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The Biosynthesis of Estrogens and Androgens
by the
Developing Chicken (Gallus gallus) Embryo

by

Derryn V. Gill BSc.

A thesis submitted to the Faculty of Graduate Studies
and Research in partial fulfilment of the requirements
for the degree of Doctor of Philosophy.

Carleton University
Ottawa, Ontario
February, 1980

The undersigned hereby recommend to the Faculty of Graduate Studies and Research acceptance of this thesis, submitted by Derryn V. Gill, BSc., in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Celite column chromatography was combined with a sensitive radioimmunoassay to fractionate and quantitate the reproductive steroids present in the allantoic and amniotic fluids of the chicken embryo. The estrogens, estrone, 17β -estradiol, and 17α -estradiol, were found to be present only as conjugates in the allantoic fluid of the female. Neither conjugated nor unconjugated estrogens were detectable in the allantoic fluid of the male embryo. Testosterone and dihydrotestosterone were present only as conjugates in the allantoic fluid of both male and female embryos. No estrogen or androgen, either conjugated or unconjugated, was detectable in the amniotic fluid of either sex. 17β -estradiol glucuronide, the most abundant estrogen in the female allantoic fluid, was first detectable at stage 35 (159 pg/ml) and the concentration increased throughout development to 4210 pg/ml at stage 45. In both the male and the female, testosterone sulfate was the predominant androgen throughout development. The concentration of testosterone sulfate increased from 189 to 1691 pg/ml between stages 36 and 44 in the female. Between stages 37 and 44, the concentration of testosterone sulfate in the male increased from 235 to 1266 pg/ml.

These steroids were synthesized by the embryo as the transfer of steroids from the egg yolk to the embryonic fluids was not significant. Embryonic liver tissue, cultured with tritiated steroids demonstrated its ability to conjugate steroids both as sulfates and glucuronides.

The concentrations of steroid in the embryonic

fluids of the partially decapitated (hypophysectomized) embryo were not significantly different from those of the intact stage 41 embryo.

FORWARD

My project, as my thesis title suggests, was to study the production of sex steroids by developing chicken embryos. The project was conceived during the time that I worked for Agriculture Canada. As Dr. Robertson's technician, I worked at quantitating estrogens and progestins in fetal and maternal plasma in domestic animals. A mammalian fetus must produce hormones that effect not only its own development but also convey chemical messages to the maternal system. The transport of these hormones across the placenta may be facilitated by the production of water soluble steroid conjugates. I felt at the time that it would be interesting to study a self-contained system, such as the avian egg, where, presumably, the steroids and steroid metabolites produced are solely necessary for the development of the embryo. The egg of the white leghorn chicken was chosen for the project as the development of white leghorn embryos has been closely studied in many areas and a detailed scheme of developmental stages has been drawn up so that the results of different studies can be more easily correlated.

In the avian egg, before you can be certain that you are measuring steroids that are actually synthesized de novo by the embryo, you must be certain that there is no large depot of stored maternal steroids from which the embryo can draw. This became the premise of my original line of attack on the project. I planned to quantitate the steroids present in the fertile, unincubated egg yolk and then compare these results to the results obtained by analysing the homogenized whole egg contents as the embryo developed. I felt that a sensitive radioimmunoassay could be used to estimate the small quantities of steroid expected in the egg yolk

if an extraction method could be developed that would produce clean steroid extracts. This proved to be an almost insurmountable task and it was therefore felt that assaying whole egg contents over the developmental period would not be practical.

The focus of the study then turned to assaying the steroids in the embryonic fluids; plasma, amniotic fluid and allantoic fluid. Of these, allantoic fluid proved to be the most readily accessible over the developmental period stage 35 to 45. Thus the major thrust of the study became the analysis of steroids in the allantoic fluid of the intact chicken embryo.

INTRODUCTION

An abundance of evidence exists to show that the gonads of the mature domestic fowl produce steroids. Woods and Domm (1966) using a direct histological visualization technique showed that the testis and both the left and right ovaries of the light-brown Leghorn fowl contain Δ^5 - 3β -hydroxysteroid dehydrogenase, a key enzyme in steroid hormone biogenesis. The plasma of immature female chickens has irregular peaks of estrogen concentration that suggest a circadian rhythmicity varying from 188 to 1190 pg/ml of plasma (Yu et al, 1974). Peterson and Common (1972) using a radioimmunoassay to assess the steroid concentration of laying hen plasma showed peak plasma concentrations of 88 pg/ml and 180 pg/ml for estrone and estradiol respectively between 2 and 6 h before ovulation. The time of this estrogen peak has been confirmed by Shahabi et al (1975). Radioimmunoassay was also used to estimate the total androgen concentration, expressed in testosterone equivalents, in laying hen plasma. The total androgen concentration reached a peak of 200 to 500 pg at 2 to 8 h before ovulation (Peterson et al, 1973; Shahabi et al, 1975).

As the peaks of plasma steroid concentration in the laying hen coincide with a time of rapid synthesis and laying down of yellow yolk components in the ovarian follicle and as steroid hormones have a chemical structure that is similar to cholesterol which is abundant in both plasma and yolk, it has long been thought that the egg yolk should contain measurable quantities of steroids. Using a thin layer chromatography procedure capable of detecting the presence of an individual steroid estrogen as low as

0.33 ug per yolk, Hertelendy and Common (1965) were unable to detect estrogen in fertile or infertile egg yolks. Arcos (1972), by injecting radiolabeled estrogens intravenously into hens, demonstrated that circulating steroids are transferred into the egg yolk. Using an endogenous estradiol concentration in the hen plasma of 300 pg/ml and assuming the transfer rates that she found to be representative and steady state, she calculated a concentration of 18 pg estradiol per egg yolk.

The early embryonic chicken cell is capable of ingesting droplets of yolk. Later in development the yolk sac endodermal enzymes are capable of digesting the yolk's contents and causing the products to be transported through the circulatory system. Thus the chicken embryo has a potential reservoir of steroids in the egg yolk.

An ever increasing volume of evidence shows that the chicken embryo is capable of endogenous production of steroids. Since 1966, many of these studies have involved the use of radioisotope labeled precursors cultured in vitro with embryonic gonadal tissue. Cedard and Haffen (1966) incubated day 7 and 10 embryos for 3 days with (^{14}C)-dehydroepiandrosterone and found that from this precursor embryonic ovaries synthesized estrone and estradiol while embryonic testes produced testosterone. Weniger et al (1967) cultivated day 7 embryonic ovaries with sodium acetate for 24 h and demonstrated the production of estrone and estradiol. They repeated this experiment with duck embryos showing that day 12 embryonic ovaries cultured for 24 h with (^{14}C)-sodium acetate produced estrone and estradiol (Akram and Weniger, 1967). Similarly, day 14 embryonic chicken right ovaries cultured for 24 h with sodium acetate were capable of synthesizing estrone, estradiol, estriol and epi-estriol (Weniger, 1968a). Weniger

(1968b) cultured undifferentiated embryonic day 5 and 6 gonads and day 7 testes and ovaries with (^{14}C) -progesterone. The day 6 gonads and day 7 ovaries produced measurable quantities of estrone and estradiol, while the day 5 gonads and day 7 testes did not. Akram and Weniger (1969) demonstrated that embryonic duck ovaries and feminized embryonic chicken testes secrete estrone and estradiol when cultured with sodium acetate. Repeating his earlier work, Weniger (1969) found that day 5 undifferentiated gonads could form estrone and estradiol from progesterone. Both the right and left day 7 embryonic chicken ovary when cultured with sodium acetate were capable of producing estrone, estradiol, estriol and epi-estriol. Ten, fourteen and nineteen day embryonic testes were cultured with sodium acetate but in no case was testosterone identified in the culture medium.

Cedard et al (1970), using both progesterone and pregnenolone as radioisotope labeled precursors, found that both the embryonic testes and ovaries were capable of producing testosterone between day 7.5 and 18. The production of labeled testosterone by the testes was greater than the production by the ovaries and increased with time. The ovarian testosterone production remained relatively constant from day 7.5 to 18 which led the authors to suggest that testosterone was produced as an intermediate in the estrogen biosynthetic pathway.

Weniger (1971) used (^{14}C) -sodium acetate as a precursor for day 4, 5 and 6 undifferentiated embryonic gonads and progesterone as the precursor with day 4 gonads and found that under these conditions only the day 6 undifferentiated gonads were capable of synthesizing estrogen. His conclusion that estrogens

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are not produced by the gonads before day 6 may be questioned as he considerably shortened the incubation times used for culture of the younger gonads and his recrystallization system may not have been sensitive enough to pick up the smaller amounts of estrogens produced.

The biosynthetic pathways of steroid synthesis were further clarified by the work of Galli and Wasserman (1972, 1973). Using radioisotope labeled progesterone as a precursor, day 7, 10 and 15 embryonic gonads were cultured for 30 min. 20α -dihydroprogesterone, 20β -dihydrotestosterone, 17α -hydroxyprogesterone, androstenedione and testosterone were synthesized by the left ovary and the testes at all three ages studied. The ovary also produced 17β -estradiol and estrone. Between day 7 and 10 the testes and left ovary showed an important increase in steroidogenic capability. During this interval the major progesterone metabolite changed from 20β -dihydroprogesterone to 17α -hydroxyprogesterone, suggesting that a shift in enzymatic activity occurred at this time. (See Appendix D).

Guichard, Cedard and Haffen (1973) incubated embryonic gonads at a number of different ages with a variety of radioisotope labeled precursors. They demonstrated that embryonic gonads were capable of producing estrogens and androgens from sodium acetate.

In 1977 Guichard et al. extended their work by using radioimmunoassay to measure the amount of steroid produced by cultured embryonic chicken gonads. They found that total steroid production increased from day 7 to 18. The female gonadal explants produced relatively more estrogen than androgen, while with the testes explants the opposite was true. Estradiol,

testosterone and dihydrotestosterone were found mainly in the culture media, whereas progesterone, dehydroepiandrosterone and estrone remained in the gonadal tissue. This suggests that the former group are secretory products, while the latter group are biosynthetic intermediates.

Teng and Teng (1977) used radioimmunoassay to show that at day 15 the gonads were major producers of estrogens and androgens. The adrenal gland and kidney produced trace amounts, but no detectable amounts were found in the liver. The estradiol production by the embryonic left ovary increased from 50 to 252 pg/ovary during culture between day 8 and 18.

Another approach to examining the endogenous production of steroids by the embryonic chicken gonad is to establish the presence of 3 β -hydroxysteroid dehydrogenase. Narbaitz and Kolodny (1964) could not identify hydroxysteroid dehydrogenase in the tissue of undifferentiated gonads before day 8 of incubation. By day 9 the enzyme was present in all the ovaries tested. Sheib and Haffen (1967) studied gonads between day 6 and 8 and found that hydroxysteroid dehydrogenase was present in the tissue of both ovaries and testes at day 6.5. The amount of enzyme in both tissues increased by day 8.5. Domm and Ericson (1972), using a histochemical technique for detection of 3 β -hydroxysteroid dehydrogenase reported an increase in enzymatic activity in the embryonic right ovary between day 7 and 10.

Of course, the most conclusive way to demonstrate endogenous steroid production is to directly measure the steroid in the tissues of the chicken embryo. Woods and Podczaski (1974) have approached this by using a fluorescent antibody method that demonstrated the

presence of androgens in the cells of the undifferentiated embryonic gonad at day 3.5. The amount of histochemically demonstrable androgen in the gonads rises throughout incubation. By day 19.5, the embryonic testes contain twice the amount of androgen present in the left ovary. Woods and Erton (1978) using an immunocytochemical technique were able to demonstrate the presence of estrogens in the undifferentiated embryonic gonad at day 3.5. The intensity of specific fluorescence was measured to give results that suggest that the estrone concentration in the left ovary and testis increased rapidly until day 13.5 and then levelled off until hatching. The concentration of estradiol in ovarian tissue increased steadily from day 3.5 to day 19.5. However, the testicular estradiol concentration did not increase above the day 3.5 level. These results concur with those of Guichard et al (1977) which suggest that estrone, more than estradiol, is retained in the gonadal tissue.

Using fluorometric methods, Gallien and Le Foulgoc (1957) demonstrated measurable amounts of estrogen in extracts of left ovarian tissue. The concentration increased from 45 to 145 to 680 ng/ovary at day 10, 13 and 21, respectively.

Also using a fluorometric method, Ozon (1965) measured the unconjugated and conjugated estrogen content of embryonic chicken blood, allantoic fluid and amniotic fluid. In day 21 blood, he found 14 ng/ml estrone, 8.2 ng/ml estradiol and 45 ng/ml estriol. The day 13 allantoic fluid contained between 0.2 and 0.5 ng/ml of each of estrone, estradiol and estriol and their conjugates. Amniotic fluid at day 10 was found to contain approximately 8 ng/ml each of estrone and estrone conjugates, 7 ng/ml each of estradiol and estradiol conjugates and 19 ng/ml of estriol conjugates.

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Kalliecharan and Hall (1974) quantitated the progesterone in embryonic chicken plasma using a competitive protein binding assay. The concentration of progesterone, shown by Guichard et al (1973) to be an important precursor in estrogen and androgen biosynthesis, increased from a low of 2.5 ng/ml at day 9 to a peak of 13.2 ng/ml at day 20.

Woods et al (1975) using a radioimmunoassay technique found that testosterone was detectable in male embryonic chicken plasma at day 7.5 and peaked at a concentration of 281 pg/ml at day 13.5; after which it declined. The testosterone concentration in female embryonic plasma was also detectable at day 7.5 and it rose to a peak of 116 pg/ml at day 15.5, after which it declined dramatically. No dihydrotestosterone was detectable in these samples.

Thus it would seem that the embryonic chicken gonad is capable of synthesizing estrogens and androgens in vitro and in vivo. The extent to which this synthesis is dependent upon gonadotropin regulation by the adenohypophysis is still under investigation.

The effect of exogenous gonadotropins on embryonic gonadal steroid synthesis has been studied using radioisotope labeled precursor experiments. Haffen et al. (1969) demonstrated that human chorionic gonadotropin augments the production of cholesterol from sodium acetate in day 10 gonads of both sexes and in day 18 testes. Cedard et al. (1968) showed that human chorionic gonadotropin increased the production of estrogen by embryonic ovaries supplied with sodium acetate or dehydroepiandrosterone at day 10 or 18.

The production of both estradiol and testosterone

in the embryonic ovary from day 8 to 18 was increased by the addition of human chorionic gonadotropin (20 i.u./ml) to the culture medium (Teng and Teng, 1977). This stimulatory effect of human chorionic gonadotropin appeared to be mediated through cyclic AMP.

Although human chorionic gonadotropin appears to have a dramatic effect on gonadal steroidogenesis in vitro, this effect may in no way mimic the in vivo regulation of steroid synthesis. The inter-species effect of human chorionic gonadotropin may be different from those of any endogenous gonadotropins which may be present.

One technique used to monitor the effect of the suspected endogenous gonadotropin is ablation of the gonadotropin-secreting tissue, the adenohypophysis. The preferred method for accomplishing adenohypophysectomy is partial decapitation (Betz, 1975). It should be noted that this also removes the source of neurosecretions such as releasing factors.

Partial decapitation at 33 to 38 h of incubation has been shown to modify the development of the embryonic gonads (Fugo, 1940; Vogel, 1957). Although differentiation into recognizable testes or ovaries is not dependent upon the presence of the pituitary gland, the gonads that develop are smaller and less dense than those of intact embryos after day 13.

Woods and Weeks (1969) showed that the concentration of histochemically demonstrable hydroxysteroid dehydrogenase in both the ovaries and the testes of partially decapitated chicken embryos was reduced by as much as 70% when compared to the concentration in normal embryonic gonads after day 13.5. Adenohypophyseal

transplants to the chorioallantoic membrane of the hypophysectomized embryos restored gonadal enzyme activity to that characteristic of intact embryos of the same age.

Vogel (1957) observed that in the hypophysectomized chicken embryo the free tissue cholesterol content per unit weight of ovaries and testes is decreased. As cholesterol is a precursor of sex hormones, he suggested that hormone production may be reduced in hypophyseoprivic embryos.

Akram and Weniger (1969) cultured ovaries and testes of day 18 partially decapitated embryos in combination with normal embryonic chicken Mullerian duct and testes respectively. The Mullerian ducts regressed and the testes underwent feminization indicating that the gonads of hypophysectomized chicken embryos possess some steroidogenic activity that is independent of pituitary control.

Akram et al (1972) cultured the day 16.5-left ovaries of partially decapitated embryos with (^{14}C)-sodium acetate. Following chromatographic separation, it was shown that the ovaries of the hypophyseoprivic embryos synthesized estrone and estradiol as do the ovaries of normal embryos. When the experiment was repeated with hypophysectomized day 24 duck embryos, the results show that hypophysectomy depressed estradiol synthesis nearly completely and reduced estrone synthesis markedly. The difference between the results of these two experiments suggests that further work needs to be done.

The object of the present study was to clarify some of the contradictions posed by the earlier work on steroidogenesis by the intact and hypophyseoprivic

chicken embryo. A comprehensive study of the production of the active gonadal steroids and their metabolites throughout embryonic development was planned.

MATERIALS AND METHODS

1. Animals

A) Experimental Animals

Fertilized White Leghorn eggs (McEwen Farm, North Gower, Ontario; strain 934 F) were incubated at $38^{\circ} \pm 0.5^{\circ} \text{C}$ and $60 \pm 5\%$ relative humidity. When appropriate, hypophysectomy of the embryo was carried out by the partial decapitation procedure described by Betz (1975).

B) Collection and Treatment of Samples

Samples of allantoic fluid and amniotic fluid were collected from the embryos at Hamburger and Hamilton (1951) stage 34 to stage 45, inclusive. Allantoic fluid was collected, using a syringe fitted with a 20 gauge needle, through the membranes beneath the air space. Amniotic fluid was likewise collected through the amniotic membrane after the other membranes had been opened up. Samples were immediately placed on ice and subsequently stored at -20°C . The embryo was examined for sex and Hamburger and Hamilton (1951) stage of development. See Appendix C for the relationship between the stage of development and the days of incubation.

11. Analytical Procedures

A) Radioactive Materials

(2,4,6,7- ^3H) estrone (98.5 Ci/mM), (2,4,6,7- ^3H) 17 β -estradiol (91.3 Ci/mM), (6,7- ^3H) estrone sulfate, ammonium salt (47.9 Ci/mM), (6,7- ^3H) 17 β -estradiol glucuronide (40 Ci/mM), (1,2,6,7- ^3H) testosterone (95.2 Ci/mM), (1,2- ^3H) testosterone sulfate (45.2 Ci/mM), (1,2- ^3H) testosterone glucuronide (50 Ci/mM) and (1,2,4,5,6,7- ^3H) dihydrotestosterone (1130 Ci/mM) were obtained from New England Nuclear (Lachine, Quebec). Estrone glucuronide (7.59×10^6 dpm/M) was kindly provided by Dr. D. G. Williamson (University of

Ottawa). All were stored in benzene-methanol at 4°C . The necessary dilutions were made with methanol and kept at -20°C .

B) Nonradioactive Steroids

Nonradioactive crystalline estrogens and androgens (Steraloids, Inc., Pawling, N.Y.) were checked for purity by thin layer chromatography. Standard solutions were prepared in methanol and stored at -20°C .

C) Solvents

All solvents, benzene, tetrahydrofuran, ethyl acetate, iso-octane (2,2,4-trimethylpentane), propylene glycol, hexane and ethylene glycol (Matheson, Coleman and Bell, Mississauga, Ontario) were spectroquality.

D) Other Chemicals and Solutions

Celite 545 (Johns Manville, Montreal, Quebec), an analytical filter aid, was heated in a muffle furnace at 600°C for 18 h to remove any organic contaminants prior to use. Celite is a diatomaceous earth product.

The charcoal (norit A) was obtained from Matheson, Coleman and Bell (Mississauga, Ontario) and the Dextran (T80) from Pharmacia (Dorval, Quebec).

The phosphate buffer, (buffer A, pH 7.0) used throughout the estrogen assay was prepared in the following manner; 2.17 g/l $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.72 g/l $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$.

The bicarbonate solution used in the extraction of sulfates was prepared in the following manner; 106 g/l NaHCO_3 . The solution was warmed and NaCl was added to saturation. The solution was cooled to room temperature before use.

E) Estrogen Antibody

Dr. B. V. Caldwell (Yale University) kindly provided the estrogen antibody used in this research (sheep antiserum to 17β -estradiol hemisuccinyl bovine

serum albumin; no. 029-18). This antibody also cross-reacted with estrone (65-70%). It was diluted 1:100 in buffer A and stored at -20°C . For use it was further diluted 1:300 with the same buffer; a working dilution of 1:30,000.

F) Testosterone Antibody

Freeze dried testosterone antisera was purchased from New England Nuclear (Lachine, Quebec). The dry antisera was stored at 4°C . The contents of one vial of antisera was reconstituted at the time of use with 10 ml of buffer A. This antisera cross-reacted with dihydrotestosterone (100%).

G) Treatment of Glass

All glassware was washed thoroughly in detergent (Decon-75 concentrate, BDH) and rinsed many times in tap water, distilled water and methanol. The columns, culture tubes and pasteur pipettes used were siliconized prior to use to discourage adsorbance of the estrogens and androgens. Since the glassware used for the collection and storage of fluids was not siliconized, there could have been some undetermined loss of steroids during this stage of the experimental procedure.

H) Liquid Scintillation Counting

The scintillation fluid contained 100 g naphthalene and 8 g Omnifluor (98% PPO and 2% Bis-MSB, New England Nuclear, Lachine, Quebec, /1 1,4 dioxane). A Beckman (Etobicoke, Ontario) LS-250 scintillation spectrometer with a counting efficiency of 40% for (^3H) was used to measure the radioactivity in each sample. It counted the internal standards to an error of $\pm 3\%$ and the assay samples to $\pm 2\%$.

I) Radioimmunoassay

Berson and Yalow (1958) were the first to point out the remarkable sensitivity possible when unlabeled antigens are measured by their ability to inhibit competitively the binding of radioactive antigens by

antibodies. Most radioimmunoassays use radioactive isotope labeled antigens and depend on the ability of unlabeled antigen (Ag) to inhibit the binding of labeled antigen (Ag*) when limited amounts of specific antibody (Ab) are present. This process may be viewed as a simple competition in which Ag reduces the amount of free Ab thus decreasing the availability of Ab to Ag*.

In performing the assay Ag* and Ab are incubated in the presence and absence of samples containing Ag. After reaching or approaching equilibrium, free Ag* and antibody-bound Ag* are separated and one or the other is determined by radioactive counting. The antigen concentration in the unknown is measured by comparing the diminution of Ag* binding produced by Ag in the sample to that of a standard curve obtained by adding graded, known amounts of Ag to the assay system (Fig. 1).

Few other assay procedures provide the combination of sensitivity, specificity, versatility and convenience that immunoassays offer. However, immunoassays can present serious problems in development and can be misused, thus yielding inadequate or misleading information.

The basis of a convenient radioimmunoassay is a highly sensitive, high-affinity antibody. A high-affinity antibody forms a relatively stable complex with antigen, with limited dissociation of the bond under the conditions of the assay. Quantitatively this situation is reflected by a high value for the association constant (K_a).

$$K_a = \frac{(Ab \cdot Ag)}{(Ab)(Ag)}$$

where: K_a = association constant

Ab = molar concentration of antibody

Ag = molar concentration of antigen

$Ab \cdot Ag$ = molar concentration of
antibody-antigen complex

The highest affinity antibodies are obtained to large structurally complex, hydrophobic haptens. To stimulate antibody production, steroids must be conjugated to large protein molecules. Conjugation of the molecule through the region that is least important for immunological recognition is important if antisera of maximal specificity are to be obtained. Useful antisera should be as free as possible from immunologic cross-reactivity. Cross-reactivity is evaluated by running simultaneous, separate standard curves with the homologous and cross-reacting antigens. Unexpected cross-reactions are a potential source of error in radioimmunoassay.

Apparent cross-reactivity may, in fact, be due to antibody heterogeneity which may be caused in part by the use of non-homogenous antigens for immunization and in part by individual differences in the way lymphoid cells perceive and respond to antigenic stimulus.

The sensitivity of the assay is directly proportional to the association constant. Under conditions in which 50% of the total antigen is antibody bound; $Ab \cdot Ag = Ag$, and the equation reduces to

$$K_a = \frac{1}{(Ab)}$$

In other words, the concentration of free antibody that must be maintained in order to bind 50% of the antigen is inversely proportional to K_a . As an initial approximation, the K_a of the antibody determines the maximal dilution of antiserum that can be used to obtain adequate binding of the marker. If a high concentration of antibody is needed, the ability of small quantities of unlabeled antigen to compete with labeled antigen for antibody is decreased, thereby,

lowering the sensitivity of the assay. Thus it is desirable to conduct the assay at very low concentrations of antibody.

In general the association constant increases as the temperature is lowered and antigen binding is maximal at or near 4° C. Due to the low temperature and the low concentration of antibody, the approach to equilibrium is slowed because of the decreased frequency of collision between antigen and antibody. Thus sufficient time must be allowed for the attainment of equilibrium.

111. Methods

A). Estrogen Radioimmunoassay

1) Unconjugated estrogens

The unconjugated estrogens, estrone and 17 β -estradiol were measured in embryonic fluids using the method described by Robertson, Smeaton and Durnford (1972) with some modifications where necessary.

The volume of fluid extracted ranged from 0.5 to 3.0 ml depending upon the steroid concentration present. At the earlier stages it was necessary to run pooled samples. Where the original fluid sample was of sufficient volume, all samples were run in duplicate. To each sample was added approximately 1,000 cpm each of (³H)estrone and (³H)17 β -estradiol (in the form of 4 or 5 pg of either steroid) which was later used to estimate recovery. Each sample was extracted with 10 volumes (at least 8 ml) of benzene for 2 min and centrifuged at 1400 rpm for 5 min. Since it was found that 90-95% of the estrone and estradiol was recovered in a single extraction with benzene from embryonic fluid without adding 0.1 ml of 0.1 N NaOH, this step was omitted. The benzene extracts were transferred, along with 1 ml benzene rinse of the extraction tube, to siliconized culture tubes and the contents were evaporated under nitrogen in a water bath at 40° C. The dried extracts

were dissolved in 0.5 ml iso-octane for transfer to celite columns.

Celite was thoroughly mixed with ethylene glycol (2:1, w:v) and dry-packed using vacuum suction and a glass ramping rod in a 5 ml disposable pipette to a height of 4 cm. A glass bead held the contents in each column. Usually 24 columns were prepared and run simultaneously.

The columns were washed twice with 5 ml iso-octane and the sample applied to each in 1.0 ml iso-octane. They were rinsed with 3.5 ml iso-octane. Estrone was eluted using 4.0 ml 15% ethyl acetate in iso-octane. 17 β -estradiol was eluted with 4.5 ml 30% ethyl acetate in iso-octane.

The column eluates were evaporated under nitrogen and 1.1 ml buffer A added. The tubes were mixed, allowed to stand at room temperature for 1 h, mixed again and 0.5 ml transferred to scintillation counting vials for the determination of recovery. 0.5 ml of the buffer A remaining in each culture tube was transferred to another siliconized culture tube. To this was added 0.1 ml of (³H)17 β -estradiol (about 12,000 cpm) in buffer A and 0.1 ml of a 1/30,000 dilution of the antiserum. The standard curves for estrone and 17 β -estradiol were obtained from tubes containing 0 to 200 pg of either estrone or 17 β -estradiol which underwent the same incubation procedure as the samples.

Incubation was carried out overnight at 4° C. A solution of 0.5% charcoal and 0.05% dextran in buffer A was used to separate unbound estrogen from bound. A 0.5 ml aliquot of this solution was added to each tube, which was mixed and left for 10 min in an ice bath. The tubes were then centrifuged at 4° C for 2 min to remove the charcoal-steroid complex. The supernatant, containing the antibody bound steroid, was transferred to a scintillation vial and the radioactivity determined.

2) Estrogen sulfate

Following the benzene extraction of unconjugated estrogens, conjugated estrogens, in the form of estrogen sulfate and 17 β -estradiol sulfate were measured in the embryonic fluids using the methods of Robertson and King (1974). The method was used originally only for the measurement of estrone sulfate, however, it was expanded in this work to obtain estimates of the concentration of estrone sulfate and 17 β -estradiol sulfate.

Hence, in addition to the internal standards for estrone and 17 β -estradiol, about 1500 cpm of (^3H) estrone sulfate were added to each sample prior to extraction. Since only a limited amount of radioactively labeled 17 β -estradiol was available, an internal standard for this steroid could not be routinely included.

After the unconjugated estrogens had been removed, 10 volumes (not less than 12 ml) of a 1:1 (v:v) solution of tetrahydrofuran and ethyl acetate were used to extract the estrogen sulfates from each sample. The tubes were mixed for 2 min, 2.0 ml of 1.0 M NaHCO_3 saturated with NaCl were added, the tubes were mixed again and centrifuged for 15 min at 1400 rpm. The tetrahydrofuran-ethyl acetate (THF-EA) extract was transferred to 15 ml tubes and evaporated under nitrogen. Hydrolysis was accomplished by adding 1.0 ml of 10% glacial acetic acid (in the 1:1 THF-EA mixture) to each tube and incubating overnight in a shaking water bath maintained at 50° C. The hydrolysis mixture was thoroughly dried down under nitrogen and the residue was redissolved in iso-octane (0.5 ml per sample). The preparation and running of the columns for separation of estrone and 17 β -estradiol obtained by the hydrolysis of the sulfo-conjugates were identical to that used for the benzene extracted estrogens. The rest of the assay was

carried out as previously described for the unconjugated estrogens.

3) Estrogen glucuronides

Conjugated estrogens, in the form of estrogen glucuronide and 17 β -estradiol glucuronide were hydrolysed in embryonic fluids shown to have no unconjugated estrogens using β -glucuronidase (Type 2, Sigma Chemical Co., St. Louis, Missouri). This β -glucuronidase was free of sulfatase activity under the conditions of the assay. After the addition of the fluid sample to the extraction tube, the phosphate buffer pH 6.8 (buffer G) was added to make a volume of 1 ml. The tubes were mixed and placed in a 37° C water bath for 5 min. To each tube 0.5 ml of the β -glucuronidase solution (2mg/ml buffer G) was added and the samples were incubated at 37° C for 4 h, shaking gently.

Each sample was extracted with 10 volumes of benzene and the rest of the assay was carried out as described for unconjugated estrogens.

4) 17 α -Estradiol

The concentration of 17 α -estradiol in the embryonic fluids was measured using the method of Robertson, Smeaton and Durnford (1972). An hydroxysteroid dehydrogenase preparation (Sigma Chemical Co., St. Louis, Missouri, grade 111) was used to selectively convert 17 β -estradiol to estrone, leaving the majority (95%) of the 17 α -estradiol unchanged.

The 30% ethyl acetate/iso-octane fraction, obtained from the celite column, was collected in a 45 ml stoppered centrifuge tube, dried down under nitrogen and the residue dissolved in 1.0 ml of sodium pyrophosphate buffer (0.034 M, pH 9.0). The solution was mixed and left at 37° C for 30 min to ensure that all the steroids present were dissolved. Then 0.2 ml of hydroxysteroid dehydrogenase solution (1.2 mg in 10 ml 0.03 M Tris buffer) was added, and the samples were

incubated at 37°C for 30 min in a gently shaking water bath. At the end of 30 min, the estrogens were extracted for 2 min with 10 ml of benzene and centrifuged for 10 min at 1400 rpm. The benzene extract was washed with water and evaporated under nitrogen. The rest of the assay was carried out as described for unconjugated estrogens with the addition of a 17 α -estradiol standard curve.

B) Androgen Radioimmunoassay

1) Unconjugated androgens

The unconjugated androgens, testosterone and dihydrotestosterone were measured in the embryonic fluids using the method employed in the laboratory of H. A. Robertson and celite columns modified from the method of Coyotupa, Parlow and Abraham (1972).

The volume of fluid extracted ranged from 0.5 to 3.0 ml depending upon the concentration of androgen present. At the earlier stages, it was necessary to run pooled samples. Where the sample was of sufficient volume, all samples were run in duplicate.

In order to estimate recovery, 1,500 cpm each of (^3H) testosterone and (^3H) dihydrotestosterone (in the form of 4 or 5 pg of either steroid) was added to each sample.

Each sample was extracted with 10 volumes (at least 8 ml) of benzene for 2 min and centrifuged at 1400 rpm for 5 min. The benzene extracts were transferred, along with 1 ml benzene rinse of the extraction tube, to siliconized culture tubes and the contents were evaporated under nitrogen. The dried extracts were solubilized in 0.5 ml iso-octane for transfer to celite columns.

Celite was thoroughly mixed in a 4:1:1 (w:v:v) ratio with ethylene glycol and propylene glycol and dry packed using vacuum suction and a glass tamping rod in a 5 ml disposable pipette to a height of 4 cm. A glass

bead held the contents in each column. Usually 24 columns were prepared and run simultaneously.

The columns were washed twice with 5 ml of iso-octane and a sample applied to each in 1.0 ml of iso-octane. They were then rinsed with 3.5 ml of iso-octane. Testosterone was eluted using 4.5 ml of 15% ethyl acetate in iso-octane. Dihydrotestosterone was eluted with 5.0 ml of 30% ethyl acetate in iso-octane.

The column eluates were evaporated under nitrogen and 1.1 ml buffer A added. The tubes were mixed, allowed to stand at room temperature for 1 h, mixed again and 0.5 ml transferred to scintillation counting vials for the determination of recovery. 0.5 ml of the buffer A remaining in each tube was transferred to another siliconized culture tube. To this was added 0.1 ml of (^3H)testosterone in buffer A (about 15,000 cpm per 0.1 ml) and 0.1 ml of the reconstituted antiserum. The standard curves for testosterone and dihydrotestosterone were obtained from tubes containing 0 to 200 pg of either testosterone or dihydrotestosterone which underwent the same incubation procedure as the samples.

Incubation was carried out overnight at 4° C and the assay was completed as for unconjugated estrogens.

The standard curve for dihydrotestosterone was obtained by displacing (^3H)testosterone.

2) Androgen sulfates

Following the benzene extraction of unconjugated androgens, conjugated androgens in the form of testosterone sulfate and dihydrotestosterone sulfate were extracted from the embryonic fluids using a procedure similar to that used for the estrogen sulfo-conjugates. The hydrolysis procedure used for the androgen sulfo-conjugates was a modification of the methods of Burstein and Lieberman (1959).

In addition to the internal standards for testosterone and dihydrotestosterone, about 1500 cpm of testosterone sulfate and dihydrotestosterone sulfate were added to each sample prior to extraction.

After the tetrahydrofuran-ethyl acetate (THF-EA) extract was dried down under nitrogen, hydrolysis was accomplished by adding 4 ml of ethyl acetate and 4 ml of acid solution (1 N H_2SO_4 ; 3 M NaCl). The tubes were shaken and incubated at 50° C in a shaking water bath for 20 h. The tubes were shaken again and then centrifuged for 5 min at 1400 rpm. The acid phase was pipetted off and the ethyl acetate phase washed with 1 ml of 1% $NaHCO_3$ and then 1 ml of distilled H_2O . The water layer was removed and the ethyl acetate fraction dried down under nitrogen. The residue was taken up in 0.5 ml of iso-octane for transfer to celite columns.

The separation on the celite columns and the assay were carried out as for the unconjugated androgens.

3) Androgen glucuronides

Conjugated androgens, in the form of testosterone glucuronide and dihydrotestosterone glucuronide, were hydrolysed in embryonic fluids shown to contain no unconjugated androgens using β -glucuronidase (Sigma Chemical Co., St. Louis, Missouri Type 2). This β -glucuronidase has no sulfatase activity under the conditions of the assay.

An internal standard of approximately 1500 cpm testosterone glucuronide and dihydrotestosterone glucuronide was dried under nitrogen in the extraction tubes before the addition of the sample.

The hydrolysis procedure used for androgen glucuronides was the same as that used for estrogen glucuronides. After hydrolysis, each sample was extracted with 10 volumes of benzene. The testosterone and dihydrotestosterone were separated on a celite column and the assay was carried out as for unconjugated

androgens.

C) Incubation of Tissues

1) Collection of tissues

Stage 42 embryos were removed from their shells and decapitated. The livers were excised, weighed, labeled and placed on ice as quickly as possible. The sex of the embryo was recorded.

2) Culture

Individual livers weighing approximately 300 mg were minced and placed in 25 ml Erlenmeyer flasks. Control flasks contained no tissue. To each flask 4 ml Medium 199 with 25mM Hepes buffer (Grand Island Biological Co., Grand Island, N.Y.) containing 250,000 cpm/ml of one steroid (estrone, estrone sulfate or testosterone) were added. The flasks were flushed with 95% O₂-5% CO₂, stoppered with cotton and incubated at 39° C in a gently shaking water bath for 3 h.

The contents of the flasks were homogenized and extracted with 20 ml of 1:1 THF-EA for 2 min. Two ml of 1 N NaHCO₃ saturated with NaCl were added and the samples were mixed and then centrifuged at 1400 rpm for 15 min. The solvent layer was evaporated under nitrogen and the residue was redissolved in 1.0 ml of methanol. From this a 0.25 ml aliquot was taken, dried down, redissolved in 2 to 3 drops of methanol and used to spot the thin layer chromatography plates.

3) Thin Layer Chromatography

Aluminum sheets precoated with silica gel (Brinkman Instruments Ltd., Toronto, Ontario) were spotted with the samples and radioactively labeled steroid standards and run in either of two solvent systems: (a) dichloromethane:ether, which was used to separate the unconjugated steroids and (b) 10:10:2 ethanol:ethyl acetate: ammonium hydroxide, which was used to separate the conjugated steroids. Each plate

was run twice with the solvent front moving 15 cm from the origin.

After the plates were dried, the radioactive peaks were scanned using a thin layer scanner (RTLS-1A by Panax Equipment Ltd., Redhill, Surrey, England).

D) Tritium Labeling Experiments

1) Injection of steroid

Unincubated eggs were wiped with 70% ethanol and a 1 mm diameter hole was punched through the shell over the air space. Through this hole approximately 100,000 cpm of steroid (estradiol, estrone sulfate or testosterone) in 0.001 ml ethanol was injected into the center of the yolk. The hole was covered with tape and the eggs incubated to stage 42.

2) Recovery of steroids

The allantoic fluid was collected quantitatively through the membranes under the air space using a sharpened pasteur pipette. The amniotic fluid was collected quantitatively through the amniotic membrane using a syringe. The volume of the fluids and the stage and sex of the embryo were recorded.

The unconjugated and conjugated estrogens and androgens were extracted and separated on celite columns as described in the radioimmunoassay method sections except that the fractions off the column were collected in scintillation vials. The column eluates were dried down, dissolved in scintillation cocktail and counted for radioactivity.

F) Calculations

1) Precision

The precision of the assay method was determined using the following formula (Gaddum, 1953).

$$\lambda = s/b \quad \text{where: } \lambda = \text{precision (pg)} \\ s = \text{standard error (pg)}$$

b = slope of the regression line

2) Sensitivity

The following formula was used to calculate the sensitivity of the assay method (Abraham, 1974).

$$S = \frac{2 \times S. D.}{R \times F} \quad \text{where } S = \text{sensitivity (pg)}$$

S. D. = mean standard deviation
of all blank values (pg)

R = recovery (%)

F = fraction of sample used
in assay

RESULTS

1. Analyses of the Methods

A) Standard Curves

Typical standard curves for the estrogens, estrone, 17 β -estradiol and 17 α -estradiol, are shown in Fig. 1 and for the androgens, testosterone and dihydro-testosterone, in Fig. 2.

B) Recovery of Internal Standard Steroid

1. Allantoic fluid and amniotic fluid

The mean recoveries of unconjugated estrogens throughout all of the assays were found to be: estrone - 70%, 17 β -estradiol - 76% and 17 α -estradiol - 73%. As it was found that 90-95% of each steroid was removed during the initial benzene extraction, the rest of the procedural losses were probably due to the transfer of the samples to and the removal of the eluates from the celite columns (Table 1).

The overall recovery of estrone sulfate from the fluids was found to be 61%. It was found that this could be broken down into a 75% recovery from THF:EA extraction and a subsequent 75-80% recovery from acid hydrolysis and separation on celite columns. The overall recovery of 17 β -estradiol sulfate was slightly lower than that of estrone sulfate (about 58%);

Fig. 1. The estrogen standard curve: (^3H)-17 β -estradiol (cpm) remaining bound to antibody as a function of estrone (▲), 17 β -estradiol (■) or 17 α -estradiol (●) added (pg). A 1/30,000 dilution of antibody was added.

Fig. 2. The androgen standard curve: (^3H)-testosterone (cpm) remaining bound to antibody as a function of testosterone (■) or dihydrotestosterone (●) added (pg).

Table 1Recovery of Internal Standards from Embryonic Fluids
in the Estrogen and Androgen Radioimmunoassay

<u>Steroid</u>	<u>% Recovery</u>	<u>Steroid</u>	<u>% Recovery</u>
E ₁ *	70	DHT *	67
17 β -E ₂	76	T	76
17 α -E ₂	73	DHTS	43
E ₁ S	61	TS	44
E ₂ S	58	DHTG	70
E ₁ G	61	TG	76
E ₂ G	63		

* The abbreviations used in this and all following tables are defined in Appendix B.

however, labeled 17 β -estradiol sulfate was not available to use routinely as an internal standard, therefore, the recovery of (^3H)estrone sulfate was used to estimate the procedural losses for both estrone sulfate and estradiol sulfate.

Using (^3H)estrone glucuronide, it was determined that only 9% of this compound was recovered using the extraction method for estrogen sulfates and of this 9%, no significant amount was hydrolysed by the treatment with 10% glacial acetic acid. However, the glucuronides were routinely hydrolysed and extracted with benzene from the sample before the sulfates were extracted.

The overall recoveries of estrogen glucuronides were found to be: estrone glucuronide - 61% and 17 β -estradiol glucuronide - 63%. As it was found that 80% of the steroid was recovered after hydrolysis and benzene extraction, the rest of the losses were probably incurred during the running of the celite columns.

Using (^3H)estrone sulfate, it was determined that only 3% of this compound was recovered after the hydrolysis and extraction method used for estrogen glucuronides.

The mean recoveries of unconjugated androgens in embryonic fluids were found to be: testosterone - 76% and dihydrotestosterone - 67%. The procedures that resulted in losses were the same as those for the estrogen assays.

The overall recoveries of androgen sulfates were found to be: testosterone sulfate - 44% and dihydrotestosterone sulfate - 43%. It was found that this could be broken down into a 73% recovery from the THF:EA extraction and a subsequent 60% recovery from the acid hydrolysis and separation on celite columns.

Using (^3H)testosterone glucuronide, it was

determined that only 2% of this compound was recovered using the extraction and hydrolysis method for androgen sulfates. However, the glucuronides were routinely hydrolyzed and extracted with benzene from the sample before the sulfates were extracted.

The overall recoveries of the androgen glucuronides were found to be: testosterone glucuronide - 76% and dihydrotestosterone glucuronide - 70%. As it was found that 83% of the steroid was recovered after hydrolysis and benzene extraction, the rest of the losses were probably incurred during the running of the celite columns. As dihydrotestosterone glucuronide was not available to be used routinely as an internal standard, the recovery of (^3H)testosterone glucuronide was used to calculate the procedural losses of both steroids.

Using (^3H)testosterone sulfate, it was determined that only 1% of this compound was recovered after the hydrolysis and extraction method used for androgen glucuronide.

C) Reagent Blank Values

Reagent blank values were determined by including in each assay two tubes containing only the dried internal standards which were taken through the whole procedure of extraction, separation on columns and incubation with antibody. These values were subtracted from the estimation for each sample as obtained from the standard curve before the corrections for the procedural losses were made. Examples of the blank values obtained are shown in Table 2.

D) Accuracy

The accuracy of the measurement was consistent over the entire experimental range. Known amounts (0, 50, 100 and 200 pg) of each of estrone, 17β -estradiol, estrone sulfate, 17β -estradiol sulfate, 17β -estradiol glucuronide, testosterone, dihydrotestosterone, testosterone sulfate, dihydrotestosterone sulfate, and

Table 2a

Reagent Blank Values in Estrogen Radioimmunoassay

	17 β -		17 β -		17 α -	17 β -		17 α -
	E ₁	E ₂	E ₁ S	E ₂ S	E ₂ S	E ₁ G	E ₂ G	E ₂ G
Mean Blank Value	3.3	2.7	2.9	7.3	7.3	4.5	4.7	8.0
(pg)								
Standard Deviation	2.3	2.5	3.4	4.1	2.0	4.1	3.5	4.6
(pg)								
N	15	15	15	15	6	15	15	6

Table 2b

Reagent Blank Values in Androgen Radioimmunoassays

Steroid	T	DHT	TS	DHTS	TG	DHTG
Mean Blank Value	12.8	3.2	13.1	6.3	18.1	6.5
(pg)						
Standard Deviation	3.7	6.8	6.5	5.2	5.9	3.1
(pg)						
N	10	10	15	15	15	15

testosterone glucuronide were added to non-cycling ewe plasma and carried through the entire procedure including correction for methodological losses as indicated by the recovery of internal standards. The results of two separate assays for each steroid are shown in Table 3. Regression equations were determined (Table 4). Correlation coefficients did not differ significantly from unity.

E) Precision

The precision with which the unconjugated and conjugated steroids could be measured was calculated using the same cold recovery assays as for the accuracy determinations. Measurements of precision were made at the 50, 100 and 200 pg levels for the unconjugated steroids and at the 50, 100 and 200 pg levels for the conjugated steroids (Table 5).

F) Sensitivity

The sensitivity of the method was calculated using the 0 to 100 pg standard curve. It was found to be: estrone - 14 pg, 17 β -estradiol - 15 pg, estrone sulfate - 25 pg, 17 β -estradiol sulfate - 31 pg, estrone glucuronide - 30 pg, 17 β -estradiol glucuronide - 25 pg, testosterone - 22 pg, dihydrotestosterone - 45 pg, testosterone sulfate - 66 pg, dihydrotestosterone sulfate - 54 pg, testosterone glucuronide - 35 pg and dihydrotestosterone glucuronide - 20 pg (Table 6).

11. Experimental Results

To compare the concentration of the steroids on an equivalent basis, the values for the sulfo-conjugates and the glucuronide conjugates are given in terms of the unconjugated steroid equivalent.

A) Estrogen Radioimmunoassay

(i) Allantoic Fluid

(1) Unconjugated estrogens

(a) Female embryo

The concentrations of both estrone and

Accuracy in Determining Estrogen Concentration

* All values are recorded as pg of free steroid.

Table 3b

Accuracy in Determining Androgen Concentrations

Amount added (pg)	T		DHT		TS		DHTS		TG		DHTG	
	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2	#1	#2
50	47.2	57.0	55.6	54.6	53.2	53.2	56.2	56.6	54.8	53.8	57.6	55.2
	4.7	6.8	6.9	5.1	3.3	7.5	4.6	3.5	9.3	6.8	6.6	6.0
100	92.4	99.8	106.0	109.0	105.4	113.0	116.0	112.2	98.4	100.0	118.0	111.0
	8.6	12.2	6.8	11.9	7.2	7.6	9.4	7.0	5.6	4.8	8.6	5.6
200	205.0	195.4	217.0	202.4	218.2	217.8	217.8	222.0	226.8	210.4	219.2	204.8
	15.5	17.0	2.1	16.8	9.1	13.9	3.0	6.7	21.0	13.6	10.6	10.6

* All values are recorded as pg of free steroid.

Table 4

Correlation Between Steroid Added and Steroid Measured
by Radioimmunoassay

	Regression Equation		Correlation Coeff.	
	Assay #1	Assay #2	Assay #1	Assay #2
E ₁	y = 1.02x + 1.90	y = 0.97x + 14.20	0.994	0.997
17 β -E ₂	y = 0.97x + 12.1	y = 0.91x + 17.90	0.993	0.985
E ₁ S	y = 0.95x + 17.3	y = 0.97x - 2.20	0.985	0.993
E ₂ S	y = 0.98x + 4.80	y = 1.03x - 2.80	0.988	0.995
E ₁ G	y = 1.03x + 4.10	y = 1.03x + 1.80	0.987	0.995
E ₂ G	y = 1.10x - 4.70	y = 1.04x - 1.40	0.996	0.994
17 α -E ₂	y = 1.03x - 0.90		0.990	
E ₂ -3S	y = 0.99x + 1.50		0.988	
T	y = 1.06x - 9.10	y = 0.93x + 9.20	0.989	0.981
DHT	y = 1.08x + 0.10	y = 0.98x + 7.90	0.997	0.984
TS	y = 1.11x - 3.40	y = 1.09x + 0.80	0.996	0.991
DHTS	y = 1.07x + 5.40	y = 1.10x + 1.70	0.996	0.997
TG	y = 1.17x + 9.40	y = 1.05x - 1.40	0.983	0.991
DHTG	y = 1.07x + 7.00	y = 0.99x + 8.30	0.992	0.993

Table 5a

Precision of Radioimmunoassay for Estrogens

Steroid.	Level of Estrogen (pg)	Intra-assay Precision (pg)		Coefficient of Variation (%)	
		Assay #1	Assay #2	Assay #1	Assay #2
E ₁	50	2.48	1.78	4.96	3.56
	100	2.39	1.84	2.39	1.84
	200	4.53	1.56	2.27	0.78
17 β -E ₂	50	3.39	2.64	6.78	5.38
	100	5.13	5.76	5.13	5.76
	200	1.84	4.10	0.92	2.05
E ₁ S	50	7.58	2.33	15.16	4.66
	100	3.37	2.56	3.37	2.56
	200	3.37	2.96	1.68	1.48
E ₂ S	50	5.21	1.64	10.42	3.28
	100	5.17	2.54	5.17	2.54
	200	3.85	3.11	1.92	1.56
E ₁ G	50	4.15	2.12	8.30	4.24
	100	4.53	2.80	4.53	2.80
	200	5.83	3.11	2.91	1.56
E ₂ G	50	2.95	2.61	5.50	5.22
	100	3.29	3.08	3.29	3.08
	200	2.02	4.53	1.01	2.27
17 α -E ₂	50	2.24		4.48	
	100	5.31		5.31	
	200	5.01		2.51	
E ₂ -3S	50	0.00		10.00	
	100	1.78		1.78	
	200	6.13		3.07	

n = 5

Table 5b

Precision of Radioimmunoassay for Androgens

Steroid	Level of Androgen (pg)	Intra-assay Precision (pg)		Coefficient of Variation (%)	
		Assay #1	Assay #2	Assay #1	Assay #2
T	50	1.97	3.25	3.94	6.00
	100	3.60	5.83	3.60	5.83
	200	6.50	8.13	3.25	4.07
DHT	50	2.84	2.32	5.68	4.64
	100	2.80	5.40	2.80	5.40
	200	0.86	7.62	0.43	3.81
TS	50	1.32	3.06	2.64	6.12
	100	2.88	3.10	2.88	3.10
	200	3.64	5.67	1.82	2.83
DHTS	50	1.91	1.42	3.82	2.84
	100	3.91	2.83	3.91	2.83
	200	1.24	2.71	0.62	1.36
TG	50	3.53	2.88	7.06	5.76
	100	2.13	2.03	2.13	2.03
	200	7.97	5.75	3.99	2.88
DHTG	50	2.74	2.70	5.48	5.40
	100	3.57	2.52	3.57	2.52
	200	4.40	4.76	2.20	2.38

n = 5

Table 6

Sensitivity of Radioimmunoassay for Estrogens and Androgens

Steroid	Sensitivity (pg)*
Estrone	14.38
17 β -Estradiol	14.71
Estrone sulfate	25.19
17 β -Estradiol sulfate	31.42
17 α -Estradiol sulfate	15.33
Estrone glucuronide	29.87
17 β -Estradiol glucuronide	24.69
17 α -Estradiol glucuronide	28.01
Testosterone	21.64
Dihydrotestosterone	45.10
Testosterone sulfate	65.66
Dihydrotestosterone sulfate	53.75
Testosterone glucuronide	34.50
Dihydrotestosterone glucuronide	19.68

* recorded as pg of free steroid

estradiol were below the detectable levels in all the samples assayed (Table 7).

(b) Male embryo

The concentration of both estrone and estradiol were below the detectable levels in all samples assayed (Table 8).

(c) Conclusion

Unconjugated estrogens were undetectable in the allantoic fluid of both the female and male embryo.

(2) Estrogen sulfates

(a) Female embryo

Estrone sulfate was first detectable in the allantoic fluid of the female at stage 36. The concentration increased to a peak of 537 pg/ml at stage 41. This increase was significant at the 1% level (Table 9, Fig. 3).

The concentration of estradiol sulfate was first detectable at stage 35 and increased significantly to a peak at stage 44 of 1505 pg/ml. The increase in the logarithm of concentration of 17 β -estradiol sulfate versus developmental stage may be linear (Table 9, Fig. 4).

When first assayed at stage 39, the 17 α -estradiol sulfate concentration was 361 pg/ml. This level increased to 937 pg/ml at stage 44 (Table 9, Fig. 3).

(b) Male embryo

Both estrone sulfate and estradiol sulfate were undetectable in all the samples assayed (Table 10).

(c) Partially decapitated embryo

The day 17.5 concentration of estrone sulfate in the partially decapitated female was 138 pg/ml. The corresponding values of 17 β -estradiol sulfate and 17 α -estradiol sulfate were respectively 1212 pg/ml and 1045 pg/ml. These values were not

Table 7

Concentration of Unconjugated Estrogens in the
Allantoic Fluid of the Female Chicken Embryo

Stage	E ₁		E ₂	
	Mean	SEM	Mean	SEM
	(pg/ml)*		(pg/ml)*	
		n		n
34	<15	3	<15	3
35	<15	5	<15	5
36	<15	5	<15	5
37	<15	6	<15	6
38	<15	6	<15	6
39	<15	5	<15	5
40	<15	6	<15	6
41	<15	5	<15	5
42	<15	4	<15	4
43	<15	4	<15	4
44	<15	4	<15	4
45	<15	4	<15	4

* The values are recorded as pg of free steroid.

Table 8

Concentrations of Unconjugated Estrogens in the
Allantoic Fluid of the Male Chicken Embryo

Stage	E ₁		n	E ₂		n
	Mean	SEM *		Mean	SEM *	
34	<15		3	<15		3
35	<15		3	<15		3
36						
37						
38						
39						
40						
41						
42	<15		5			
43						
44	<15		6	<15		7
45						

* All values are recorded as pg/ml of free steroid.

Table 9

Concentration of the Estrogen Sulfates in the Allantoic Fluid of the Female Chicken Embryo

Stage	E ₁ S		n	E ₂ S		n
	Mean	SEM *		Mean	SEM *	
34	<25		10	<31		10
35	<25		5	146	48.9	5
36	76	11.9	3	78	12.6	5
37	122	12.0	11	338	27.5 ^a	12
38	140	43.5	4	458	50.4	6
39	109	32.3	6	470	27.9 ^b	9
40	244	85.2	11	371	50.0	15
41	537	159.6	13	886	85.8 ^a	15
42	483	214.4	10	614	125.0 ^b	17
43	201	37.8	6	1627	363.5 ^a	7
44 ^a	336	106.3	12	1505	296.1 ^b	20
45	543	109.8 ^a	4	1359	305.3 ^a	5

* All values are recorded as pg/ml of free steroid.

a Statistically significant from stage 36 at the 1% level as determined by 't' test.

b Statistically significant from stage 36 at the 5% level as determined by 't' test.

E₁S and E₂S are undetectable in the male embryo.

Therefore statistical comparison could not be carried out.

Fig. 3. The mean concentration of estrone sulfate (Δ), 17β -estradiol sulfate ($\square$), 17α -estradiol sulfate ($\circ$), estrone glucuronide ($\blacktriangle$), 17β -estradiol glucuronide ($\blacksquare$) and 17α -estradiol glucuronide ($\bullet$) in the allantoic fluid of the female chicken embryo during development. Concentrations are recorded as ng of unconjugated steroid.

Table 10.

Concentrations of Estrogen Sulfates in the
Allantoic Fluid of the Male Chicken Embryo

Stage	E ₁ S		n	E ₂ S		n
	Mean	SEM *		Mean	SEM *	
34	< 25		3	< 31		4
35	< 25		3	< 31		3
36						
37						
38						
39						
40	< 25		5	< 31		5
41						
42	< 25		7	< 31		5
43						
44	< 25		8	< 31		8
45						

* All values are recorded as pg/ml of free steroid.

Fig. 4. The logarithm of the mean concentrations of total estrogen conjugates plus androgen conjugates (—), total estrogen conjugates (— · —) and total androgen conjugates (— —) in the allantoic fluid of the chicken embryo during development. Concentrations are recorded as ng of unconjugated steroid.

Table 11

Concentrations of α -configuration Estrogen
Conjugates in the Allantoic Fluid of the
Female Chicken Embryo

Stage	17 α -E ₂ S		n	17 α -E ₂ G		n
	Mean	SEM *		Mean	SEM *	
34						
35						
36						
37						
38						
39	361	145.3	3	231	57.9	4
40	392	110.1	5	335	50.2	5
41	959	174.8	6	904	152.2 ^a	7
42	588	128.0	5	1325	275.5	5
43						
44	937	291.9	3	944	156.4 ^a	3
45						

* All values are recorded as pg/ml of free steroid.

a Statistically significant from stage 39 at the 1% level as determined by 't' test.

17 α -E₂S and 17 α -E₂G are undetectable in the male embryo.

Therefore statistical comparison could not be carried out.

significantly different from the normal female stage 41 values (Table 12).

(d) Conclusion

Only the female chicken embryo synthesizes detectable quantities of estrogen sulfates and the amount produced by intact embryos is not significantly different from the amount produced by partially decapitated embryos.

(3) Estrogen glucuronides

(a) Female embryo

From stage 36, estrone glucuronide rises to a significantly increased value of 1825 pg/ml at stage 45. The 17 β -estradiol glucuronide concentration increased from stage 35 to a peak at stage 45 of 4210 pg/ml. This peak is a significant increase from stage 36. The increase in the logarithm of the 17 β -estradiol glucuronide concentration value versus the developmental stage may be linear (Table 13, Fig. 3).

When first assayed at stage 39, the concentration of 17 α -estradiol glucuronide was 231 pg/ml. The concentration increased with time to a peak of 1325 pg/ml (Table 13, Fig. 3).

(b) Male embryo

The concentrations of estrogen glucuronides were below the level of detection in all the samples assayed (Table 14).

(c) Partially decapitated female embryo

The day 17.5 concentration of estrone glucuronide in the partially decapitated female embryo was 528 pg/ml. The corresponding values of 17 β -estradiol glucuronide and 17 α -estradiol glucuronide were 897 and 538 pg/ml. These values were not significantly different from the normal female stage 41 values.

(d) Conclusion

Only the female chicken embryo synthesized estrogen glucuronides in detectable

Table 12

Concentrations of the Steroids in the Allantoic Fluid
of the Partially Decapitated Chicken Embryo at Day 17.5

Steroid	Concentrations in pg/ml					
	Female			Male		
	Mean	SEM *	n	Mean	SEM *	n
E ₁ S	138	27.4	6			
17 β -E ₂ S	1212	421.5	10			
E ₁ G	528	132.3	9			
17 β -E ₂ G	897	156.0	11			
17 α -E ₂ S	1045	657.2	3			
17 α -E ₂ G	538	155.7	3			
TS	1292	272.5	6	922	134.2	8
DHTS	448	128.0	6	372	57.0	8
TG	225	41.1	3	215	45.8	4
DHTG	221	64.4	4	223	57.7	4

* All values are recorded as pg/ml of free steroid.

- d Statistically significant at the 5% level as determined by 't' test. Comparisons were made with intact, untreated embryos at stage 41.
- e Statistically significant at the 5% level as determined by 't' test. Comparisons were made between sexes at any given stage.

Table 13

Concentrations of Estrogen Glucuronides in the
Allantoic Fluid of the Female Chicken Embryo

Stage	E ₁ G		n	E ₂ G		n
	Mean	SEM *		Mean	SEM *	
34	<30		8	<25		6
35	<30		5	159	40.9	5
36	97	7.0	2	387	154.2	4
37	124	19.1	6	270	16.9 ^b	6
38	151	21.3	5	478	110.4 ^b	5
39	97	9.3	11	507	109.4 ^b	10
40	164	22.4	14	628	108.8 ^b	14
41	612	83.7 ^a	14	990	180.0 ^b	16
42	955	125.3 ^a	14	2103	208.6 ^a	12
43	1143	241.4 ^a	11	3501	684.3 ^a	11
44	1102	182.7 ^a	14	2016	219.4 ^a	17
45	1825	355.8 ^a	4	4210	733.9 ^a	4

* All values are recorded as pg/ml of free steroid.

a Statistically significant from stage 36 (E₁G) or 35 (E₂G) at the 1% level as determined by 't' test.

b Statistically significant from stage 36 (E₁G) or 35 (E₂G) at the 5% level as determined by 't' test.

E₁G and E₂G are undetectable in the male embryo.

Therefore statistical comparison could not be carried out.

Table 14

Concentrations of the Estrogen Glucuronides in the Allantoic Fluid of the Male Chicken Embryo

Stage	E ₁ G		n	E ₂ G		n
	Mean	SEM *		Mean	SEM *	
34	<30		3	<25		3
35	<30		3	<25		4
36						
37						
38						
39						
40	<30		4	<25		5
41						
42	<30		6	<25		5
43						
44	<30		8	<25		9
45						

* All values are recorded as pg/ml of free steroid..

quantities. The amount of estrogen glucuronide produced by the hypophyseoprivic embryo was not significantly different from the amount produced by the intact embryo.

(ii) Amniotic fluid

The concentrations of all the steroids tested in amniotic fluid in both female and male embryos were below the level of detection (Table 15).

B) Androgen Radioimmunoassay

(i) Allantoic fluid

(1) Unconjugated androgens

(a) Male embryo

The concentrations of both testosterone and dihydrotestosterone were below the level of detection in all samples assayed (Table 16).

(b) Female embryo

The concentrations of both testosterone and dihydrotestosterone were below the level of detection (Table 17).

(c) Conclusion

Unconjugated androgens were not present in detectable amounts in the allantoic fluid of either male or female embryos.

(2) Androgen sulfates

(a) Male embryo

Testosterone sulfate was first detectable at stage 37 of development and the concentration increased significantly to a concentration of 1266 pg/ml at stage 44 (Table 18, Fig. 5).

From a value of 79 pg/ml at stage 37, the concentration of dihydrotestosterone sulfate increased significantly to a peak of 842 pg/ml at stage 41 (Table 18, Fig. 5).

(b) Female embryo

The concentration of testosterone

Table 15

Concentrations of Steroids in the Amniotic Fluid
of the Chicken Embryo^a

Steroid	Male		Female	
	Concentration *	n	Concentration *	n
E ₁	<15	15	<15	12
17 β -E ₂	<15	13	<15	11
E ₁ S	<25	39	<25	21
17 β -E ₂ S	<31	39	<31	20
E ₁ G	<30	40	<30	21
17 β -E ₂ G	<25	47	<25	20
T	<22	12	<22	11
DHT	<45	10	<45	10
TS	<66	10	<66	19
DHTS	<53	10	<53	17
TG	<35	11	<35	16
DHTG	<20	1	<20	14

^a The samples assayed cover the developmental interval stage 35 to stage 44.

* All values are recorded as pg/ml of free steroid.

Table 16

Concentrations of the Unconjugated Androgens in the Allantoic Fluid of the Male Chicken Embryo

Stage	T		n	DHT		n
	Mean	SEM *		Mean	SEM *	
34	<22		3	<45		4
35						
36						
37						
38						
39						
40	<22		5	<45		5
41						
42						
43						
44	<22		3	<45		3
45						

* All values are recorded as pg/ml of free steroid.

Table 17

Concentration of Unconjugated Androgens in the
Allantoic Fluid of the Female Chicken Embryo

Stage	T			n	DHT			n
	Mean	SEM	*		Mean	SEM	*	
34								
35								
36	<22			2				
37								
38	<22			6				
39	<22			2				
40	<22			8	<45			3
41	<22			2				
42	≥22			3				
43								
44	<22			4	<45			4
45								

* All values are recorded as pg/ml of free steroid.

Table 18

Concentrations of Androgen Sulfates in the
Allantoic Fluid of the Male Chicken Embryo

Stage	TS		n	DHTS		n
	Mean	SEM *		Mean	SEM *	
34						
35						
36	<66		3	<54		2
37	236	56.0	5	79	14.0	3
38	746	231.5 ^b	2			
39	145	15.0	4	135	21.5 ^b	3
40	960	288.5 ^b	5	758	112.2 ^{a,c}	3
41	567	612.7 ^b	3	842	349.4 ^b	3
42	784	105.0 ^a	8	272	96.7	9
43	1266	169.2 ^a	10	561	96.7 ^b	11
44	783	247.1 ^b	3	202	68.5	2

* All values are recorded as pg/ml of free steroid.

a Statistically significant from stage 37 at the 1% level as determined by 't' test.

b Statistically significant from stage 37 at the 5% level as determined by 't' test.

c Statistically significant at the 5% level as determined by 't' test. Comparisons were made between sexes at a given stage.

Fig. 5. The mean concentrations of testosterone glucuronide (•), dihydrotestosterone glucuronide (▲), testosterone sulfate (◦) and dihydrotestosterone sulfate (△) in the allantoic fluid of the male and female chicken embryo during development. Concentrations are recorded as ng of unconjugated steroid.

sulfate increased from 189 pg/ml at stage 36 to a peak of 1691 pg/ml at stage 45 (Table 19, Fig. 5).

Dihydrotestosterone sulfate is first detectable at a level of 76 pg/ml at stage 36. The dihydrotestosterone concentration increased significantly to a peak of 2048 pg/ml at stage 44 (Table 19, Fig. 5).

The values for androgen sulfate concentrations in the female embryo were not significantly different from those of the male embryo.

(c) Partially decapitated male embryo

The concentration of testosterone sulfate and dihydrotestosterone sulfate at day 17.5 in the partially decapitated male embryo were 922 and 372 pg/ml respectively. These values were not significantly different from the normal male stage 41 values.

(d) Partially decapitated female embryo

The day 17.5 concentration of testosterone sulfate in the partially decapitated female embryo was 1292 pg/ml. The corresponding value for dihydrotestosterone sulfate was 448 pg/ml. These values were not significantly different from the values for the intact female embryo at stage 41.

(e) Conclusion

Both male and female chicken embryos synthesized sulfated androgens. The difference in the concentrations between male and female embryos and between partially decapitated and intact embryos is not significant.

(3) Androgen glucuronides

(a) Male embryo

The testosterone glucuronide concentration rose from a first detectable level of 123 pg/ml at stage 38 to a stage 44 value of 626 pg/ml (Table 20, Fig. 5).

From a first detectable level of 95 pg/ml at stage 38, the concentration of dihydrotestosterone glucuronide rose to a stage 44 level of 471 pg/ml (Table 20, Fig. 5).

Table 19

Concentrations of the Androgen Sulfates in the Allantoic Fluid of the Female Chicken Embryo

Stage	TS		n	DHTS		n
	Mean	SEM *		Mean	SEM *	
34						
35						
36	189	39.2	4	76	8.5	3
37	128	6.3	3	54		3
38	424	49.5	3	81	19.0	3
39	653	191.3	4	305	113.0	4
40	1346	274.4 ^b	3	303	51.0 ^{b,c}	4
41	1150	355.1	5	409	163.4	5
42	606	147.8	5	280	37.7 ^a	5
43	1100	137.2 ^a	5	845	255.3 ^b	5
44	1691	993.2	5	2048	799.5 ^b	3
45						

* All values are recorded as pg/ml of free steroid.

a Statistically significant from stage 36 (TS) and 38 (DHTS) at the 1% level as determined by 't' test.

b Statistically significant from stage 36 (TS) or 38 (DHTS) at the 5% level as determined by 't' test.

c Statistically significant at the 5% level as determined by 't' test. Comparisons were made between sexes at a given stage.

Table 20

Concentrations of Androgen Glucuronides in the Allantoic Fluid of the Male Chicken Embryo

Stage	TG		n	DHTG		n
	Mean	SEM *		Mean	SEM *	
34						
35						
36	<34		2	<20		2
37	<34		6	<20		7
38	123	28.1	4	95	2.5	2
39	96		1			
40	299	29.3 ^a	5	187	39.7	7
41	288	64.9	5	239	33.5 ^a	5
42	224	151.2	6	149	27.2	5
43	508	181.7	10	88	12.5 ^b	5
44	626	397.0	4	471	262.6	3
45						

* All values are recorded as pg/ml of free steroid.

a Statistically significant from stage 38 at the 1% level as determined by 't' test.

b Statistically significant from stage 38 at the 5% level as determined by the 't' test.

c Statistically significant at the 5% level as determined by the 't' test. Comparisons were made between sexes at a given stage.

(b) Female embryo

The concentration of testosterone glucuronide increased significantly from an initial level of 66 pg/ml at stage 36 to a peak of 1394 pg/ml at stage 42 (Table 21, Fig. 5).

Dihydrotestosterone glucuronide was first detectable at stage 40 at a level of 111 pg/ml and did not change significantly thereafter (Table 21, Fig. 5).

The concentrations of androgen glucuronides in the female embryo were not significantly different from the concentrations in the male embryo.

(c) Partially decapitated male embryo

The day 17.5 concentration of testosterone glucuronide in the partially decapitated male embryo was 215 pg/ml. The dihydrotestosterone glucuronide value at day 17.5 was 223 pg/ml. These values were not significantly different from the normal stage 41 values nor were they different from the values for the partially decapitated female.

(d) Partially decapitated female embryo

The day 17.5 concentration of testosterone glucuronide in the partially decapitated female embryo was 225 pg/ml. The corresponding value for dihydrotestosterone glucuronide concentration was 221 pg/ml. These values were not significantly different from the normal stage 41 values.

(ii) Amniotic fluid

The concentrations of all the androgens tested in the amniotic fluid in both female and male embryos were below the level of detection (Table 15).

C) Incubation of Liver Tissue in vitro with Radioactive Steroids

(1) Estrone sulfate as precursor

(a) The embryonic female liver tissue in vitro was capable of deconjugating estrone sulfate to estrone. The estrone produced was reduced to estradiol which in

Table 21

Concentrations of the Androgen Glucuronides in the Allantoic Fluid of the Female Chicken Embryo

Stage	TG		n	DHTG		n
	Mean	SEM *		Mean	SEM *	
34						
35						
36	66	15.5	2			
37				<20		4
38	136	13.6	12			
39				<20		4
40	214	29.0 ^b	15	111	3.0	4
41	298	60.1 ^b	5	91	16.9	5
42	1394	1005.4 ^b	4	140		1
43	298	125.6 ^a	5	131	40.7	5
44	1089	590.4 ^b	6	129	32.5	3
45						

* All values recorded as pg/ml of free steroid.

a Statistically significant from stage 38 (TG) or 41 (DHTG) at the 1% level as determined by 't' test.

b Statistically significant from stage 38 (TG) or 41 (DHTG) at the 5% level as determined by 't' test.

c Statistically significant at the 5% level as determined by 't' test. Comparisons were made between sexes at a given stage.

turn could be reconjugated to estradiol sulfate or estradiol glucuronide (Table 22).

(b) The male embryonic liver tissue in vitro could perform the same metabolic changes as the female tissue but to a lesser degree (Table 22).

(2) 17 β - Estradiol as precursor

(a) The female embryonic liver tissue in vitro was capable of oxidizing estradiol to estrone. These estrogens could be conjugated both as sulfates and as glucuronides (Table 22).

(b) The male liver tissue was capable of producing the same metabolites as the female tissue (Table 22).

(3) Testosterone as precursor

(a) The embryonic female liver tissue in vitro was capable of reducing testosterone to dihydro-testosterone and of conjugating testosterone as a sulfate and as a glucuronide (Table 22).

(b) The male embryonic liver tissue was capable of the same metabolic changes as the female tissue. With testosterone as precursor, both the male and female showed a large proportion of unidentified steroid (Table 22).

D) Recovery of Tritium Labeled Precursor Steroids from Yolk

In this study three radioactive steroids (estrone sulfate, 17 β -estradiol and testosterone) were injected into the unincubated egg yolk. After the eggs were incubated to stage 42, the allantoic and amniotic fluids were collected and eight steroids (estrone sulfate, estradiol sulfate, estrone glucuronide, estradiol glucuronide, testosterone sulfate, testosterone glucuronide, dihydrotestosterone sulfate and dihydrotestosterone glucuronide) were extracted.

In all the steroids studied in both male and female embryos, the portion of the precursor radioactivity

Table 22

Liver Mince Metabolites of Radioactive Steroids Separated by Thin Layer Chromatography

TLC		Steroid Metabolites Identified ^a											
System	Precursor	Sex	E ₁	17 β -E ₂	E ₁ S	17 β -E ₂ S	17 β -E ₂ C	(u)	T	DHT	TS	TG	* O
Free ^b	E ₁ S	Control ^d	0	0	-	-	-	0	0	0	-	-	100
		Male	44	6	-	-	-	6	0	0	-	-	44
		Female	39	12	-	-	-	10	0	0	-	-	39
Free	17 β -E ₂	Control	0	57	-	-	-	0	0	0	-	-	43
		Male	4	37	-	-	-	0	0	0	-	-	59
		Female	3	15	-	-	-	0	0	0	-	-	82
Free	T	Control	0	0	-	-	-	14	57	0	-	-	29
		Male	0	0	-	-	-	30	30	10	-	-	30
		Female	0	0	-	-	-	31	31	7	-	-	31
Conjugate ^c	E ₁ S	Control	0	0	100	0	0	0	0	0	0	0	0
		Male	40	40	53	5	2	0	0	0	0	0	0
		Female	30	30	54	10	5	0	0	0	0	0	0
Conjugate	17 β -E ₂	Control	95	95	5	0	0	0	0	0	0	0	0
		Male	59	59	11	19	7	4	0	0	0	0	0
		Female	33	33	22	39	6	0	0	0	0	0	0
Conjugate	T	Control	0	0	0	0	0	6	94	94	0	0	0
		Male	0	0	0	0	0	32	43	43	19	6	0
		Female	0	0	0	0	0	33	34	34	27	6	0

^a Scored as a percentage of the total steroid on the plate. 0 represents the amount of steroid that stayed at the origin. - indicates that this steroid stayed at the origin. (u) represents the amount of unidentified steroid.

^b TLC system: 5:2; MeCl:Ether c TLC system: 90:90:18; EtOH:EtAc:NH₄ OH

^d Control flasks contained no liver tissue.

Table 23

Percentage Recovery of Radioactive Steroids (following Injection into the Yolk before Incubation) from Allantoic and Amniotic Fluid of the Male and Female Chicken Embryo at Stage 42 and 43

Sex	Fluid	Injected		E ₁ S	17 β -E ₂ S	E ₁ G	17 β -E ₂ G	TS	DHTS	TG	DHTG
		Isotope									
Female	Amniotic	E ₁ S		.01 (2) ^a	.02 (3)	.01 (2)	.01 (3)				
Female	Amniotic	17 β -E ₂		.01 (2)	.01 (4)	.01 (2)	.01 (4)				
Female	Amniotic	T		.11 (2)							
Female	Allantoic	E ₁ S		.19 (6)	.23 (7)	.07 (6)	.19 (7)				
Female	Allantoic	E ₂		.05 (10)	.29 (10)	.03 (10)	.13 (10)				
Female	Allantoic	T		.08 (3)	.65 (7)	.01 (3)	.03 (3)	.10 (3)	.09 (3)	.06 (3)	.22 (3)
Male	Amniotic	E ₁ S			.93 (1)		.20 (1)				
Male	Amniotic	E ₂			.15 (1)						
Male	Allantoic	E ₁ S		.22 (3)	.31 (3)	.15 (3)	.26 (3)				
Male	Allantoic	E ₂		.05 (3)	.32 (7)	.02 (3)	.17 (3)				
Male	Allantoic	T		.10 (2)	.10 (2)	.02 (1)	.03 (1)	.09 (2)	.08 (2)	.08 (2)	.27 (2)

a Numbers, in parentheses indicate sample size.

recovered was always less than 1% (Table 23).

DISCUSSION

Although considerable work has been done on studying the production of steroids by the embryonic gonads of the chicken, a number of contradictions remain in the data.

Repeated studies have demonstrated the capacity of embryonic female gonads greater than 7 days old to produce estrone, estradiol and estriol in vitro (Weniger, 1967, 1969, 1971; Guichard et al, 1973, 1977). In the ovarian tissue, the concentration of estrone may be higher than that of estradiol (Woods and Erton, 1978; Teng and Teng, 1977) whereas in the medium surrounding cultured ovarian tissue, the concentration of estradiol exceeds that of estrone. However, the amounts of these steroids produced by the gonads in vivo had not, until the present study, been quantitated.

Some in vitro studies have suggested that the embryonic testis is capable of estrogen biosynthesis (Cedard, 1970; Guichard, 1977), while others have suggested that the testis either is incapable of estrogen synthesis or only develops estrogen synthetic capacity late in the incubation period (Weniger, 1968b, 1971; Galli and Wasserman, 1972, 1973).

Although the work of Weniger and Zeis (1976) suggests the contrary, both embryonic ovaries and testes have been shown to produce testosterone (Galli and Wasserman, 1972, 1973; Cedard, 1970) but, while Guichard et al (1977) demonstrated that the gonads in vitro could produce dihydrotestosterone as well as

testosterone, Woods et al. (1975) found no dihydrotestosterone in either male or female embryonic plasma.

Previous studies have concentrated on establishing the presence of unconjugated steroids in the avian embryo. One exception is the work of Ozon (1965) which demonstrated measurable concentrations of estrone, estradiol and estriol conjugates in embryonic allantoic fluid. He used an acid hydrolysis before extraction, but did not determine the type of conjugate present.

A number of factors combine to suggest that conjugated steroids may be important in the chicken embryonic fluids. Firstly, estrogen sulfates have been found in the plasma of mature hens (Peterson and Common, 1972). Thus the chicken must have a functioning steroid sulfotransferase. Secondly, estrone sulfate has been found to be the predominant estrogen present in the fetal fluids of the sheep (Carnegie and Robertson, 1978), the cow (Robertson and King, 1979) and possibly the pig (Robertson and King, 1974). Thirdly, Dutton and Ko (1966) demonstrated the presence of an UDP-glucuronyl-transferase in the embryonic chicken liver from at least day 8 of incubation. The water-soluble glucuronides are thought to be the major urinary excretion product of estrogens. As the allantois is the repository of waste products from the kidney during incubation, the estrogens found in the allantoic fluid would presumably be in the form of conjugates; glucuronides or sulfates. This has been found to be true in the present study.

"The actual secretion of a steroid by an endocrine gland cannot be proven only by the study of the biosynthesis of steroid hormones by the gland from exogenous precursors nor by the demonstration of the

presence of hormones in the gland. The proof of endocrine secretion in the first place requires an analysis of the venous blood draining the gland".

Proof of the gonadal production of sex steroids using this definition of Ozon (1972) would present difficulties due to the small size and delicacy of the chicken embryo. Therefore, an accumulation of circumstantial evidence must be used. Teng and Teng (1977) cultured portions of tissue from the adrenal, kidney, liver, testes and right and left ovaries and found that the ovaries were the major producers of estrogen, while the testes and right ovaries were the major producers of testosterone. The steroids produced by the embryo are the same as those produced by the ovaries of the hen. I have shown (Table 23) that the potential maternal source of steroids, the egg yolk, is not a major contributor of either estrogens or androgens to the embryo. Histochemically demonstrable testosterone and 17 β -estradiol have been shown to occur in the tissues of the gonads of the chicken embryo (Woods and Podczaski, 1974; Woods and Erton, 1978). Thus the gonads would seem to be the endogenous producers of estrogens and androgens.

The conjugation of the sex steroids may also take place in the gonads. However, the presence of conjugating enzymes in the gonads has not been demonstrated for the chicken embryo as it has for the human embryo (Wengle, 1966) and the salmon (Idler et al, 1971). UDP-glucuronyltransferase has been found in the liver, kidney, gut and adrenal gland of the chicken, and the presence in the kidney and liver has been confirmed in the chicken embryo (Wishart and Dutton, 1975). Estrone sulfotransferase is found in abundance in embryonic chicken cartilage (Adams, 1963). I have shown (Table 22) that minced liver tissue in vitro is capable of conjugating free estradiol and

testosterone in both sulfate and glucuronide forms and of deconjugating and reconstituting estrone sulfate. Thus, the chicken embryo is capable of metabolizing steroids to conjugates in vivo.

In this study the steroid hormones were identified and quantitated in the allantoic fluid of the embryonic chicken. The amniotic fluid was also studied but the concentrations of sex steroids in the fluid, if any, were below the level of detection of the assay. As steroids are mainly excreted in the urine and the bile, they should not concentrate in the amniotic fluid, but rather in the allantoic fluid. A study of plasma steroid concentrations was also begun. However, the volume of plasma available during the earlier stages studied was so small that pools of plasma from a large number of chicken embryos were necessary. Such a study would not only have been extremely costly, but it would not have shown the variability in the steroid concentration among different individuals. Since Challis et al (1974) found that there were 'sizeable variations' in the steroid concentration in bovine cord blood at any given embryonic age and Attal (1969) found 'wide variation' in the androgen concentration of testes of different ovine fetuses at the same age, individual variation in the steroid production of chicken embryos was expected and shown to occur in this study. Part of this variability may be accounted for by the fact that the samples were collected at random intervals throughout the year. Guichard et al (1979) have shown that the total steroid production by chicken embryonic gonads during the summer months was significantly higher than the steroid production during the winter months.

Since unconjugated estrogens and androgens are fat soluble and the contents of the allantois are aqueous, there is, as expected, no detectable quantity of free

steroid present in the allantoic fluid of either the female or male embryo at any of the stages tested. The steroids were found to exist as esters of sulfuric or glucuronic acids. It is possible that other types of conjugates, such as double conjugates, exist in the allantoic fluid, however; the assays used would not detect them.

The aromatized steroid nucleus, of which estradiol is typical, is not broken down during the urinary or biliary excretion of estrogens. Instead the estrogen is inactivated by the formation of conjugates. Thus in an organism such as the chicken embryo where the excretion products are retained within the allantois, the concentration of estrogen conjugates in the allantoic fluid can be used as a measure of total estrogen synthesis.

When cultured with radioisotope labeled precursors, neither testes tissue (Teng and Teng, 1977) nor liver tissue (Table 22) from the male chicken embryo synthesized detectable amounts of estrogen. Therefore the androgens are the endproducts of the steroid biosynthetic pathway in the male and the concentration of their conjugates in the allantoic fluid can be used as a summation of total androgen synthesis with time.

The major steroid hormones are first detectable in the allantoic fluid at approximately stage 36. At this stage the total estrogen concentration in the allantoic fluid of the female embryo is approximately equivalent to 0.6 ng/ml of free steroid. Thereafter the logarithm of the concentration of total estrogen increases linearly with time to a peak of 7.9 ng/ml at stage 45.

The concentrations of both estrone and estradiol

conjugates increase slowly from stage 36 to stage 39 or 40, at which time the rate of increase in the concentrations rises sharply. This alteration in the rate of synthesis coincides with the change in enzyme function noted by Galli and Wasserman (1973). The concentration of estradiol conjugates at any given time is greater than the concentrations of estrone conjugates. This relationship might be expected as the ratio of 17 β -estradiol to estrone is greater than unity in the urine of non-laying hens (Common et al, 1969). Also the concentrations of the glucuronides are greater than the concentrations of the sulfates of either estrogen throughout development. This may reflect the function of the glucuronides as the final excretion products.

Unlike the estrogens which are produced by female embryos but not by the males, the androgens are produced by both sexes during development. The androgens reach a detectable level in the allantoic fluid at approximately stage 36. Of the androgens studied, in both male and female embryos throughout development testosterone sulfate appears in the greatest concentration. Both of the dihydrotestosterone conjugates are detectable in lesser quantities than are their testosterone equivalents. This confirms the results of Guichard et al (1977) who found that both ovaries and testes in culture produced more testosterone than dihydrotestosterone until stage 44. These data conform to the idea that testosterone is the circulating form of the hormone which is only converted at the site of action to dihydrotestosterone.

The androgen sulfates are present in the allantoic fluid at higher concentrations than are the glucuronides. The logarithm of the concentration of

total androgens in either male or female embryos is linear throughout development and the concentration of total androgen is not significantly different between males and females. These data differ from those of Woods et al (1975) who found that in the male the unconjugated testosterone concentration in plasma was significantly higher than the concentration in the female. They were, however, unable to detect dihydrotestosterone in the plasma sample.

As chicken embryos of both sexes synthesized similar quantities of androgens the difference in the endocrine environment between the male and female embryo was due to the estrogen synthesized by the female. This differs from the mammalian embryonic condition where it has been found that throughout most of the gestation period there is no difference between the estrogen concentrations in the male and female embryos (Challis et al, 1974; Robertson and King, 1978), whereas the difference between the testosterone concentrations of males and females is significant (Challis et al, 1974).

The results of the present study can not offer evidence as to whether this difference in steroid synthesis is responsible for sexual differentiation. Although the histochemical work of Woods et al (1974, 1978) suggests that steroids occur in the gonads before sexual differentiation occurs, Weniger (1968b) has demonstrated that only differentiated ovarian or testicular tissue is capable of biosynthesis of sex steroids from sodium acetate. In the present study, the sex steroids were not detectable in the allantoic fluid until after sexual differentiation determined by gross observation of the gonads had taken place.

The pattern of steroid synthesis is consonant with the view that the sex steroids are important in the

differentiation of the gonaducts (Price et al, 1975). In the male embryo, the right and left Müllerian ducts regress between stages 35 and 39 at a time when increasing concentrations of androgens are being synthesized by the embryo. Similarly in the female embryo the regression of the right Müllerian duct starts at stage 35 and is concluded by stage 42. During this period the female synthesizes an ever increasing amount of androgen. This correlation does not prove that an androgen is the Müllerian duct inhibiting substance but it does concur with other evidence that suggests that androgens are involved in the normal Müllerian duct regression (Lutz-Ostertag, 1976). Contrary data suggest that the Müllerian duct inhibiting factor is a protein and that neither testosterone nor dihydrotestosterone is capable of causing regression in vitro (Weniger and Zeiss, 1973, 1976). Weniger, however, bases this hypothesis partially on his conclusion that the embryonic testes are incapable of synthesizing testosterone. A conclusion that the present study and the work of Woods et al (1975) prove to be unfounded.

The left Müllerian duct of the female embryo does not regress, but continues to develop, eventually becoming the functional oviduct. From stage 34 to 38, the weight and length of the left Müllerian duct increases only slightly. From stage 40 until hatching, the left Müllerian duct grows rapidly. This pattern of growth parallels the pattern of the increase in estrogen synthesis by the female embryo. The number of estrogen binding sites in the presumptive oviduct increases rapidly between stage 34 and 38 and then levels off (Teng and Teng, 1975). This increase in estrogen receptor sites may be caused by an autonomous mechanism endogenous to Müllerian duct cells or it may be controlled by the endogenous estrogen secreted from

the ovary. Here again, the correspondence between oviduct development and estrogen concentration does not establish that estrogen is the causal agent.

The steroids which are involved in differentiation of the gonaducts have not been identified. With reference to the implication of androgens in the regression of the Mullerian ducts, tissue culture experiments conducted to investigate this phenomenon have used testosterone as the androgenic agent. Although it has been shown in mammalian embryos that dihydrotestosterone appears to be the active androgenic agent in Mullerian duct development, Weniger and Zeiss (1973) have shown that dihydrotestosterone has the feminizing effect of causing Mullerian duct maintenance in male chicken embryos in vivo and in vitro. The present study shows that the concentration of testosterone conjugates is generally higher than the concentration of dihydrotestosterone conjugates throughout development in both male and female embryos (Fig. 5). This duplicates the results of Guichard et al (1977) who found that both ovaries and testes in culture produced more testosterone than dihydrotestosterone until stage 44. As the concentration of hormone localized in a tissue may be greatly different from the concentration of the same hormone in the extracellular fluids, the allantoic fluid, plasma or culture medium concentrations may not be useful in identifying the causal agents in gonaduct development.

The mechanism of control of gonadal steroid synthesis has not yet been fully elucidated. The timing of the abrupt rise in the curve of estrogen or androgen concentration in the allantoic fluid corresponds to the proposed beginning of the influence of the adenohypophysis on gonadal secretions (Woods and Weeks, 1960). However, the rise in concentration

of estrogen conjugates in the allantoic fluid is linear when graphed against the increase in ovarian weight during development (Fig. 6). This suggests that the rise of estrogen synthesis may be endogenously controlled by the ovarian cells and that total estrogen output may be determined by the number or size of differentiated cells in the gonad. The curve of weight gain by the ovary approximates the shape of the curves of increase in a number of important developmental parameters, such as oxygen consumption or total body weight (Romanoff, 1960). This may suggest that the increase in estrogen synthesis is controlled by a broader spectrum trophic hormone such as growth hormone rather than by a specific gonadotrophin.

In this study, embryos that were partially decapitated at 33 h were sampled at day 18. At this time the 'hypophysectomized' embryos had developed to the size of stage 41 embryos rather than to the size of intact stage 44 embryos. The concentrations of estrogens and androgens in the allantoic fluid of the partially decapitated embryos were not significantly different from the concentrations in normal stage 41 embryos. These data concur with the suggestion that steroid synthesis is related to gonadal growth. However, the mechanism of control of steroid synthesis can only be clarified by performing assays for gonadotrophins in vivo coupled with assays of the effect of purified avian gonadotrophins on steroid synthesis by gonads in vivo and in vitro.

The estrogens and androgens have been implicated in the development of a limited number of extra-gonadal tissues (reviewed in Freeman and Vince, 1974). Chondroitin sulfate synthesis in cartilagenous bone may be stimulated by testosterone and reduced by estradiol. Epiphysis tissue from the femora or tibia

Fig. 6. The logarithm of the mean concentrations of estrone sulfate (— —), 17 β -estradiol sulfate (—), estrone glucuronide (— · —) and 17 β -estradiol glucuronide (— · —) in the allantoic fluid of the female chicken embryo vs the logarithm of the wet weight of the left ovary. Concentrations are recorded as ng of unconjugated steroid.

of the day 13 chick embryo is capable of metabolizing testosterone to 5 β -androstane derivatives. Thus androgen metabolites may be influential in bone and cartilage development. Also, the pigmentation of sex-linked feathering of hybrid female chicken embryos is sex hormone dependent.

Testosterone serves as a precursor for 5 β -androstane derivatives which induce delta-aminolevulinic acid synthetase, the rate limiting enzyme in porphyrin synthesis in blastoderm blood island tissue. Thus these androgens may be influential in erythropoietic activity (Parsons, 1970; Gill and Betz, 1979).

In conclusion, the female chicken embryo is capable of synthesizing the estrogens; estrone, 17 α -estradiol, and 17 β -estradiol and the androgens; testosterone and dihydrotestosterone. These steroids are found in the allantoic fluid conjugated as sulfates and glucuronides. Throughout most of the incubation period, 17 β -estradiol glucuronide is the most abundant estrogen and testosterone sulfate is the most abundant androgen. The male chicken embryo does not synthesize measurable amounts of estrogens, but does produce testosterone and dihydrotestosterone conjugates, of which testosterone sulfate is the most abundant. The steroid concentrations in the 'hypophysectomized' embryos are not significantly different from those of the intact embryo.

Steroid Concentrations in the Unincubated Egg Yolk

INTRODUCTION

The peripheral plasma concentrations of unconjugated estrogens in the laying hen reach peaks of 300 pg/ml estradiol and 138 pg/ml estrone before ovulation (Peterson and Common, 1972). The total androgen concentration, expressed as testosterone equivalents, attained peak values that ranged from 125 to 1100 pg/ml at from 8 to 2 h before ovulation (Peterson et al, 1973). Riddle and Dunham (1942) and Arcos (1972) have shown that estrogens are transferred from the blood of the ring dove and domestic hen to the yolk of the egg. Arcos (1972) isolated unconjugated and conjugated (sulfate) estrone and 17 β -estradiol. She estimated that estrone and estradiol would probably be present in equal amounts which she calculated to be only 18 pg/egg yolk. Hertelendy and Common (1965) found no estrogen in either fertile or infertile incubated eggs, and estimated that the egg yolk contains less than 1 ug of estrogen. Marlow and Richert (1940) using a rat estrus bio-assay found that the yolk from freshly laid eggs contained 5 rat units of estrogenic material per kg of yolk. Hertelendy and Common (1965) suggest that if 1 rat unit is assumed to equal 0.1 microgram of steroid, then the estrogenic activity found by Marlow and Richert could be explained by the presence of as little as 8 ng/yolk of estradiol or 6.6 ng/yolk of estrone. Hertelendy and Common (1965) also give a fine review of the bio-assay methods that have been used to detect estrogenic activity in egg yolk. These experiments using biological assay techniques gave results ranging from no detectable estrogenic activity to 10 ug estrogen/yolk.

It seemed possible that the expected low levels of estrogens and androgens in the egg yolk might be measurable by a sensitive radioimmunoassay method.

MATERIALS AND METHODS

1. Collection and Treatment of Samples

Fertilized White Leghorn eggs (strain 934 F) were kept refrigerated until used.

11. Analytical Procedures

The general procedures did not vary from those used for the estimation of embryonic fluid samples.

111. Methods

A) Extraction Method #1

The extraction method outlined in the paper of Arcos (1972) was tried. The method was found to be very slow because of the formation of emulsions. The resulting extracts were not clean enough to run on celite columns.

B) Extraction Method #2

The method of Arcos was modified as follows:

a) One egg yolk, plus appropriate internal standards, was poured into 150 ml of vigorously stirring MeOH.

b) The supernatant was poured off into a Buchner funnel containing Whatman No. 1 filter paper. An additional 50 ml MeOH was added to the egg yolk material and the mixture was stirred briefly. The mixture was poured into the Buchner funnel and filtered.

Unconjugated Estrogen

c) The filtrate was poured into a 500 ml separatory funnel and 50 ml H₂O was added. The mixture was swirled to mix and then extracted with 50 ml of petroleum ether four times. The petroleum ether was discarded.

d) The MeOH fraction was dried down under nitrogen until only the water remained. The water was

made up to 5% NaHCO_3 .

e) The NaHCO_3 was extracted with 50 ml of benzene. At this point the mixture was left in the refrigerator overnight to allow the emulsion to break down.

f) The benzene was transferred to a 50 ml tube and dried down under nitrogen.

Conjugated Estrogens

g) 18 g of NaCl was added to the NaHCO_3 and the mixture was swirled until the NaCl dissolved.

h) The NaCl fraction was extracted three times with 30 ml ethyl acetate.

i) The three ethyl acetate extracts were combined and dried down under nitrogen.

This method was subsequently altered in the following ways:

e) The NaHCO_3 was extracted twice with 30-ml benzene. This to some extent eliminated the formation of emulsions.

h) The extraction with ethyl acetate was replaced with three extractions of 30 ml of chloroform. However, the extracts were still not clean enough to run in a consistent manner on celite columns.

C) Extraction Method #3

The above extraction method was altered to include a step to 'freeze-out' the lipid component of the sample.

a) After the methanol extraction and the filtration as in b) above, the extracts were placed in the freezer at -20°C for four h.

b) As no noticeable amount of lipid had surfaced after this time, the sample was immersed in an acetone-dry ice bath for five min. The sample was then drawn through a Buchner funnel that had similarly been cooled in acetone-dry ice.

As very little lipid was removed by this method, the procedure was altered as follows:

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a) After the methanol extraction and the filtration, the extracts were immersed in an acetone-dry ice bath for five min.

b) The frozen samples were centrifuged at -20°C for 15 min at 5000 rpm.

Once again, only a small amount of lipid material was removed by this method and the procedure was altered as follows:

a) After the methanol extraction and the filtration, the extracts were placed in the freezer at -20°C overnight.

b) The frozen samples were centrifuged at -20°C for 15 min at 5000 rpm.

Although this method did remove a small amount of white fatty material, it did not remove the bulk of the yellow coloured substances which were largely responsible for inconsistent celite column elution.

D) Extraction Method #4

Some aspects of the previously described methods were combined in the following method.

a) Egg yolks (each weighing approximately 17 g) were separated from the whites of the eggs.

Approximately 2000 cpm of each of the appropriate internal standards were added to each yolk. The yolk samples were mixed and allowed to sit for 5 min.

b) Each yolk was dripped slowly into 140 ml of rapidly stirring methanol and left to stir for 1 h.

c) The yolk mixture was then filtered through Whatman No. 1 paper under suction. The precipitate was washed with 40 ml MeOH.

d) The filtrate was made to 70% MeOH with distilled water and cooled to -20°C .

e) The cooled extract was centrifuged at -20°C for 1 h at 10,000 rpm (16,300 g).

f) The supernatant was aspirated and extracted twice with 50 ml of petroleum ether. The petroleum ether was discarded and the 70% methanol was dried down

using a rotary evaporator to remove the MeOH.

g) The remaining aqueous solution was made to 5% NaHCO_3 (2.5 g/50 ml H_2O) and extracted twice with 30 ml of benzene.

h) The benzene extracts were combined, washed with 5 ml of 5% NaHCO_3 and then taken to dryness under nitrogen in a 40° C waterbath.

i) Separation of the estrogens by celite column chromatography was done by the method of Robertson, Smeaton and Durnford (1972).

This method was varied as follows:

a) The extraction technique of 'Extraction Method #4' was followed through to the end of step (f).

b) The remaining aqueous solution was made to pH 13 with 1 N NaOH.

c) The alkaline solution was extracted with 30 ml of 1:1 (v:v) benzene:petroleum ether. The benzene:petroleum ether extract was discarded.

d) The aqueous solution was neutralized with HCl and made to 5% NaHCO_3 .

e) The NaHCO_3 was extracted twice with 30 ml of benzene.

f) The extraction was completed as in steps (h) and (i) of 'Extraction Method #4'.

This variation was found not to significantly improve the results.

The recoveries of radiolabeled internal standards using this method were low and variable. This problem may have originated in two separate places: (1) The benzene extracts often emulsified. Although the samples were centrifuged to break down the emulsions, some of the benzene fraction was not recoverable. (2) The elution of the samples from the celite columns was inconsistent. In fact, the concentration of impurities in some samples was so great that the columns were completely plugged and the samples were lost. This occurred even with samples that did not appear to be

heavily contaminated.

The preliminary results, however, suggested that the concentrations of estrogens in the egg yolk were sufficiently high (estrone = 577 pg/ml, estradiol = 368 pg/ml, estrone sulfate = 836 pg/ml, n = 9) that a reduced volume of egg yolk could be used in future assays.

E) Extraction Method #5

a) The egg yolk was separated from the white and weighed. One gram of yolk was placed in a 45 ml centrifuge tube and mixed with the appropriate internal standards.

b) Fifteen ml MeOH was added and the mixture was mixed on a vortex mixer for five min.

c) The methanol fraction was recovered and mixed with 5 ml of distilled water. The mixture was cooled to -20°C and centrifuged for 1 h at 10,000 rpm and -20°C .

d) The supernatant was pipetted into a clean tube and the methanol was evaporated under nitrogen.

e) The remaining water was made to 5% NaHCO_3 and extracted twice with 5 ml of benzene.

f) The benzene was recovered and dried down under nitrogen.

g) Separation of the estrogen by celite column chromatography was done by the method of Robertson, Sméaton and Durnford (1972).

The reduction in the sample size made the assay less expensive, less time consuming and less cumbersome but it did not greatly improve the precision of the assay as the problem of inconsistent elution of the columns remained. Preliminary results yielded a value for the concentration of estrone of 223 pg/g (n = 25).

F) Extraction Method #6

As the attempts to further purify the egg yolk sample had failed to yield consistent results, it was felt that a change in chromatography column matrix and eluting solvents might improve the precision of the

assay.

a) One gram of egg yolk, one ml of water and appropriate internal standards were placed in a 50 ml centrifuge tube and mixed. The tube was swirled on a vortex mixer for 2 min.

b) Six ml of water was added and the tube was spun for one h at 10,000 rpm and -20° C.

c) The supernatant was removed to another 50 ml tube and extracted with 10 ml of petroleum ether. The tube was centrifuged at 5000 rpm for 10 min. The petroleum ether fraction was removed and discarded.

d) The 70% methanol fraction was dried down under nitrogen until only water remained.

e) The aqueous fraction was made to 5% NaHCO_3 and extracted with 10 ml of benzene and washed with 2 ml benzene.

f) The benzene fractions were combined and dried down under nitrogen. The residue was dissolved in 0.2 ml of column eluting solvent. The column eluting solution used was 62:20:18 (v:v) iso-octane:benzene:methanol.

g) One gram of LH20 Sephadex was expanded in methanol. The excess methanol was decanted and the LH20 was washed with column eluting solution. The LH20 slurry was packed in a 10 ml disposable pipette and washed with 30 ml of eluting solution.

h) The sample was layered on the column. An additional 0.2 ml of column eluting solution was used to rinse the sample tube and was also layered on the column.

i) The column was eluted with the above solution in the following manner:

4 ml - discard

4 ml - estrone fraction

0.5 ml - discard

4 ml - estradiol fraction

j) The appropriate fractions were dried down under nitrogen and assayed.

The Sephadex LH20 columns were less convenient for the operator than the celite columns used in earlier assay methods and they did not seem to increase the precision of the assay. In some cases the columns ran unexpectedly slowly or became completely clogged. The values for the concentrations of estrogens given by this assay were: estrone = 304 pg/g and estradiol = 99 pg/g (n = 8).

G) Extraction Method #7

As the lengthy extraction techniques that had been previously tried gave low and variable recoveries of radiolabeled internal standard, it was felt that an abbreviated procedure might improve the consistency of the assay.

a) One half gram of egg yolk plus 1 ml of water and appropriate internal standards were placed in a 50 ml centrifuge tube and mixed.

b) Ten ml of benzene were added slowly and the tube was swirled on a vortex mixer for 2 min.

c) The mixture was centrifuged at 5000 rpm for 20 min.

d) The benzene fraction was removed and the residue was rinsed with 4 ml of benzene.

e) The two benzene fractions were combined and washed twice with 1 ml of 5% NaHCO_3 . The NaHCO_3 fractions were discarded.

f) The benzene fraction was dried down under nitrogen.

For these samples the residue after extraction was highly coloured and tended to clog the columns. However for the samples that were eluted from the columns the recovery of the radioisotope-labeled internal standard was higher than in previous assays but still inconsistent. The values for the mean concentrations of estrogens given by this assay were:

estrone = 585 pg/g and estradiol = 230 pg/g (N = 24).

H) Extraction Method #8

1) Unconjugated estrogens and androgens

As the steroids in the egg yolk are mixed with many benzene-soluble lipids, it was necessary to add 'clean-up' steps to the extraction procedure used for embryonic fluids.

A mixture of 0.5 ml egg yolk and 0.5 ml of buffer A (plus appropriate internal standards) was gently shaken with 15 ml benzene for 2 min and centrifuged at 1400 rpm for 30 min. The benzene phase was transferred to a 15 ml tube and dried down under nitrogen. Three ml of 1N NaOH and 3 ml of chloroform: carbon tetrachloride (1:5 v:v) were added, the tube was shaken for 2 min, and the tube was centrifuged at 1400 rpm for 5 min. The NaOH phase was transferred to another 15 ml tube and each phase was washed with 1 ml of the other phase.

The chloroform:carbon tetrachloride fraction plus the wash was evaporated under nitrogen and redissolved in 0.5 ml iso-octane. The celite columns and assay were run as for unconjugated androgens from embryonic fluids.

The NaOH fraction was neutralized with 5 N HCl and extracted with 10 volumes of benzene. The assay was completed as for unconjugated estrogens from embryonic fluids.

2) Estrogen sulfates and androgen sulfates

Following the benzene extraction of unconjugated steroids, the sulfates were extracted with 10 volumes of tetrahydrofuran:ethyl acetate (THF:EA, 1:1 v:v). After the tubes were mixed for 2 min, 2.0 ml of 1.0 M NaHCO₃ saturated with NaCl were added. The tubes were mixed again and centrifuged for 15 min at 1400 rpm. The THF:EA extract was transferred to 15 ml tubes and evaporated under nitrogen. 3.0 ml of 1 N NaOH and 3.0

ml of chloroform:carbon tetrachloride (1:5 v:v) were added, the tubes were shaken for 2 min and the tubes were centrifuged at 1400 rpm for 5 min. The NaOH phase was transferred to another 15 ml tube and each phase was washed with 1 ml of the other phase.

The NaOH fraction was neutralized with 5 N HCl and extracted with 10 volumes of THF:EA (1:1 v:v) and 2 ml 1.0 M NaHCO_3 saturated with NaCl. The hydrolysis, celite column separation and assay were completed as for sulfo-estrogens in the embryonic fluids.

The chloroform:carbon tetrachloride fraction was dried down and then partitioned between 3 ml of distilled water and 3 ml hexane. The hexane fraction was transferred to another tube and each fraction was washed with 1 ml of the other fraction. The hexane fraction was discarded. The water fraction plus water wash were extracted with 15 ml THF:EA (1:1 v:v) and 2 ml 1 N NaHCO_3 saturated with NaCl. The THF:EA fraction was evaporated under nitrogen. The hydrolysis, celite column chromatography and assay were completed as for sulfo-androgens in embryonic fluids.

RESULTS

1. Analysis of Methods for Extraction Method #8

The methods used were the same as those for the assay of the embryonic fluids. The only difference occurred in the recovery of internal standard steroids.

A) Recovery of Internal Standard Steroids

Due to an increase in the number of purification steps in the extraction procedure and a decrease in the efficiency of the hydrolysis methods in samples containing increased amounts of protein and lipid, the mean recoveries of steroid from egg yolk were lower than those from the embryonic fluids. The recoveries are listed in Table A1.

Table A1

Recovery of Internal Standards in Estrogen and Androgen
Radioimmunoassays

Steroid	Percentage Recovery
Estrone	51
Estradiol	46
Estrone sulfate	25
Dihydrotestosterone	37
Testosterone	48
Testosterone sulfate	15

11. Experimental Results

Table A2

Concentration of Steroids in the Unincubated Egg Yolk

Steroid	Concentration (pg/ml)		
	Mean	S. E. M.*	n
Estrone	969	228	19
Estradiol	335	274	5
Estrone sulfate	622	146	4
Estradiol sulfate	95	190	5
Testosterone	812	958	17
Dihydrotestosterone	1272	798	20
Testosterone sulfate	1993	173	9
Dihydrotestosterone sulfate	1831	613	9

* recorded as pg of free steroid

A) Radioimmunoassay of Steroids in the Unincubated
Chicken Egg Yolk

a) Unconjugated steroids

1) Estrogens

The mean concentrations of estrone and estradiol were 969 and 335 pg/ml respectively (Table A2).

2) Androgens

Testosterone was present in the egg yolk at a mean concentration of 812 pg/ml while dihydrotestosterone was present at a concentration of 1272 pg/ml (Table A2).

b) Steroid sulfates

1) Estrogen sulfates

The mean concentration of estrone sulfate was 622 pg/ml and of estradiol sulfate was 95 pg/ml (Table A2).

2) Androgen sulfates

The mean concentration of testosterone sulfate in egg yolk was 1993 pg/ml and the concentration of dihydrotestosterone sulfate was 1831 pg/ml (Table A2).

DISCUSSION

The composition of the mature yellow yolk of the chicken egg has a very low percentage of water compared to other physiological fluids. The percent compositions of protein, neutral fats and phosphatides are unusually high: 28%, 50% and 21% respectively. Much of the neutral fat is in the form of cholesterol and cholesterol derivatives. Thus any extraction procedure designed to remove steroids would also extract a variety of lipid substances. As the contaminants are present in the yolk at much higher concentrations than are the steroids, any attempt to produce a relatively pure extract of estrogen or androgen is fraught with difficulty.

None of the extraction methods listed nor any of the countless minor variations thereof produced extracts that were consistently clean enough to yield sufficiently precise elution from the chromatography columns. The hydrolysis method used for the liberation of estrogens and androgens from sulfo-conjugates in the embryonic fluids did not give a high recovery of free steroid when used on extracts of egg yolk.

However, all the methods tried suggest that the estrogens and their sulfo-conjugates are present in concentrations between 0.1 and 1 ng/ml of yolk while the androgens and their sulfo-conjugates seem to be present in concentrations between 1 and 2 ng/ml of yolk. This gives an approximate concentration of 15 ng of estrogen and 30 ng of androgen per egg yolk.

APPENDIX B

Steroid Nomenclature

The systematic nomenclature used in this appendix is taken from the 'Definitive Rules for Nomenclature of Steroids' issued by the IUPAC Commission on the Nomenclature of Organic Chemistry and the IUPAC-IUB Commission on Biochemical Nomenclature (reprinted in 'Pure and Applied Chemistry (1972) 31, 285-322').

<u>Trivial</u>	<u>Systematic Name</u>
Androstenedione	4-Androstene-3,17-dione
Dehydroepiandrosterone	3 β -Hydroxy-5-androsten-17-one
20 α -Dihydroprogesterone	20 α -Hydroxy-4-pregnen-3-one
20 β -Dihydroprogesterone	20 β -Hydroxy-4-pregnen-3-one
Dihydrotestosterone (DHT)	17 β -Hydroxy-5 α -androstane-3-one
Dihydrotestosterone	5 α -Androstane-3-one-17 β -yl-
glucuronide (DHTG)	glucopyranosiduronic acid
Dihydrotestosterone sulfate (DHTS)	5 α -Androstane-3-one-17 β -yl-sulfate
Epi-estriol (epi-E ₃)	1,3,5(10)-Estratriene-3,16 β ,17 β -triol
17 α -Estradiol (17 α -E ₂)	1,3,5(10)-Estratriene-3,17 α -diol
17 β -Estradiol (17 β -E ₂)	1,3,5(10)-Estratriene-3,17 β -diol
17 β -Estradiol 3-glucuronide (E ₂ G)	1,3,5(10)-Estratriene-3-yl-17 β -ol-glucopyranosiduronic acid
17 β -Estradiol 3-sulfate (E ₂ S)	1,3,5(10)-Estratriene-3-yl-17 β -ol-sulfate
Estriol (E ₃)	1,3,5(10)-Estratriene-3,16 α ,17 β -triol
Estrone (E ₁)	3-Hydroxy-1,3,5(10)-estratrien-17-one
Estrone glucuronide (E ₁ G)	1,3,5(10)-Estratrien-17-on-3-yl-8-D-glucopyranosiduronic acid
Estrone sulfate (E ₁ S)	1,3,5(10)-Estratrien-17-on-3-yl-sulfate
17 α -Hydroxyprogesterone	17 α -Hydroxy-4-pregnen-3,20-dione
Pregnanediol	5 β -Pregnane-3 α ,20 α -diol
Pregnenolone	5-Pregnen-3 β -ol-20-one

APPENDIX B - continued

TrivialSystematic Name

Progesterone (Pro)

4-Pregnen-3,20-dione

Testosterone (T)

17 β -Hydroxy-4-androsten-3-oneTestosterone glucuronide
(TG)4-Androsten-3-one-17 β -yl-
glucopyranosiduronic acid

Testosterone sulfate (TS)

4-Androsten-3-one-17 β -yl-sulfate

APPENDIX C

Relationship of Stages of Development and Days of Incubation

"The shortcomings of a classification based on chronological age are obvious to every worker in the field, for enormous variations may occur in embryos even though all eggs in a setting are placed in the incubator at the same time. Many factors are responsible for the lack of correlation between chronological and structural age. Among these are genetic differences in the rate of development of different breeds, seasonal differences in the viability and vigor of embryos, differences in the stage of development when incubation is started, differences in the 'freshness' of eggs, i.e., the lapse of time between laying and incubation, differences in the temperature of eggs when placed in the incubator, and in the size of the individual egg, differences in the temperature of incubation and in the type and size of incubator" (Hamburger and Hamilton, 1951). For all these reasons, the relationship between stages of development and days of incubation given in the following table can only be approximate.

Table C1

Relationship of Stages of Development and Days of Incubation

<u>Stage</u>	<u>Day</u>	<u>Stage</u>	<u>Day</u>
34	8	40	14
35	9	41	15
36	10	42	16
37	11	43	17
38	12	44	18
39	13	45	19

APPENDIX D

Biosynthesis of Androgens and Estrogens in the
Chicken Embryo

Fig. D1. Biosynthesis of androgens and estrogens in the chicken embryo (composed from the data of Galli and Wasserman (1973)). 1, cholesterol; 2, pregnenolone; 3, progesterone; 4, 20 α -dihydroprogesterone; 5, 20 β -dihydroprogesterone; 6, 17 α -hydroxyprogesterone; 7, androstenedione; 8, testosterone; 9, dihydrotestosterone; 10, estrone; 11, 17 α -estradiol; 12, 17 β -estradiol.

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