androstenedione. Crossed lines indicate standard errors (n=P).


90
80
70
60
50
40
30
20
10
PERCENTAGE BOUND
0
50
100
150
200
WEIGHT OF ANDROGEN IN PICOGRAMS
2.
1.
3.
4.

Since absolute specificity was not achieved, the data have been reported as total androgen in terms of testosterone equivalents.

(b) Accuracy:- amounts of testosterone of 50, 100 and 200 pg were added in duplicate to 0.2 ml portions of the same laying-hens' plasma, which were then assayed together with the corresponding duplicate blank samples. The resultant recoveries are presented in Table 12.

Table 12. Recoveries of testosterone added to 0.2 ml samples of laying-hens' plasma.

Amount Added pg	No. Duplicates N	Recovery pg ± SE	%
50	8	43.7 ± 7.0	87.4
100	8	83.7 ± 8.8	83.7
200	8	171.8 ± 9.1	85.9

(c) Precision:- an average value for the precision was estimated from 30 duplicate assays taken at random from all available data for ovulating and non-ovulating hens. These values ranged from 150 to 850 pg/ml. The standard deviation, calculated from $SD = \sqrt{(\Sigma d^2/2\ N)}$, was 12.6 pg/ml.

(d) Sensitivity:- the least amount distinguishable

from zero amount at P=0.05 was 24.6 pg.

IV.3.3. Levels of Circulating Androgens During the Ovulatory Cycle

The procedure for relating times of blood sampling to ovulation has already been described. Data were obtained for 12 ovulations within a laying sequence, for six comparable intervals where ovulation did not accompany oviposition and for six birds that did not lay during the experimental period. The androgen values were plotted as percentages of the highest value observed for the individual cycle in question, as previously outlined for the progesterone and estrogen assays. The data for total androgen concentrations from ovulating birds are presented in Figures 32 and 33. The corresponding data for non-ovulating and non-laying birds are presented in Figures 34 and 35, respectively.

The average concentrations (±SE) of total androgens, uncorrected for procedural losses, during six-hour intervals of the ovulatory cycle for both ovulating and non-ovulating birds are shown in Table 13. The corresponding data for androstenedione levels (±SE) are shown in Table 14.

The total androgen concentration in birds where ovulation accompanied oviposition displayed a cyclic variation

Figure 32. Plasma total androgen concentrations plotted against time before (-) or after (+) oviposition accompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

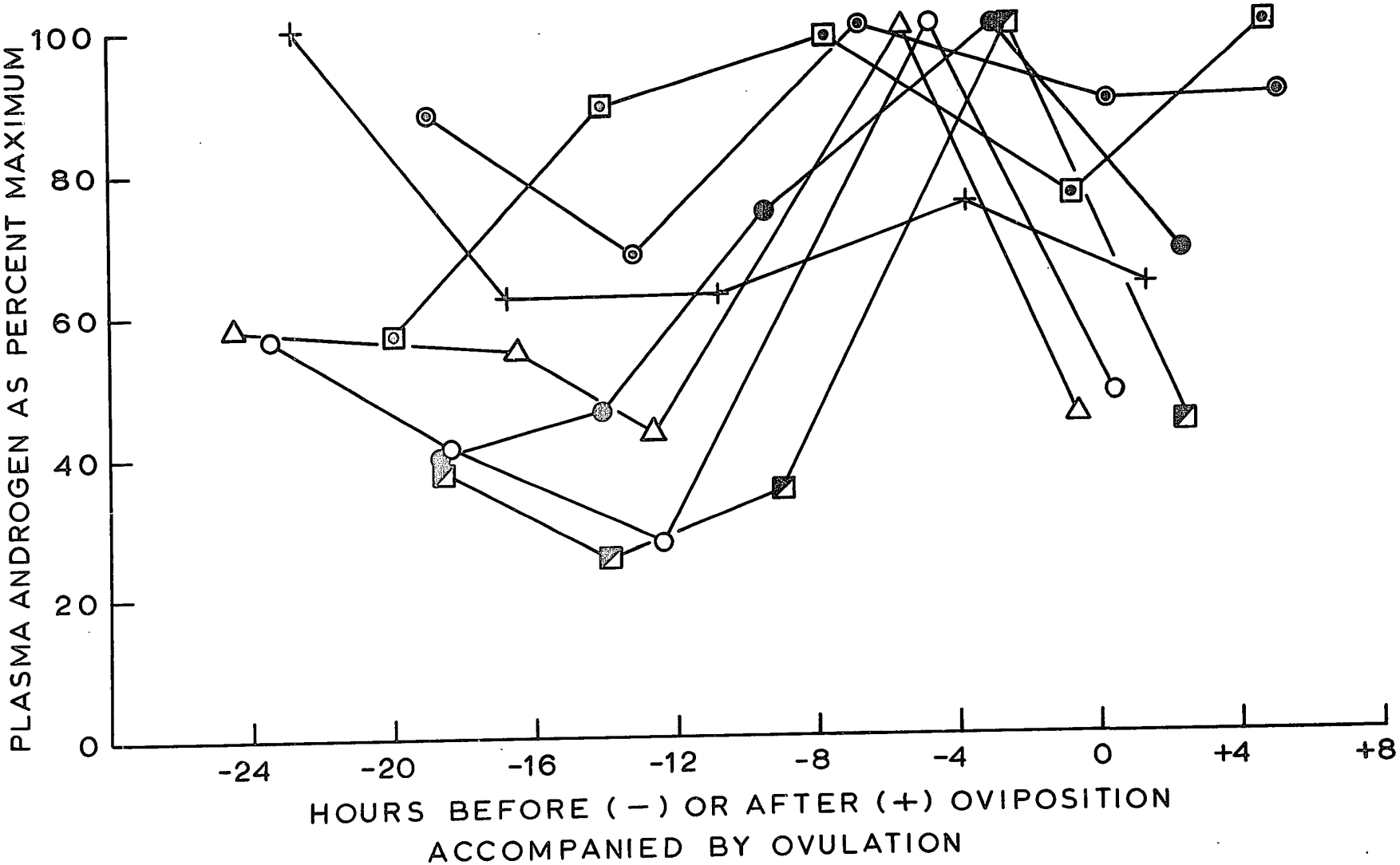

PLASMA ANDROGEN AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (−) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 33. Plasma total androgen concentrations plotted against time before (-) or after (+) oviposition accompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

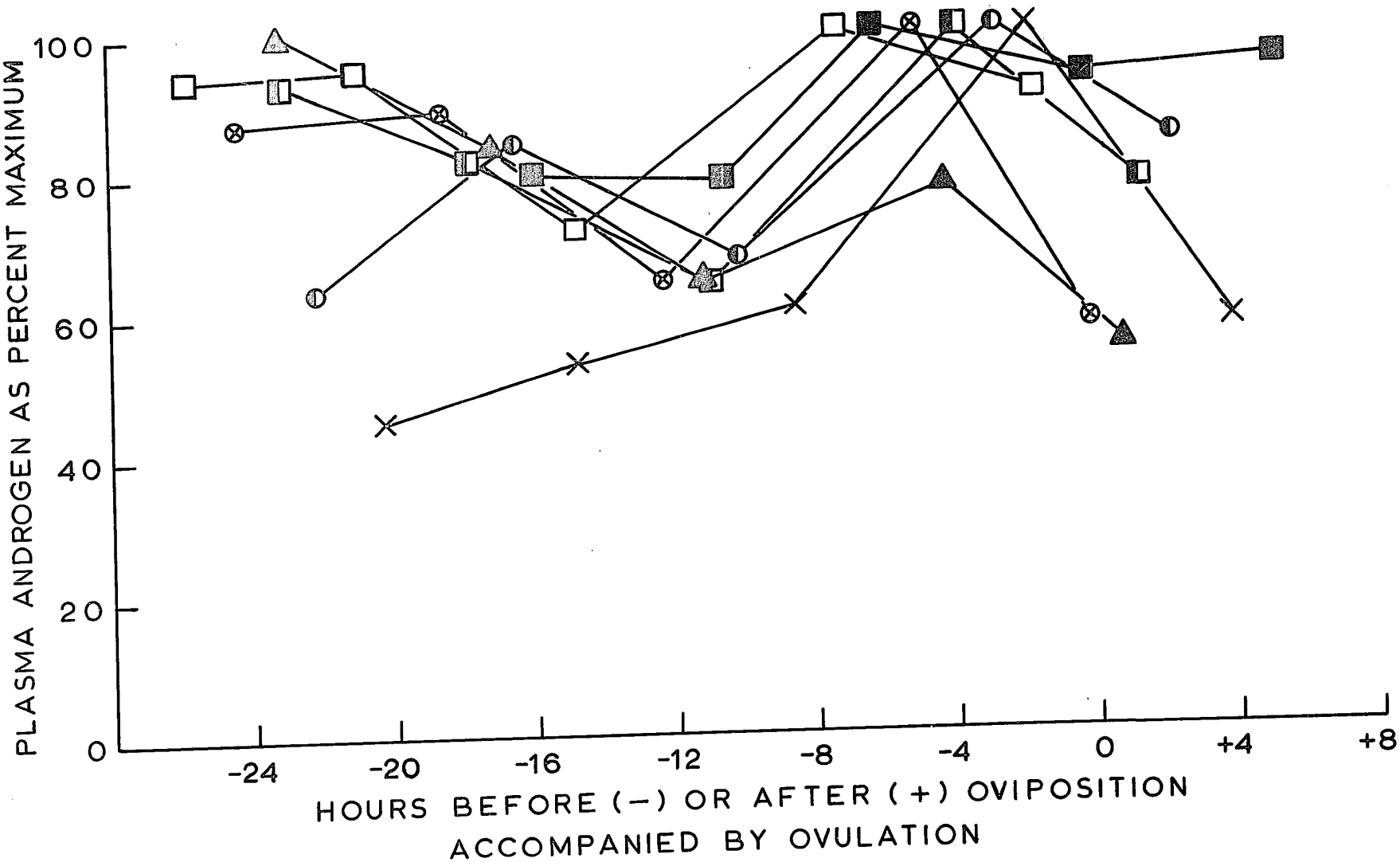

PLASMA ANDROGEN AS PERCENT MAXIMUM
100
80
60
40
20
0
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (−) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 34. Plasma total androgen concentrations plotted against time before (-) or after (+) oviposition unaccompanied by ovulation. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

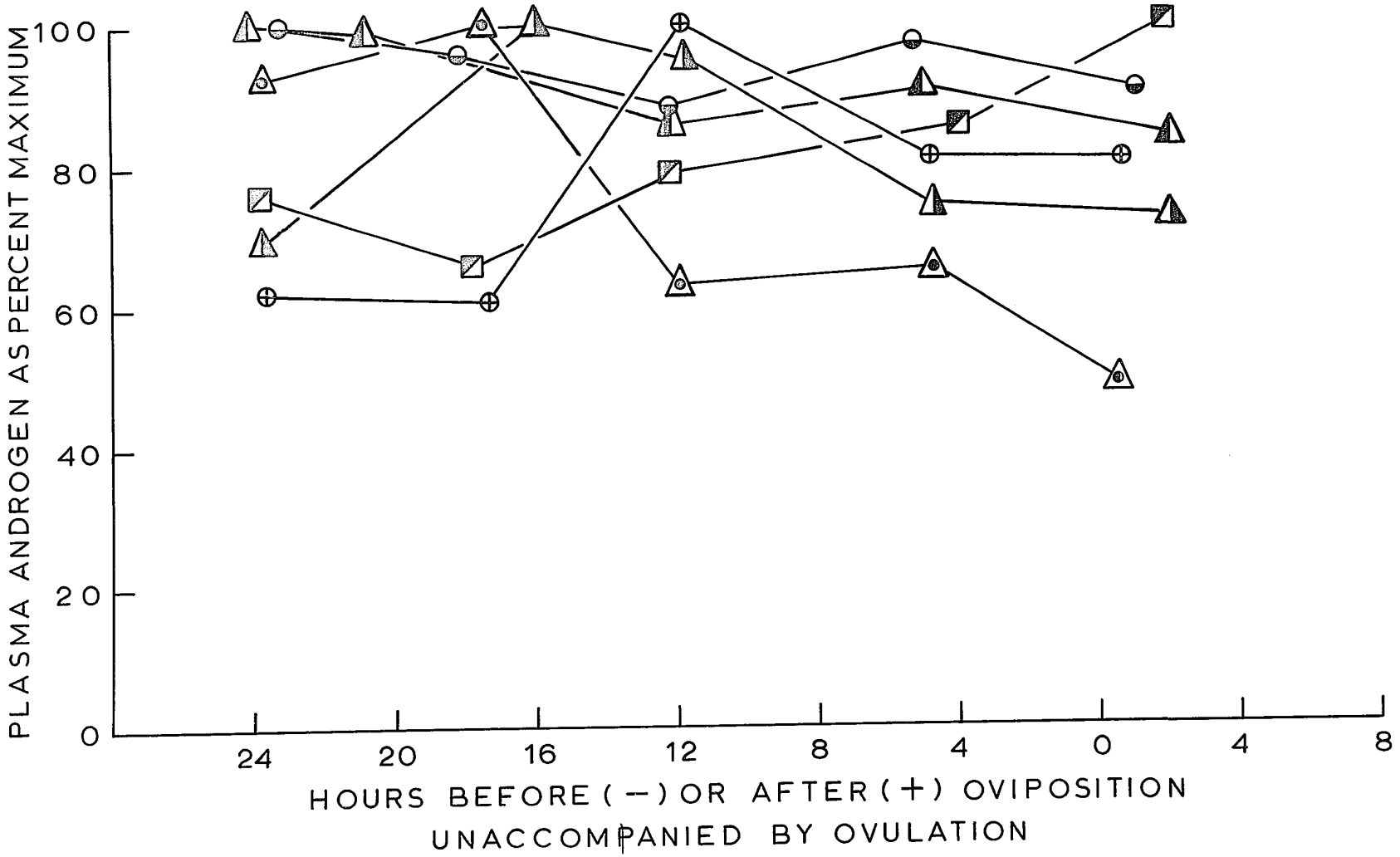

PLASMA ANDROGEN AS PERCENT MAXIMUM
100
80
60
40
20
0
24
20
16
12
8
4
0
4
8
HOURS BEFORE (−) OR AFTER (+) OVIPOSITION
UNACCOMPANIED BY OVULATION

Figure 35. Plasma total androgen concentrations plotted against times of sampling for birds which did not lay during the experimental period. Values for each individual hen expressed as percentages of the maximal value observed during the period for that bird.

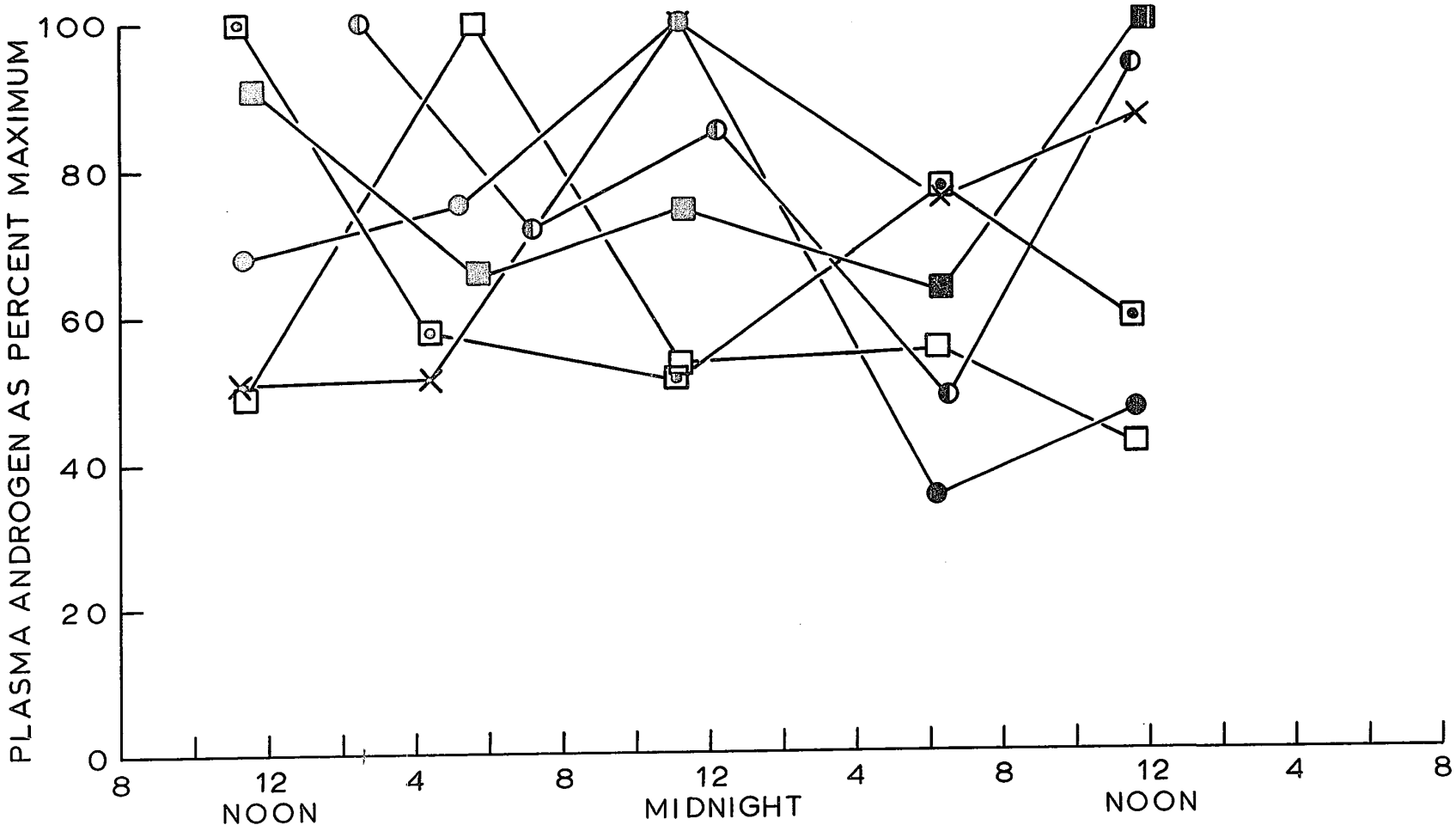

PLASMA ANDROGEN AS PERCENT MAXIMUM
100
80
60
40
20
0
8
12
NOON
4
8
12
MIDNIGHT
4
8
12
NOON
4
8

Table 13. Average concentration for total androgen in testosterone equivalents during six-hour intervals over the ovulatory cycle.

Time Interval Before Oviposition (hours)	Accompanied by Ovulation Concentration (pg/ml ± SE)*	Unaccompanied by Ovulation Concentration (pg/ml ± SE)*
-26 to -20	463.3 ± 82.4^{abcd}	499.4 ± 77.4^{a}
-20 to -14	356.2 ± 43.6^{ab}	500.6 ± 83.2^{a}
-14 to -8	320.0 ± 28.1^{a}	495.0 ± 47.2^{a}
- 8 to -2	563.8 ± 75.3^{d}	428.1 ± 61.1^{a}
- 2 to +4	385.3 ± 37.3^{abc}	470.0 ± 69.7^{a}

*Means carrying same superscript letter are not significantly different (P=0.05).

Table 14. Average androstenedione concentrations during six-hour intervals over the ovulatory cycle.

Time Interval Before Oviposition (hours)	Accompanied by Ovulation Concentration (pg/ml ± SE)*	Unaccompanied by Ovulation Concentration (pg/ml ± SE)*
-26 to -20	300 ± 120	295 ± 118
-20 to -14	289 ± 109	257 ± 104
-14 to -8	250 ± 125	286 ± 112
- 8 to -2	404 ± 130	228 ± 123
- 2 to +4	295 ± 118	279 ± 131

* None of the group differences attained significance (P=0.05).

with low values occurring during the period of 14 to 12 hours before oviposition. The concentration then rose to significantly ($P < 0.05$) higher values at from 8 to 2 hours before ovulation. The values for androstenedione did not display any significant group difference, the standard deviations being very high owing to the fact that it was only possible to make single analyses for this steroid. It may be remarked, however, that the average values displayed a pattern similar to that of the values for total androgen.

The data for periods where oviposition was not accompanied by ovulation displayed a different pattern. There was no significant variation in plasma androgen concentration. This lack of a cyclical variation was also seen in birds that did not lay during the experimental period. There was no decline in plasma androgen concentration when there was neither ovulation nor oviposition; and in these circumstances levels remained very similar to those observed for ovulating birds apart from the peak period. Though the major result of this study relates to the occurrence, in a given bird, of a peak level of plasma androgen some eight to two hours before ovulation, the actual levels measured are of interest and are presented in Table 15. The range of androgen concentration in the blood of six mature cocks is also

given for purposes of comparison. The observed concentrations are very similar to the values reported by others.

Table 15. Androgen levels (pg/ml) in blood samples from individual hens and cocks (values not corrected for procedural losses).

	Androgen Concentration pg/ml			
	Testosterone equivalents		Androstenedione	
	Min	Max	Min	Max
Preceding an ovulation 12 hens	125	1100	98	954
Preceding a day of no ovulation 6 hens	150	900	105	879
No egg laid during the experimental period 6 hens	200	800	118	905
6 mature cocks	570	6950	475	5090

PART III

GENERAL DISCUSSION

Figures 36 and 37 summarise in a semidiagrammatic form the observed variation in levels of ovarian steroids in the peripheral plasma of the domestic hen during the ovulatory cycle. All steroids measured exhibited peak plasma values during the period two to eight hours before ovulation. Levels of all the steroids were falling before ovulation. In addition there were indications of an earlier peak for both estrone and estradiol although as seen in Figure 24 and Table 10, these peaks were not statistically significant. These fluctuations in steroid levels may now be discussed in relation to the reported variations in gonadotrophin during the ovulatory cycle.

If a hypothesis regarding the control of ovulation is advanced on the basis of variations of plasma levels of ovarian and pituitary hormones, then that hypothesis must take into consideration the following points.

1. Progesterone and androgen each exhibit a single peak some four to six hours before ovulation.

2. Estrogen also exhibits a peak level during the period four to six hours prior to ovulation but with indications of a second peak around 21 hours before ovulation.

3. The major peak of FSH occurs about 14 to 11 hours before ovulation and a minor peak occurs some 24 hours before ovulation.

Figure 36. Semidiagrammatic representation of the variation in plasma androgens (open circles) and progesterone (closed circles) during the ovulatory cycle of the domestic hen.

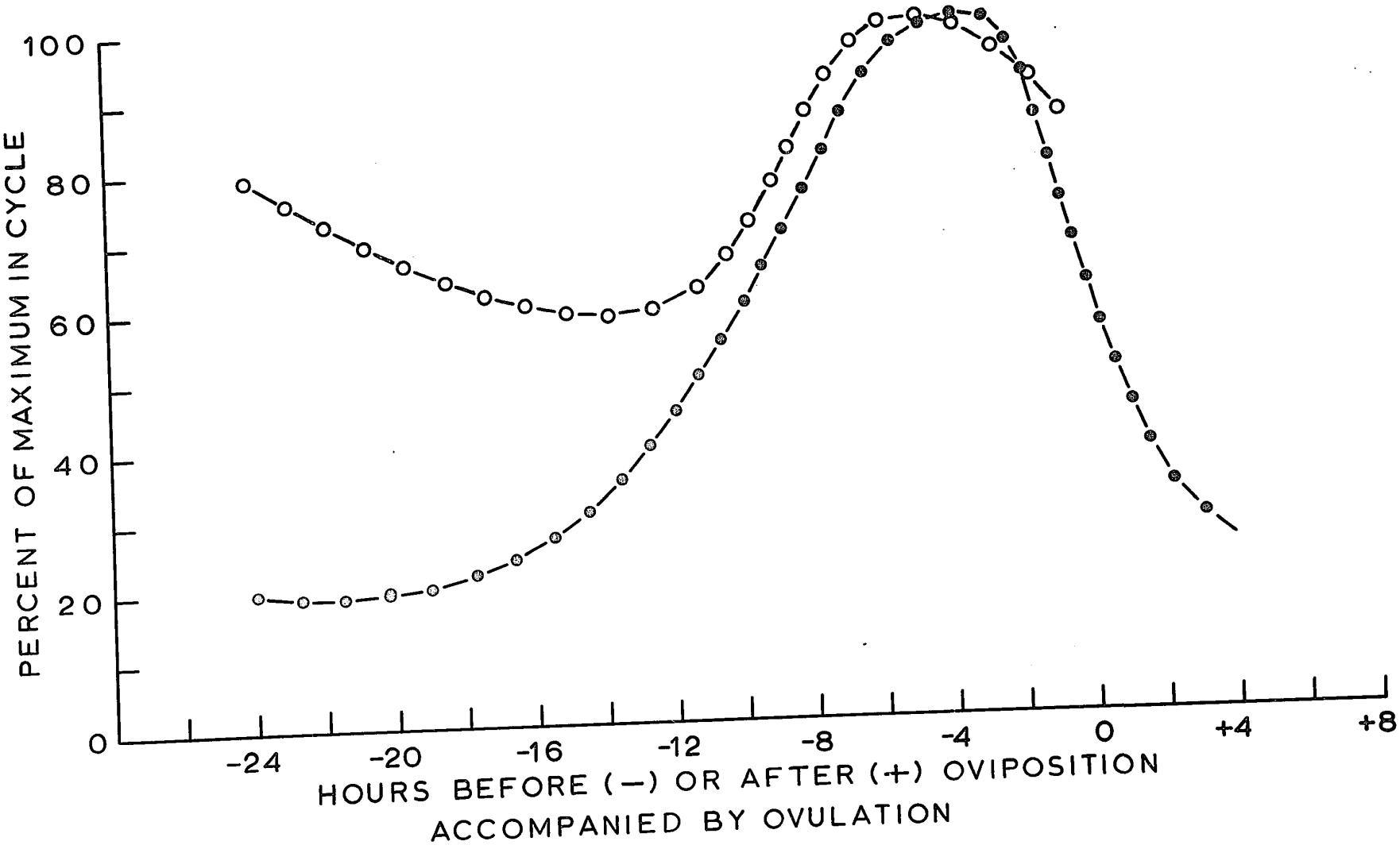
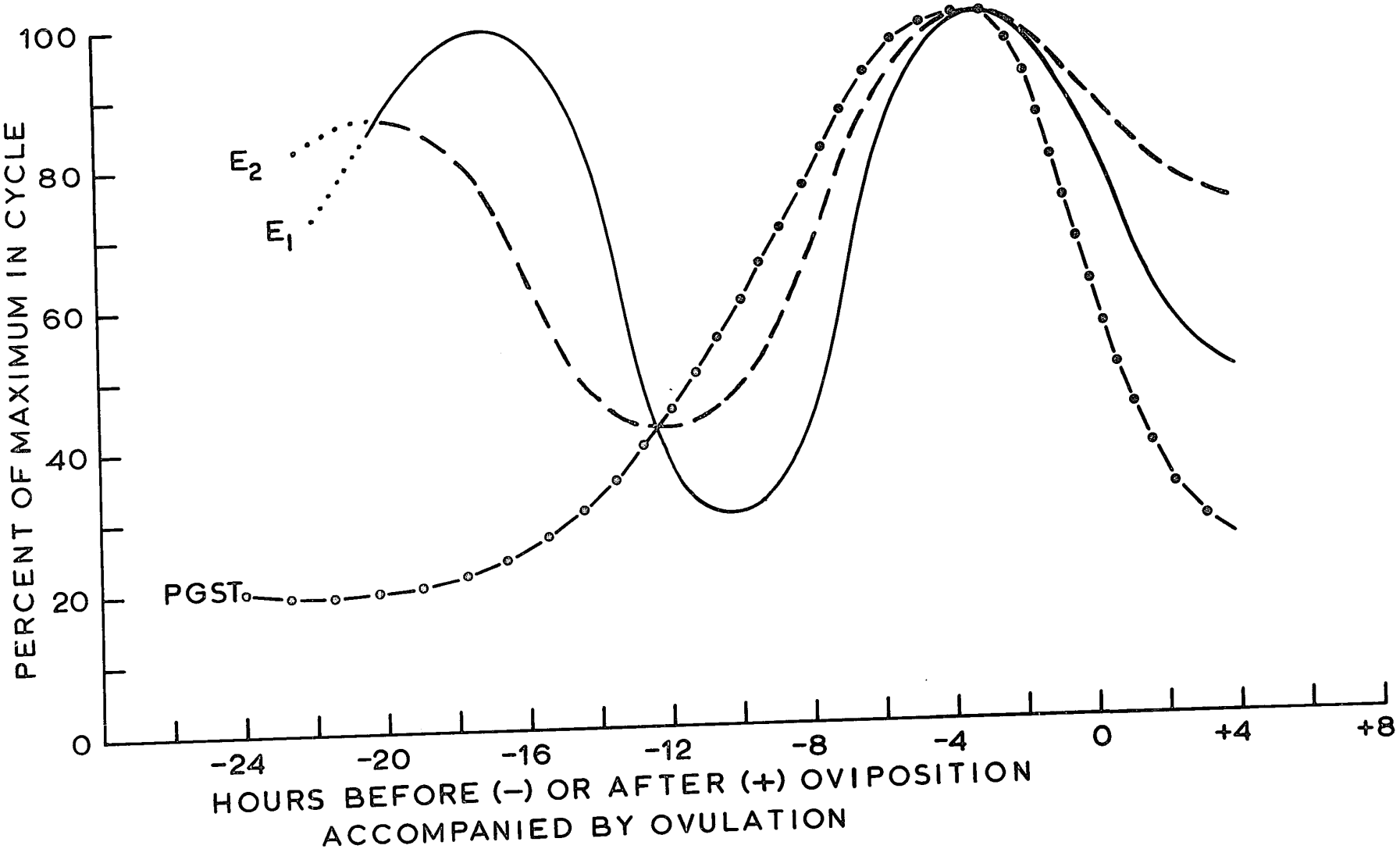

100
80
60
40
20
0
PERCENT OF MAXIMUM IN CYCLE
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (—) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

Figure 37. Semidiagrammatic representation of the variation in estrone (E_1), estradiol (E_2) and progesterone during the ovulatory cycle of the domestic hen. The dotted lines indicate the uncertainity of the early estrogen peaks.


100
80
60
40
20
0
PERCENT OF MAXIMUM IN CYCLE
E2
E1
PGST
-24
-20
-16
-12
-8
-4
0
+4
+8
HOURS BEFORE (-) OR AFTER (+) OVIPOSITION
ACCOMPANIED BY OVULATION

4. The major peak of plasma LH occurs four to seven hours prior to ovulation, with indications of a minor peak some 23 to 20 hours before ovulation.

5. No peaks of androgen, progesterone, estrogen or gonadotrophin are to be observed on days of missed ovulation.

Fraps considered that progesterone was the substance most likely to function as the 'excitation hormone' in LH release, since administration of this steroid enhanced ovulation of the hens' follicle by activating certain regions of the hypothalamus, but not the pituitary. The increase in plasma progesterone during the ovulatory cycle demonstrated in this thesis is closely associated in time with the increase in LH that occurs some seven hours before ovulation. Subsequent work by others on the levels of progesterone throughout the ovulatory cycle agrees with this observation, both in respect to the time of the preovulatory progesterone peak and to the fact that no peak was observed on the day of missed ovulation (14,55,154). These data are compatible with Fraps' theory to some extent, but the results do not support his view that the missed ovulation is due to an elevated neural threshold coincident with increasing levels of progesterone, because the level of this steroid does not increase on days of missed ovulation.

It has been implied recently that progesterone is without hormonal activity in the non-mammalian vertebrates (18). From its very definition as a ketosteroid that supports or assists pregnancy (12), it is obvious that the hormonal role of progesterone in the 'lower' vertebrates must be different from that which it plays in mammals, although an analogous function may exist in the ovoviviparous lizards. It would be imprudent, however, to propose that the steroid was initially only an intermediary metabolite and that it has been adapted by mammals to meet a new reproductive need. Granted that the corpus luteum and the role of progesterone in maintaining pregnancy are peculiar to mammals, the well-documented evidence available indicates that progesterone does have a physiological function, or functions, in the 'lower' vertebrates (124,166,221,288,304). It is for this reason that one cannot make a direct comparison of the progesterone levels and gonadotrophin release in mammals with the corresponding phenomena in the remaining vertebrates. There is no evidence at present that these two systems are homologous or even analogous.

It is more cogent to compare the effects of progesterone on ovulation as between the main groups of non-mammalian Tetrapoda. Mention has been made that a Δ^4-3-ketosteroid has been implicated in ovulation in the

Anura and evidence has been presented that suggests progesterone as the steroid most likely to be involved.

There are also indications of a preovulatory surge of progestins in at least one species of amphibia. The available data for reptiles suggest that progesterone is antigonadal in action since intrahypothalamic implants of this steroid inhibit ovarian growth and subsequent ovulation. Much more information is required before any definite conclusions can be made regarding the relationship of the progesterone-ovulation system among the 'lower' vertebrates.

There has been some controversy over the ability of the post-ovulatory follicle in the domestic hen to secrete steroids (103) and whether or not the ruptured follicle affects in any way subsequent ovulation. The work presented in this thesis demonstrates that the post-ovulatory follicle does not possess any secretory activity, for levels of progesterone remained low during the 24 hours after the last ovulation of the sequence. This does not preclude the effect of the post-ovulatory follicle on subsequent oviposition acting via some neural mechanism, for the ovary and follicles of the domestic hen are extensively innervated (60,105).

There is also experimental evidence that estrogens may affect ovulation in the domestic hen. Fraps (90) observed that injection of estradiol benzoate 28 to 11 hours before an expected ovulation suppressed that ovulation in 55% of the treated hens. The injected dose per bird ranged from 1 to 2.5 mg and in view of the plasma levels of estrogen observed in this thesis, this aspect observed by Fraps may be more pharmacological than physiological. Subject to this reservation, the artificial elevation of estrogen levels during this period suggests that the drop of plasma estrogen to relatively very low levels observed over the interval 18 hours to 10 hours before ovulation may be one of the conditions for subsequent ovulation. The results of Hawkins et al. (115) also suggest that estrogen affects the neural mechanisms associated with ovulation. They showed that there were significant cyclic accumulations of radioactivity in the pituitary of the laying hen after the injection of tritiated estradiol-17β, the greatest accumulation being in the period 16 to 12 hours before the expected ovulation. This is in contrast to the report of Morgan and Wilson (194) who found a relative lack of selective uptake of injected tritiated progesterone by the brain, including the pituitary gland. Furr and Cunningham (55)

cite some unpublished observations which suggest that estrogens may effect LH release. Significantly more LH was secreted by fowl pituitary glands incubated in vitro with whole pieces of median eminance tissue and with 0.1 μg of estradiol-17β, than from pituitaries incubated alone. No differences were detected in the amounts of LH released when the glands were incubated alone or with either estradiol-17β or median eminance tissue, respectively. They further indicated that progesterone appeared to inhibit the release of LH in this system.

The results reported in this thesis demonstrate that there is a cyclical change in the plasma concentrations of free estrone and estradiol during the ovulatory cycle of the hen. Lague (161) has obtained essentially similar results, both in respect to the preovulatory peaks of estrone and estradiol and to the observed plasma concentrations. This general pattern of estrogen variation has also been obtained in relation to the secretion of estrone and estradiol in the urine (37). It may be noted that if the early peaks of estrogen are physiologically significant, then the early maximum and subsequent minimum for plasma estrone might be expected to lag behind the corresponding maximum and minimum for estradiol because estradiol is normally

regarded as lying earlier in the metabolic pathway of phenolic steroids than does estrone.

The major peak of estrogen during the period six to four hours before ovulation may be associated with the release of pituitary LH that occurs at this time. The earlier peak of plasma estrogen, if present, overlaps in time the release of LH purported to occur some 21 hours before ovulation. The data for pituitary LH content (116) are also relevant. Increasing levels of LH in the pituitary are associated in time with decreasing free plasma estrogen. The decreases in pituitary LH levels at 21 hours and 8 hours before ovulation coincide in time with the increases in circulating free estrogen.

When this cyclic variation of plasma estrogen concentration is compared with the corresponding variation of plasma progesterone, it is seen that over the interval of about 20 to 8 hours before ovulation, estrogen concentrations are still falling whereas the progesterone concentration is beginning to rise from relatively low levels. From about eight hours to four hours before ovulation estrogen concentrations are rising sharply from their low minima to their principal maxima. Both estrogen and progesterone attain their maximum during the period six to two hours prior to ovulation. The available data are insufficient to resolve

the question of whether estrogen and progesterone concentrations attain their maxima concurrently or sequentially.

The observed levels of androgens display the same pattern of variation as do the other ovarian steroids, with a peak some eight to two hours before ovulation. The role of androgens in the ovulation of the domestic hen is uncertain. Van Tienhoven (287) has indicated that testosterone injections induce premature ovulation in 41% of treated birds although he gave no experimental details. More recently, Heald et al. (118) injected various doses of testosterone propionate into domestic hens and observed that at no concentration did the androgen affect pituitary LH levels. On the other hand, doses of 0.2 and 1.0 mg/kg severely depressed egg-laying. It is quite possible that the androgens are connected in some way with oviposition and that they exert this influence by some effect on calcium metabolism. There is evidence that androgens influence plasma calcitonin levels in both the Japanese quail and the domestic hen. Alternatively, the observed fluctuations in androgen levels during the ovulatory cycle may merely reflect the varying activity of ovarian steroidogenesis as a whole. It is obvious that the available information is completely inadequate to permit of any tentative resolution of the

relationship between ovulation and levels of circulating androgens.

Although the three classes of ovarian steroids exhibited the same cyclic variation during the ovulatory cycle, it is probable that only progesterone and estrogens have a direct effect on LH release. Heald et al. (118) have shown that intramuscular injections of progesterone and of estrogen both increased pituitary LH levels, whereas testosterone was without effect. These variations in plasma progesterone and estrogen can be interpreted in terms of inhibition or stimulation of the release of pituitary LH and FSH.

If the early peaks of estrogen and gonadotrophin are set aside because of their dubiety, then the hypothesis need only consider a single release of each hormone. Even so, if one considers individually the two gonadotrophins and the two ovarian steroids, there are eight possible stimulating or inhibiting feedback effects of estrogen and progesterone. As it is more likely that some synergism, both of action and effect, exists between the hormones, then there is at least a threefold increase in the number of possible inhibitory and stimulatory feedback effects. In this regard it has been shown recently that there is an

augmentative effect of FSH on LH-induced ovulation in the domestic hen (152). The inability to clarify which of these feedback mechanisms are operating physiologically clearly demonstrates the lack of knowledge in this field.

One may speculate that the primary stimulus for the onset of ovulation acts through some higher brain centre and activates the release of FSH-RF and subsequently the secretion of FSH from the anterior pituitary. This FSH is secreted about 14 to 11 hours before ovulation, activates the follicle and causes the increase in plasma levels of estrogen and/or progesterone. These, either singly or synergistically, inhibit further FSH secretion but stimulate ovulatory amounts of LH thus leading to ovulation. The increase in plasma LH inhibits the ovary and causes the rapid decline in the concentration of the ovarian steroids. This decline inhibits further LH secretion but removes the block to the secretion of FSH; and if there is no inhibition from the higher brain centres, a new ovulatory cycle begins.

This speculation does not consider more than one release of the hormones involved. Recent work on a possible role of the early LH peak demonstrated that ovulation was blocked in all the hens studied by phenobarbital sodium

injections 26 hours before the expected ovulation (281). The authors proposed that the drug blocked the early release of LH, but another explanation is that the drug blocked the mid-cycle release of FSH. It has also been suggested recently that not only are the two LH peaks implied in ovulation but that the second peak is stimulated by the development of the preceding ruptured follicle (82). This hypothesis fails because the ruptured follicle has no secretory activity. Until more specific and more detailed gonadotrophin assays are performed, the question as to the existence of one or two gonadotrophin peaks will remain unresolved.

Another factor which appears important in ovulation is the extensive innervation of the hen's follicle (59, 105). These nerves are both adrenergic and cholinergic and small bundles of fibres pass through the thecal layer (105) and have axon terminals in close contact with the thecal glands (59). As these thecal glands are known to be directly involved in steroidogenesis (58), these nerve fibres have been postulated as being involved in steroid synthesis, the rupture of the follicle, feedback to the central nervous system and the regulation of seasonal reproductive behaviour (59). Recent work has examined the effect of injecting anti-adrenergic drugs into the

follicular wall on subsequent ovulation (153). The results suggest that catecholamines may be involved in ovulation and that the blockade of ovulation by the anti-adrenergic drugs may be due to the inability of the nerves to release catecholamines. It was concluded that the adrenergic neurons in the hen's follicle are involved in ovulation and that the alpha receptor sites are the most prominently involved in this effect. It is of interest to note that this antiovulatory effect only acts prior to 14 hours before ovulation; if the drugs are injected some 12 to 13 hours before ovulation then ovulation occurs normally. The relationship between this neural factor and the hormonal variations in the control of ovulation is entirely unknown.

In order to resolve the mechanisms involved in the ovulation of the hen's follicle, one must determine the exact causal relationships between the ovarian steroids and gonadotrophin release. The elucidation of these relationships depends ultimately upon the simultaneous measurement of the plasma hormones involved during the ovulatory cycle. The direct autonomic innervation of the thecal glands and the resultant possibility that steroidogenesis may be under both hormonal and neurogenic control must also be considered. Until detailed studies on these topics are

done, one can only speculate as to the precise mechanisms of ovulatory control. Moreover, it is most likely that the observed phenomena of ovarian steroid variation demonstrated in this thesis only represents the "fine-tuning" of ovulatory control and that the ultimate mechanism for regulating ovulation resides in the higher brain centres and involves the entraining of external stimuli upon a genetically determined endogenous circadian rhythm.

PART III

GENERAL DISCUSSION

SUMMARY

1. The concentration of progesterone, determined by competitive protein-binding (CPB), in the peripheral blood plasma of laying hens attained a peak value four to six hours before ovulation. A peak value was not observed on days when ovulation did not occur, that is, on days when the terminal egg of a sequence was laid.

For nine ovulatory cycles the average progesterone level, uncorrected for procedural losses, was 2.45 ng/ml with a range of 0.5 to 8.8 ng/ml. For five periods covering a day of no ovulation the average was 2.95 ng/ml with a range of 0.5 to 12.5 ng/ml.

2. Estradiol and estrone concentrations were measured in peripheral plasma of laying hens by both CPB and radioimmunoassay (RIA). The bulk of the experimental work was performed using RIA although there was no difference between estrogen concentration measured by the two methods. Evidence was obtained that the estradiol measured by RIA was essentially estradiol-17β.

For 13 ovulatory cycles the average concentration of estradiol was 123 pg/ml with a range of 36 to 284 pg/ml. The corresponding average concentration for estrone was

64 pg/ml with a range of 14 to 138 pg/ml. For 12 days of no ovulation the average estradiol concentration was 142 pg/ml with a range of 42 to 364 pg/ml. The corresponding average value for estrone was 91 pg/ml with a range of 18 to 242 pg/ml. All these estrogen concentrations are uncorrected for procedural losses.

The results were interpreted as demonstrating the existence of peak levels of plasma estrone and estradiol at from six to two hours before ovulation, with the possibility of an earlier peak of both estrogens some 18 to 22 hours prior to ovulation. Such peak values were not demonstrable on days when ovulation did not occur.

3. The concentrations of androstenedione, determined by CPB and total androgens, determined by RIA, were measured in the peripheral plasma of laying hens. Both androstenedione and total androgens attained peak values at from eight to two hours before ovulation. Peak values were not observed on days when the terminal egg of a sequence was laid, nor when oviposition failed ot occur during the experimental period.

For 12 ovulatory cycles the total androgen concentration, in testosterone equivalents, ranged from 125 to 1100 pg/ml. The corresponding values for androstenedione ranged from

98 to 954 pg/ml. For six days where ovulation did not accompany oviposition, the total androgen concentrations ranged from 150 to 900 pg/ml. The corresponding values for androstenedione ranged from 105 to 879 pg/ml. For six days where oviposition failed to occur during the experimental period the concentrations of total androgen and androstenedione ranged from 200 to 800 pg/ml and 118 to 905 pg/ml, respectively. The above androgen concentrations have not been corrected for procedural losses.

CLAIMS TO ORIGINAL RESEARCH

To the best of the author's knowledge, the following are the claims to original research.

1. The first reported application of CPB to the measurement of the progesterone concentrations in peripheral plasma of laying hens.

2. The first reported observation that the progesterone concentration in the peripheral plasma of ovulating hens displays a cyclic variation with peak values occurring at from six to four hours before ovulation; and that such peak values do not occur on days of no ovulation.

3. The first reported measurement of the estrone and estradiol concentrations in the peripheral plasma of laying hens by both CPB and RIA.

4. The first reported observation that the plasma levels of estrone and estradiol in ovulating hens display a cyclic variation with peak concentrations of both estrogens occurring at from six to two hours before ovulation; and that such peak values are not present when ovulation does not occur.

5. The first reported application of RIA to measure the androgen concentration in the peripheral plasma of laying hens.

6. The first reported application of CPB to assay androstenedione in the peripheral plasma of laying hens.

7. The first reported observation that the concentration of total androgen in the peripheral plasma exhibits a cyclic variation, with maximum concentrations occurring at 8 to 2 hours before ovulation; and that such peak values do not occur on days of no ovulation.

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