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Some aspects of steroid hormones biosynthesis by ovarian tissue of the Wilson's phalarope (Steganopus tricolor)

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SOME ASPECTS OF STEROID HORMONES BIOSYNTHESIS BY OVARIAN TISSUE

OF THE WILSON'S PHALAROPE (STEGANOPUS TRICOLOR)

by

Ho-Jane Yu
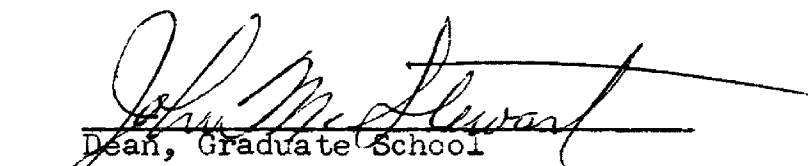
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PART A

CHARACTERIZATION AND IDENTIFICATION OF SOME POLAR PRODUCTS OF <u>IN VITRO</u>

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17α-OL-3,20-DIONE(17α-HYDROXYPROGESTERONE) METABOLISM BY WILSON'S

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I. INTRODUCTION

The family Phalaropidae consists of three species of small shore-
birds, the red, northern and Wilson's phalaropes. All three species
show a considerable degree of sex reversal in comparison to normal pas-
serine, anserine and upland game birds in which the male is the dominant
sex.

The female phalaropes have the more brilliant nuptial plumage and
are more aggressive in courtship and pair formation than the males. It
is suggested that sex hormones might be related to the development of
the breeding plumage (12). Recent investigations by Johns (54) have
shown that when phalaropes are partially plucked in the autumn, the new
feathers that grow in the plucked area are highly colored as in breed-
ing females if the birds are treated with the male sex hormone, but not
when treated with the female sex hormone.

Höhn and Cheng (49) reported that the testosterone content of the
ovaries exceeds that of the testes in breeding phalaropes. However,
only the male incubates the eggs, cares for the young and develops the
incubation patch. Pfeiffer and Johns (53) demonstrated that male sex
hormone (testosterone) and prolactin are required for the formation of a

brood patch. The reason that female phalaropes do not form brood patches is apparently due to a deficiency of prolactin, although blood levels of testosterone also may be low at this time (80).

The prenuptial molt occurs in the early spring and egg laying from May to July. Presumably testosterone secretion by the ovaries exceeds that of the testes during the time of the prenuptial molt. On the basis of the assumption concerning a high testosterone secretion by the ovary in the early spring and a decreasing testosterone production when the reproductive season approaches, the secretion of androgen by the ovaries might follow curve 1 in Figure 1. On the other hand, the secretion of androgen by the testes might follow curve 2 as indicated in Figure 1 since the male apparently does not produce sufficient androgen in early spring to stimulate the development of the bright female type breeding plumage. It is the purpose of the present experiments (Part B) to investigate this hypothesis by measuring the 17β-hydroxysteroid dehydrogenase activity present in the gonads of phalarope during the breeding season.

Recent studies have shown that the sequence of reactions of pregn-5-ene-3β-ol-20-one (pregnenolone)---$\rightarrow$ pregn-4-ene-3,20-dione (progesterone)---$\rightarrow$ 17α-hydroxyprogesterone---$\rightarrow$ androst-4-ene-3,17-dione (androstenedione)---$\rightarrow$ androst-4-ene-17β-ol-3-one (testosterone) exist in all organs which synthesize steroid hormones (Figure 2) (62,93,96). An alternate pathway of conversion pregnenolone to testosterone via 17α-hydroxypregnenolone, androst-5-ene-3β-ol-17-one (dehydroepiandrosterone), and andostenedione has been reported for mammalian endocrine tissue as well (Figure 2) (57,107). The placenta and ovary can further convert
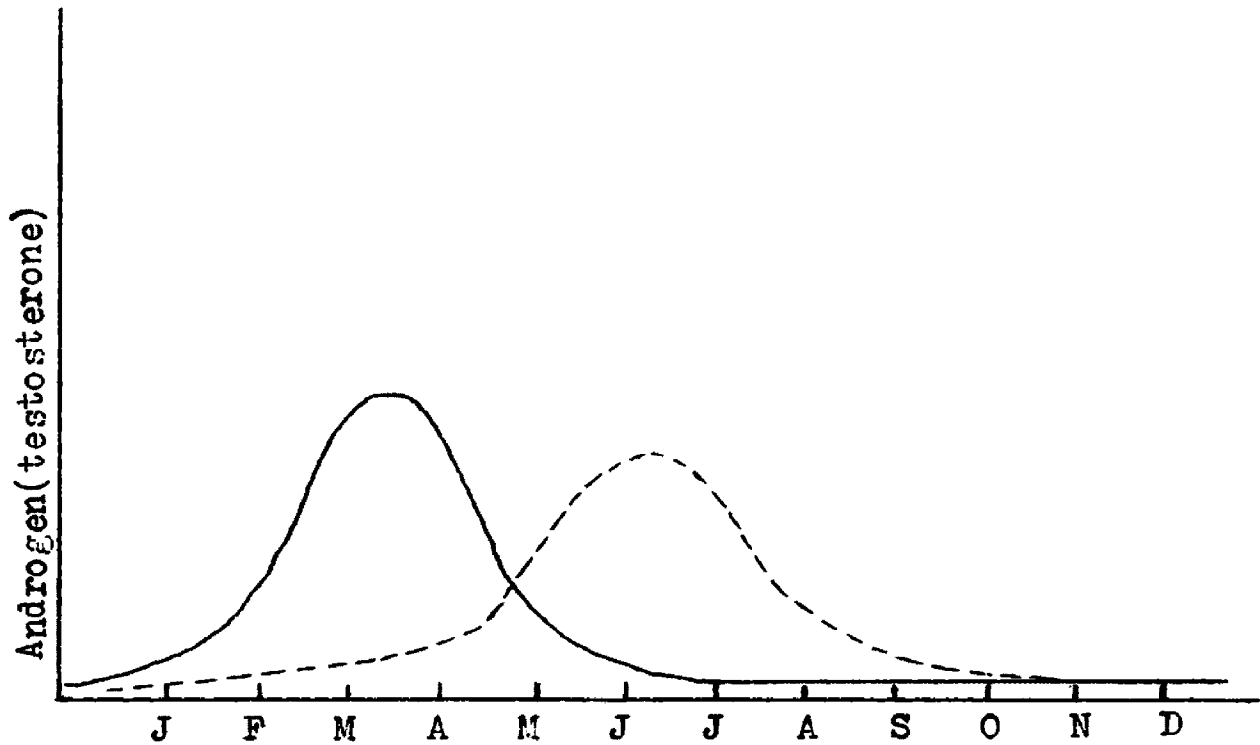
Figure 1   Seasonal change in androgen secretion by
             phalarope gonadal tissues (hypothetical
             curves).

───────────   curve 1. androgen secretion in ovarian tissue.
─ ─ ─ ─ ─   curve 2. androgen secretion in testicular tissue.

androgens to estra-1,3,5-triene-3-ol-17-one (estrone) and estra-1,3,5-triene-3,17β-diol (17β-estradiol) (6,112,83).

It has been well demonstrated by Fevold and Eik-Nes (34,35) that avian testicular tissue is capable of synthesizing both testosterone and androstenedione from progesterone _in_ _vitro_ via a pathway similar to that found for homogenated mammalian tissue (91,83,93). However, there is a major difference in progesterone metabolism between avian and mammalian testicular tissue in the extent of 20-ketone reduction (35). On the other hand, the biosynthesis of some non-phenolic polar steroids and estrogens by Wilson's phalarope ovarian tissue has not been investigated. It is the purpose of these experiments (Part A) to investigate the _in_ _vitro_ biosynthesis of some polar non-phenolic steroids and estrogens from 17α-hydroxypregnenolone and 17α-hydroxyprogesterone by ovarian tissue of Wilson's phalarope.

II. HISTORICAL REVIEW

A. Biosynthesis of pregn-4-ene-17α,20α-diol-3-one and pregn-4-ene-17α,20β-diol-3-one.

Early in 1951, pregn-4-ene-17α,20β-diol-3-one(17α,20β-dihydroxy-progesterone) was first detected as a metabolite of 17α-hydroxypro-gesterone by perfused bovine adrenals (48). After several years, Lynn and Brown (66) isolated the same steroid from testicular tissue incubated with progesterone.

Evidence of the formation of both steroid glycols (20α and 20β form) from 17α-hydroxyprogesterone by ovarian tissue was first demon-strated by Sandor and Lanthier (90). During the same year, these two steroid glycols were found by German biochemists as the intermediates of progesterone by perfused bovine (76) and porcine adrenals (77). Human fetal testicular tissue is also capable of producing both of these 20-ketone reduction products of 17α-hydroxyprogesterone (1).

It is of interest to note that these two steroid glycols can be formed by non-mammalian vertebrates as well. The 20β-hydroxy steroid has been identified in the blood of Pacific salmon (<u>Oncorhynchus nerka</u>) by Idler and his coworkers in 1960 (52). Furthermore, both 20-hydroxy epimers of reduced 17α-hydroxyprogesterone were isolated from homogenates of testes of English sparrow (Passer domesticus) incubated with pro-gesterone-4-^{14}C (34).

The formation of α-isomer or both of these steroid glycols has been reported by other investigators: the α-isomer by bovine adrenal and ovary incubated with 17α-hydroxyprogesterone (63); the 20α-isomer

from progesterone by human minced ovary (107); and both isomers from

human testicular tumor tissue after incubation with either progesterone

or 17α-hydroxyprogesterone (25).

C-20 reductase activity acting on various substrates has been

demonstrated in all steroid producing glands (115). As far as biolog-

ical function of the 20α and 20β reduced 17α-hydroxyprogesterone is

concerned, very few reports have been published. Fevold and Eik-Nes

(36) reported that the presence of 1.0 micromole of unlabeled pregn-4-

ene-17α,20α-diol-3-one in the testicular tissue of English sparrow

incubation medium, progesterone used as substrate, caused a 60% inhi-

bition of the formation of testosterone and 30% decrease in the andro-

stenedione accumulation. It is suggested that this steroid might

inhibit the 17α-hydroxyprogesterone side chain cleaving enzyme (C-17,20-

desmolase). It is also suggested that these two C-20 epimers of

reduced 17α-hydroxyprogesterone cannot be the intermediates in the path-

way of formation of androstenedione or testosterone from 17α-hydroxy-

progesterone since they are the end metabolites in bird's testicular

tissue (33).

B. Biosynthesis of estrogens.

The three main estrogens in the human are estrone,17β-estradiol

and estra-1,3,5-triene-3,16α,17β-triol(estriol). 17β-Estradiol is the

most potent estrogen in the Allen-Doisy test (2). The ovary is consid-

ered as the main site of estrogens formation under normal physiological

conditions. During pregnancy placental estrogen secretion is a 100-1000

times greater than that of the ovary. Recently it has been shown that

testes, adrenal cortices (7) and corpora lutea (51) also take part in the synthesis of estrogens.

The estrogenic hormone was first demonstrated by Doisy and Allen in 1923. A vast amount of work in isolation of estrone and 17β-estradiol eventually culminated in 1929-1930 (19,67,23,20,24).

Numerous studies have shown that estrogens may arise from acetate, cholesterol, progesterone and androgens. Heard and his co-workers (46,47) were the first to study the biogenesis of estrogens from acetate $\underline{in}$ $\underline{vivo}$. They demonstrated that acetate-1-^{14}C could serve as an efficient precusor of estrone in the pregnant mare. In later years, $\underline{in}$ $\underline{vitro}$ studies have shown that estrone and 17β-estradiol are produced from ^{14}C-labeled acetate in the ovaries of human and dog and in the testes of human, dog and cat (16). Labeled pregnenolone may act as precusor to estrone and 17β-estradiol in the ovaries of the human and the pig (21). Estrogens can also arise from progesterone in the ovaries of many species (16).

There are two pathways for the production of testosterone from pregnenolone. The first is Δ^4-pathway which consists of the formation of testosterone via progesterone, 17α-hydroxyprogesterone and andro-stenedione. The other Δ^5-pathway involves 17α-hydroxypregnenolone, dehydroepiandrosterone and androstenedione.

The Δ^4- route was first reported by Slaunwhite and Samuels (93) in testicular tissue and has been verified by several workers (28,92, 29). Solmon $\underline{et}$ $\underline{al}$. (96) also suggested that a Δ^4-pathway exists from progesterone to estrogens in bovine ovarian tissue. The Δ^5-pathway has been reported by several workers to occur in dog (28), rabbit (44) and

bovine tissue (57). In addition, androst-5-ene-3β,17β-diol(androstene-diol) has been postulated as an intermediate in the testosterone bio-synthesis in the $\triangle^5$-pathway (82,41,29,94). Ellis and Berliner (29) demonstrated that pregnenolone is biotransformed into testosterone in mouse testes through an alternative pathway which involves 17α-hydroxy-pregnenolone, dehydroepiandrosterone and androstenediol as intermedi-ates. Rosner et al. (82) reported that after equal concentrations of dehydroepiandrosterone-7-α-^{3}H and 17α-hydroxyprogesterone-4-^{14}C were incubated with rabbit testicular tissue, the ratio of ^{3}H to ^{14}C was higher in the enzymatically synthesized testosterone than in androst-enedione, indicating that dehydroepiandrosterone could form testosterone without going through androstenedione step. However, it is difficult to interpret the experimental results only by the ^{3}H/^{14}C ratio of metabo-lites obtained from the double labeled substrates without considering endogenous steroids level present in the tissue. The conversion of androstenediol into testosterone has been observed in bacterial prepara-tion (104), and homogenates of human placenta and adrenocortical tumor tissue (9,10). It is suggested that androstenedione is not an obliga-tory intermediate in testosterone biosynthesis. A generalized scheme of androgen biosynthesis is presented in Figure 2.

In 1936, Fisher expressed the opinion that testosterone could be converted to natural estrogens by removal of the angular methyl group at C-10 (16). During later years, many biochemists demonstrated the conversion of either androstenedione or testosterone into estrogens in the human placenta, ovaries, testes, adrenal cortices, and corpora lutea (21).

An important discovery in elucidating the estrogen biosynthetic pathway was proved by Meyer (73). He isolated and identified 19-hydroxy-4-androstene-3,17-dione(19-hydroxy-androstenedione) from a homogenate of bovine adrenals incubated with androstenedione and dehydroepiandrosterone. He then concluded that 19-hydroxyl compounds are formed before aromatization occurs. Additional evidence for the role of the 19-hydroxyl derivatives as intermediates in estrogens synthesis was provided by the studies of Longchampt et al. (65). Further work in this field was considerably facilitated by Ryan (84). He discovered that the human placenta contains an enzyme system which is capable of aromatizing androstenedione to estrone in yields of 50-70% when the proper enzyme-substrate ratios were observed. The aromatizing enzyme is localized in the microsomal fraction and requires oxygen and NADPH whereas NADH is ineffective. Hayano et al. (45) obtained the same yield of estrone from 19-oxo-androst-4-ene-3,17-dione(19-oxo-androstenedione) as from 19-hydroxy-androstenedione after incubation with placental microsomes. Therefore, 19-oxo-androstenedione is also a possible intermediate in the pathway of aromatization. An important contribution to the aromatizating mechanism in the estrogens biosynthesis was observed by Breuer and Grill who incubated testosterone, androstenedione and 19-hydroxyandrostenedione with microsomes from human placenta in the presence of NADPH and detected formadehyde and estrogens in the incubation medium from these substrates. The molar ratio of the two products was close to 1 (16).

Until recently, Δ^4-3-keto-C_{19}-steroids were supposed to be obligatory intermediate precusors of estrogens biosynthesis. The well known

Acetate $\longrightarrow$ $\longrightarrow$

cholesterol

progesterone

pregnenolone

17α-hydroxy-
progesterone

17α-hydroxy-
pregnenolone

androstenedione

dehydroepiandrosterone

testosterone
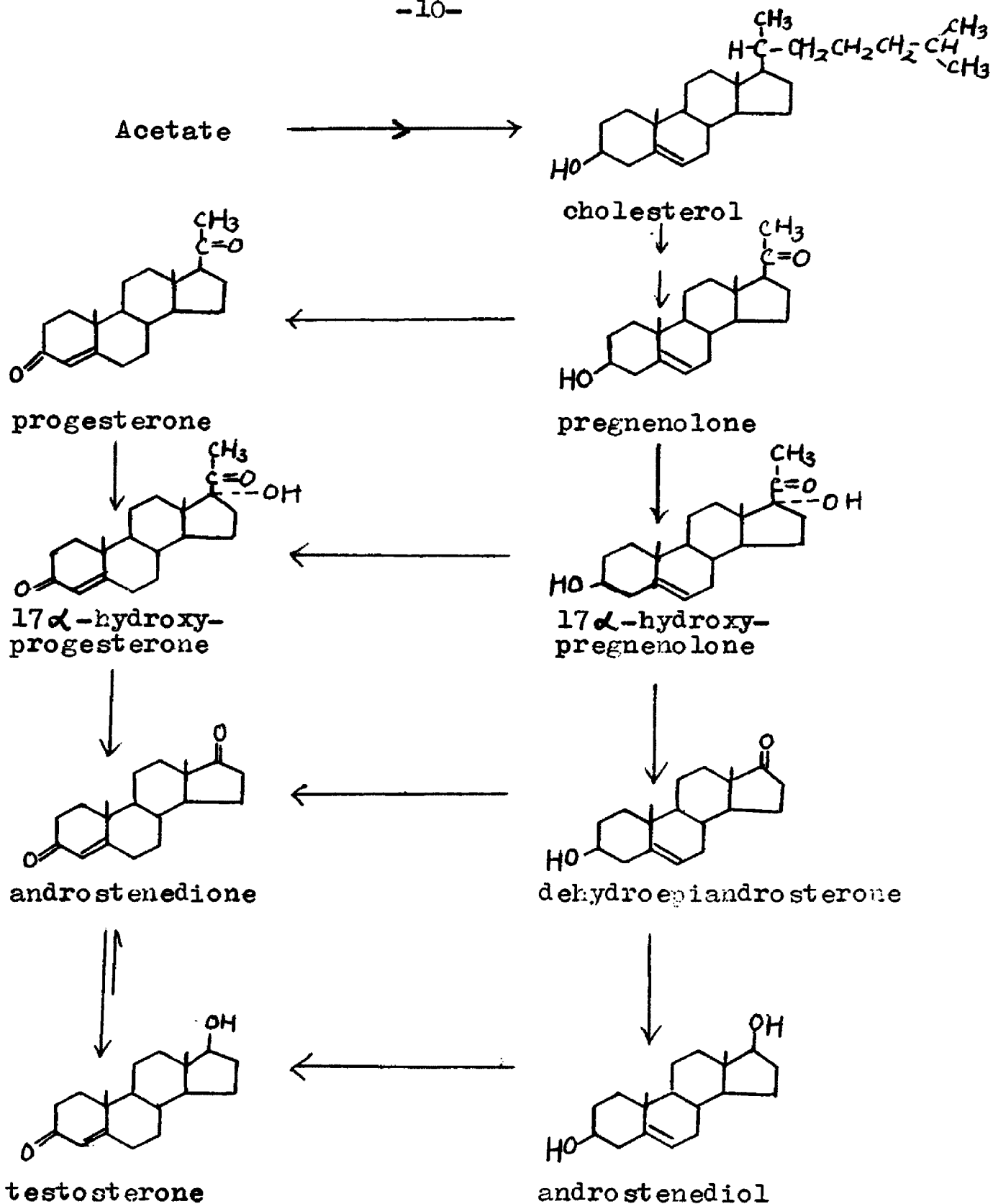
androstenediol

Figure 2 Biosynthesis of androgens

aromatization of dehydroepiandrosterone to estrogens has been explained by the reaction sequence: dehydroepiandrosterone—→ androstenedione or testosterone—→ 19-hydroxyandrostenedione or 19-hydroxytestosterone—→ estrogens. However, the ability of 19-hydroxydehydroepiandrosterone to be aromatized by placental microsomes has been proved by Wilcox and Engel (111). This fact was confirmed by the experiments of Starka and Stastny (98) who observed that the aromatization of 19-hydroxydehydro-epiandrosterone by placental cytoplasmatic and microsomal cell fraction in vitro exceeded not only the aromatization rates of dehydroepiandro-sterone or androstenedione but even that 19-hydroxyandrostenedione. Moreover, 19-hydroxydehydroepiandrosterone was identified in the poly-cystic ovarian tissue incubated with dehydroepiandrosterone (98). This indicates that aromatization of dehydroepiandrosterone may proceed by the pathway via 19-hydroxydehydroepiandrosterone, but with the evidence available it is not possible to deduce whether the conversion of 19-hydroxydehydroepiandrosterone to 19-hydroxyandrostenedione or 19-hydroxy-testosterone is an obligatory step in the formation of estrogens from this precusor.

The interconversion of 17β-estradiol and estrone is the best-known and well demonstrated reaction in vitro and in vivo under various experi-mental conditions in a large number of mammals including rats, guinea pigs, rabbits, cattle and human (16,27). The enzyme responsible for this conversion is 17β-estradiol dehydrogenase, which has been purified by Langer and Engel (60) from human placenta.

Estriol was first isolated from human pregnancy urine by Marrian in 1930. During the following years, Pincus and coworkers demonstrated

the conversion of estrone and 17β-estradiol to estriol in man (16).

Engel et al. (32) and Breuer and coworkers (15) discovered that human liver tissue has the function of converting 17β-estradiol into estriol. Recently Ryan (85) demonstrated that androst-5-ene-3β,16α-diol-17-one (16α-hydroxydehydroepiandrosterone) can be converted to estriol via 16α-hydroxytestosterone in human placenta. A 16α-hydroxy-nonphenolic compound has also been reported as the precusor of estriol in sow ovary by Kadis (55) and Ryan and Magendantz (88) have isolated 16α-hydroxy-epiandrosterone as an estriol precusor from human blood. The presence of a 16α-hydroxylase enzyme system has been reported in sow ovary (56), in swine adrenals (81) and in human ovary (106). Therefore estriol can be synthesized from 16α-hydroxylated non-phenolic compound without involving estrone and estriol.

A summary of the various possible biosynthestic pathways to estrogens are presented in Figures 2 and 3.

Several other phenolic steroids have been subsequently isolated and identified: estra-1,3,5-triene-3,16β,17β-triol(16-epiestriol) from human pregnancy urine (69,70); estra-1,3,5-triene-3,16α-diol-17-one(16α-hydroxyestrobe) in human pregnancy urine (71,72); estra-1,3,5-triene-3,16β-diol-17-one(16β-hydroxyestrone) in women's urine (16); 2-methoxy-estra-1,3,5-triene-3-ol-17-one(2-methoxy-estrone) from human placenta perfusion with 17β-estradiol (31,103).

Marrian et al. (71,72) suggested the hypothesis that 16α-hydroxy-estrone and 16β-hydroxyestrone are formed from estrone by 16α and 16β-hydroxylation and they are intermediate in the "hydration" of estrone to estriol and 16-epiestriol, respectively. This hypothesis was strength-
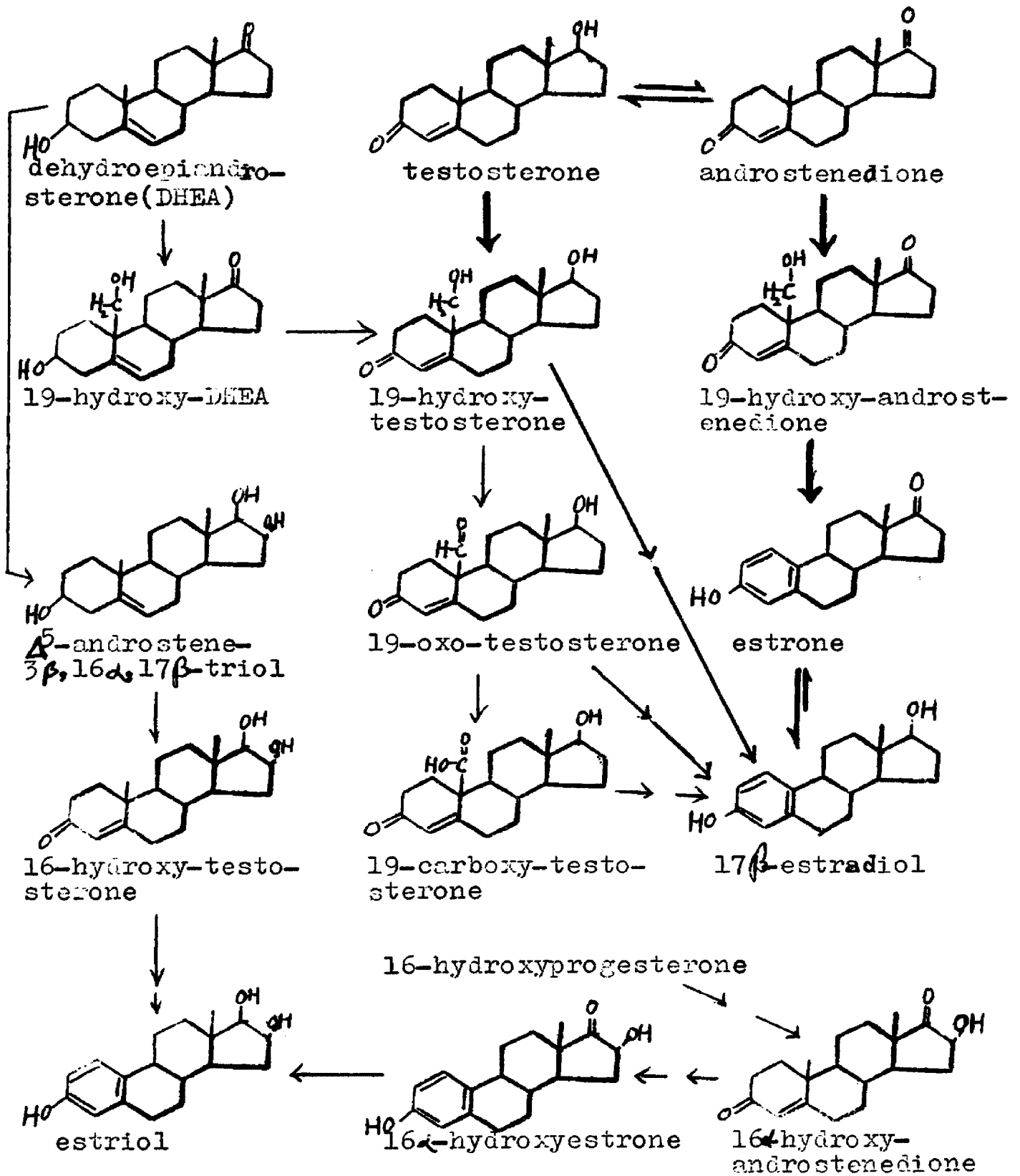
Figure 3. Biosynthesis of estrogens from C-19 steroids

ened by the finding of estriol in the urine of two persons receiving injection of 16α-hydroxyestrone (18) and by the isolation of estriol, estra-1,3,5-triene-3,16α,17α-triol(17-epiestriol) and estra-1,3,5-triene-3,16β,17α-triol(16,17-epiestriol) from human liver slice incubated with 16α-hydroxyestrone (16).

Another 16-substituted estrogen which plays an important part in the intermediary metabolism is 16-oxo-17β-estriol. This phenolic steroid was first obtained in human urine after injection of 17β-estradiol-16-^{14}C (61) and later it was isolated from pregnancy urine by Marrian (71). King (58) incubated estriol with rat kidney homogenates in the presence of NAD and NADP and isolated 16-oxo-17β-estradiol and 16-epiestriol. After incubation of 16-epiestriol in the same system, 16-oxo-17β-estradiol and estriol were detected. Therefore, he suggested that the introduction of 16-keto group most likely proceeds by way of 16α-hydroxylation and the action of a 16-dehydrogenase.

Although 16-oxo-estrone was suspected as an oxidation product of estriol (68), it is not an end product of estrogen metabolism (99,100). Estriol and 16-epiestriol were detected in the urine of a man who had received an injection of 16-oxo-estrone (101). Breuer _et al_ (14) reported that 16-oxo-estrone is a key substance in the metabolism of the 16-substituted estrogens. He found that metabolites after incubation of 16-oxo-estrone with human liver slice were 16α-hydroxyestrone, 16β-hydroxyestrone, 16-oxo-17β-estradiol, estriol, 16-epiestriol, 17-epiestriol and 16,17-epiestriol. However, little information concerning the formation of the 16-oxogenated estrogens by gonadal tissue is available. The interrelations of 16-oxo-estrone, 16-oxo-17β-estradiol and

other 16 and 17 substituted phenolic estrogens are presented in Figure 4.

The metabolic reactions involve a wide variety of transformations such as introduction of hydroxy groups, reversible oxidation-reduction of hydroxyl and carbonyl groups and methylation. In general, C-2, C-6, C-16 of the estrogens appear to be the most prominent metabolic transformation positions (31,15,72). More detailed interrelationship of metabolism of estrogens will not be discussed here.
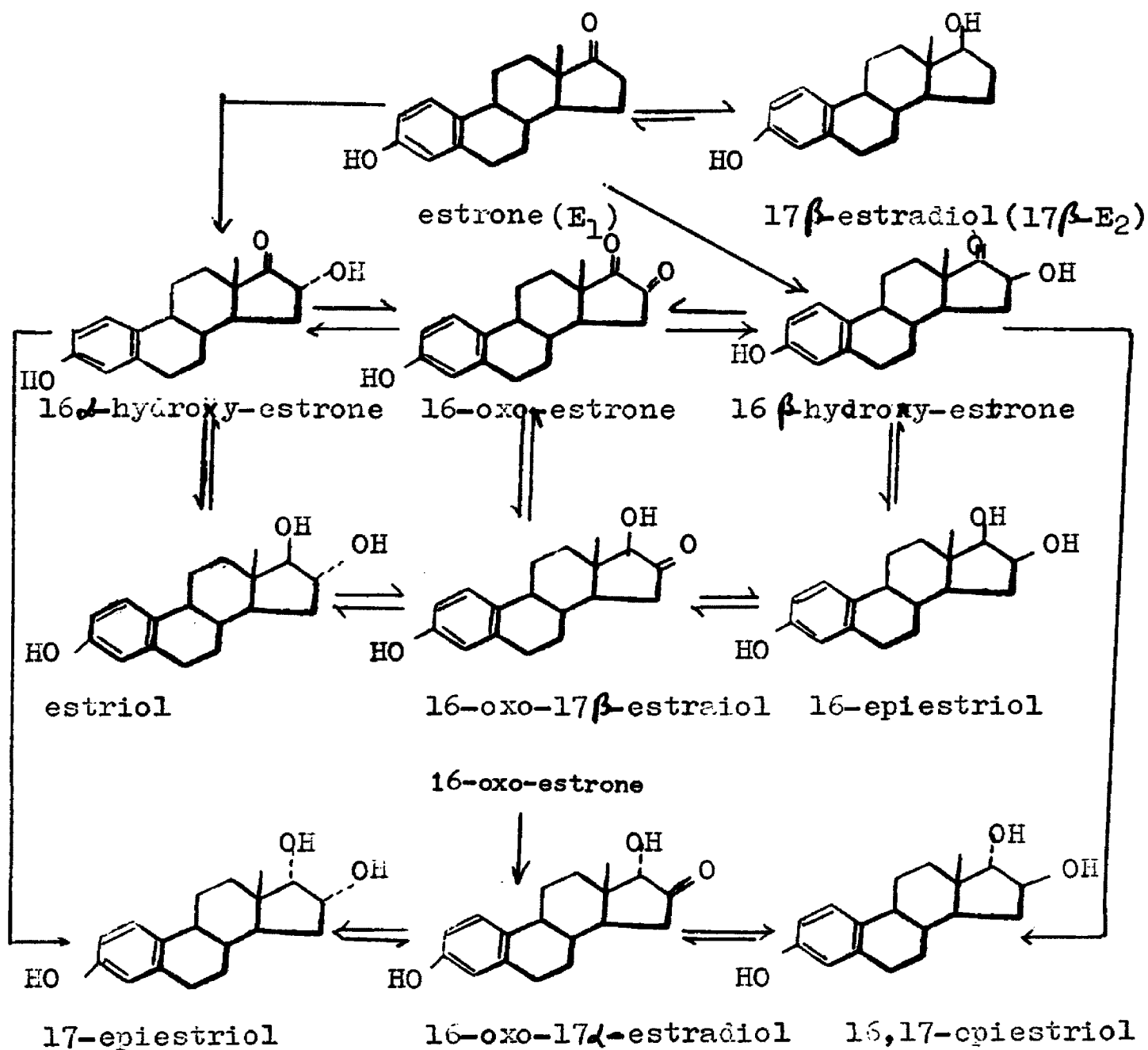
Figure 4. Interrelationship of estrone and 17β-estradiol with 16 and 17 substituted phenolic estrogens.

III. EXPERIMENTAL MATERIALS AND PROCEDURES

A. Experimental animals.

Wilson's phalarope was selected as the experimental animal since it shows a partial sex reversal behavior. The birds were obtained in Western Montana during the first two weeks of May. Within ten minutes after the birds were shot, the gonads were removed, weighed, and frozen in liquid nitrogen.

B. Chemicals.

1. 17α-Hydroxypregnenolone-7-α-^{3}H (Mann S 4890), specific activity 100 mc/mmole.

2. 17α-Hydroxyprogesterone-4-^{14}C (Nuclear England Corp., Lot No. 134-204-23a), specific activity 36.2 mc/mmole.

3. Estrone-6,7-^{3}H (TRA, 126, Batch 3, Nuclear Chicago), specific activity 500 mc/mmole.

4. 17β-Estradiol-4-^{14}C (Batch 2, CFA 320, Nuclear Chicago), specific activity 31.8 mc/mmole.

5. 20α-Reduced form of 17α-hydroxypregnenolone-7-α-^{3}H (specific activity as number 1) was prepared by reduction with LiALH$_4$ in benzene and ether solvents (personal communication, J. W. Hinman, The Upjion Co., Kalamazoo, Mich.)

All radioactive standard steroids were purified by paper chromatography in the benzene/formamide system prior to use.

6. Cofactors and additives

a. Triphosphopyridine nucleotide (TPN), Lot. No. 16-B-7002, and diphosphopyridine nucleotide (DPN), Lot No. 15-B-7260 were

obtained from the Sigma Chemical Company and used without further purification.

  b. <u>Adenosine triphosphate</u> (ATP) 99% pure, control No. 6211 was obtained from Nutritional Biochemical Corporation.

  c. <u>Sodium fumarate</u> (Fu 15684) was made by C. F. Boehringer and Soehne Gmbh Mannheim Company in Germany.

  d. <u>Nicotinamide</u> was procured from Matheson, Coleman, and Bell Company.

  7. <u>Buffer.</u> Krebs-Ringer phosphate buffer was prepared as described by Umbreit <u>et al.</u> (105).

  8. <u>Standard steroid solutions.</u> Steroid solutions were prepared by dissolving weighed quantities of crystalline steroids in redistilled ethanol and were stored at 4°C. The concentrations of solutions of Δ^{4}-3-keto steroids were determined by their ultraviolet absorbanced at 240 mμ.

  9. <u>Solvents.</u> All solvents were spectral grade or were redistilled prior to use.

  10. <u>1,2-Dihydroxypropane</u> (propylene glycol) was obtained from Matheson, Coleman and Bell Company (Lot N. 2493) and was used without further purification.

  11. <u>Chromium trioxide (CrO$_3$)</u>, Lot No. 2576, Mallinckrodt Chemical works was stored in a desiccator.

  12. <u>Pyridine</u> with a Lot No. 7180 was obtained from the same company as that of CrO$_3$ and was vacuum redistilled and stored in a desiccator.

13. A.C.S. reagent Code 1002 <u>acetic anhydride</u> was purchased from Allied Chemical Company and stored in a desiccator after vacuum distillation.

14. <u>Acetic acid.</u> Allied Chemical glacial acetic acid (A.S.C. reagent Code 1019) was vacuum redistilled over acetic anhydride and stored in a desiccator.

15. <u>Dimethyl sulfate</u> with Lot No. p 328 was obtained from Easterman Organic Chemicals and redistilled before use.

16. <u>Glucose-6-phosphate dehydrogenase</u> was purchased from Cal-Biochemical Company, with Lot No. 64891, 140 Eu/mg.

17. <u>Scintillation mixture.</u> This solution was prepared by dissolving 4 gm of 2,5-diphenyloxazole(PPO) and 50 mg of 1,4-bis-2-(5-phenyloazolyl)-benzene(POPOP) in one liter of toluene and was stored in a brown bottle.

18. <u>Silica gel.</u> Silica gel G, No. 7731, acc. to Stahl from E. Merck AG. Darmstad (Germany) Company was used for thin layer chromatography. Silica gel (No. 7734) from the same company as above with mesh size 0.05-0.20 mm was used for column chromatography.

19. <u>Chromatography paper.</u> Whatman No. 1 filter paper (46x57 cms) was used for chromatographic separation.

20. <u>Nitrogen.</u> Bottled water-pumped nitrogen was obtained from the Industrial Air Products Company and filtered through glass wool prior to use.

21. <u>Thin layer chromatography plate.</u> Thin layer chromatography was done on glass plates coated with silica gel G. Fifty milliliters

water was added to 25 gm silica gel G mixed with luminescent phosphor (Du pont 609), and the slurry was spread on various size glass plates (20x20, 8x20, 5x20 cms) at a thickness of 0.2 mm. After half an hour, the plates were heated at 110°C for 1.5 hours and placed in a desiccator (4).

22. **N,N-dimethyl formamide**, Matheson, Coleman, and Bell Co., SG 5974.

23. **Bis-(trimethylsilyl)-acetamide**, Chemical Co., Inc. 071071.

24. **Trimethylchlorosilane(CH$_3$SiCl$_3$)**, Pierce Chemical Company, stock No. 885 304.

C. Equipment.

1. **Shaking incubator** - A research Specialties Model 2156 Constant temperature shaking water bath was used for the incubations.

2. **Strip counter** - A Model RSC paper strip and thin-layer plate gas-flow counter designed by Atomic Accessories, Inc., was used to locate radioactivity on paper and thin-layer chromatograms.

3. **Scintillation counter** - A Liquid Scintillation Spectrometer (Nuclear Chicago Model No. 812830) was used to determine ^{3}H and ^{14}C quantitatively (106).

4. **Spectrophotometers** - Beckman Model DU spectrophotometer was used to determine the absorbancy of solutions at various wave lengths.

5. **Haines scanner** - Ultraviolet absorbing material on paper chromatograms was located by use of a scanning device described by Haines (1950).

6. **PH meter** - A Corning pH meter Model 12 was used to measure the pH of the buffer solutions.

7. <u>Gas Chromatography apparatus</u> - F and M Scientific Model 402 High Efficiency Gas Chromatograph equipped with dual flame ionization detectors was employed for steroid identification to analyze steroids.

8. <u>Autoclave</u> - The glassware for incubations was sterilized in an American Sterilizer (patent number 2470776) designed by Erie Company, Pennsylvania.

9. <u>Centrifuge</u> - A Model No. 76344 H centrifuge manufactured by International Equipment Co. was used to centrifuge the mixtures.

D. Procedures.

    1. <u>Paper chromatography</u>

Two centimeter strips of Whatman No. 1 filter paper were impregnated with formamide as the stationary phase, loaded with the substance and the mobile phase added last. The choice of the solvent systems was according to Zaffaroni-type systems (113,114). The chromatograms were allowed to develop in a sealed glass tank, pre-saturated with developing solvents, with descending flow technique.

    2. <u>Drying process</u>

The aliquots of samples were evaporated to dryness under a stream of nitrogen gas in a constant temperature water bath at $40^{\circ}C$. The residues were rinsed with absolute ethanol.

    3. <u>Substrate preparations</u>

Fifty-two microcuries of 17α-hydroxypregnenolone-7-α-^{3}H, 0.43 mg of non-radioactive 17α-hydroxypregnenolone, 19.5 μc of 17α-hydroxyprogesterone-4-^{14}C and 0.25 mg of non-radioactive 17α-hydroxyprogesterone were dissolved in 2.6 ml ethanol propylene glycol(1:1). The final con-

centrations were approximately 0.05 μmole and 2 uc of 17α-hydroxypreg-nenolone and 0.05 μmole plus 0.75 μc of 17α-hydroxyprogesterone per Q10 ml of ethanol:propylene glycol(1:1). The tube containing substrates was stored in a freezer until used.

4. Tissue preparation

Previously frozen ovarian tissue was thawed and placed in a glass homogenizer containing 20 ml Krebs-Ringer phosphate buffer pH 7.35 and homogenized with a Ten-Broek tissue grinder at $0^\circ C$. (Table 1).

Concentration of cofactors in the buffer were 40 mM nicotinamide: 0.4 mM DPN, ATP, and TPN; 0.1 mM sodium fumarate; and 1 mg/ml of both D-glucose-6-phosphate and β-D-glucose. The tissue concentration was 104 mg per ml.

5. Preparation of incubation flasks

All glassware used in the incubations was sterilized in an auto-clave prior to use to prevent microbiological transformations of steroid molecules. Glass stoppered or cork stoppered 25 ml Erlenmeyer flasks were used as incubation vessels.

Two tenth ml of substrates were added to each incubation flask and ethanol was evaporated. Two ml of homogenate and glucose-6-phos-phate dehydrogenase were placed into the incubation flasks. Blank incu-bation flasks were prepared in the same manner except the tissue homoge-nate was replaced by Krebs-Ringer phosphate buffer containing cofactors. The flasks were gassed for one minute with 95% O_2-5% CO_2 immediately after the addition of the homogenate and tightly stoppered.

6. Incubation

Table 1

Phalarope ovaries used for incubation

Bird No.	Date collected	Weight(mg)
66-4	5-16-66	225.0
66-9	"	195.0
66-10	"	144.8
66-17	"	446.0
66-21	"	136.4
66-31	"	242.0
66-32	"	220.0
65-6	5-7-65	130.8
65-41	6-21-65	182.8
65-65	6-21-65	158.4
65-62		

Total wt. 2081.2 mg

Remark: Each incubation flask contained about 208.1 mg homogenized tissue.

Each flask was placed in the constant temperature shaking water bath and incubated at $41^{o}C$ for three hours. The reaction was stopped by adding 5.0 ml diethyl ether:ethyl acetate (4:1, v/v), mixing thoroughly and freezing at $-20^{o}C$.

7. Extraction

a. The incubation media were thawed, transferred into citric acid tubes and the incubation flasks were rinsed with 3.0 ml glass-redistilled water. The resulting 5.0 ml aqueous phase was extracted with four 10.0 ml volumes of ethyl ether:ethyl acetate (4:1, v/v). The extraction tubes were centrifuged for ten minutes at 2500 rpm after each extraction to separate emulsions. The four volumes of organic extract were combined and evaporated to dryness.

b. One tenth microcurie of estrone-6,7-^{3}H, 0.04 µc of estradiol-4-^{14}C and 0.01 µc of 20α-reduced form of 17α-hydroxypregnenolone-7-α-^{3}H were added in 5 ml of water to each of two citric acid tubes. Extraction of these standards was carried out at the same time and in the same manner as were the incubation samples. The results from these extractions were used for the calculation of the percent recovery of the added compounds.

8. Product purification, isolation and identification

a. Separation of neutral steroids from phenolic steroids

Separation of the phenolic and non-phenolic steroid was accomplished by partitioning the extracts between 1.0 N NaOH and toluene (30). The neutral steroid fraction was chromatographed on paper in the

heptane/formamide system. The chromatograms were allowed to run for one hour after the mobile phase had reached the end of the strips and were then removed from the tank. After the heptane was evaporated from the paper, the strips were further developed in the heptane:benzene(1:1)/formamide system to front. Fifty micrograms each of authentic testosterone, androstenedione and progesterone were chromatographed on a separate strip with each sample chromatogram.

The phenolic steroids were chromatographed in the benzene/formamide system to front. Fifty micrograms each of authentic estrone and 17β-estradiol were chromatographed simultaneously with each chromatogram of samples. The ultraviolet absorbing compounds located on the dry chromatograms were visulized by means of the Haines scanner and the non-radioactive standard estrogens were detected by running Barton's reaction (8) on the dried standard strip. The radioactive steroids on the chromatogram were located by scanning on a strip counter.

Areas of the chromatograms containing radioactive metabolites were eluted with methanol. Like fractions were pooled and evaporated to dryness for further identification.

  b. Further paper chromatographic separations were employed in the following systems:

1. benzene/formamide   2. heptane:benzene(1:1)/formamide

3. heptane/formamide   4. chloroform:benzene(1:1)/formamide

5. chloroform/formamide   6. toluene/propylene glycol

Each isolated substance was repeatedly chromatographed in these systems to establish homogeneity.

c. Acetylation

After the separation of steroids appeared to be complete, acetylation were performed. Two tenth ml of pyridine:acetic anhydride (4:1 v/v) were placed in the tube containing the unknown metabolite. The tube was tightly stoppered and placed in a dark hood overnight (8-16 hours). The reaction was stopped by the addition of one ml of absolute ethanol. The ethanol, pyridine and acetic anhydride were evaporated. The acetate derivatives of unknown and standard steroids were applied to paper strips and the chromatogram developed in several systems as mentioned above (33).

d. Saponification

Since the amount of metabolites available was small, the acetate derivatives were saponified to regain the original compound. Saponification was performed by dissolving the acetate derivatives in 0.5 ml ethanol to which 0.5 ml of 2.5% sodium carbonate was added. The reaction was allowed to proceed at room temperature overnight. At the end of this period, the ethanol was evaporated; the solution diluted to 5 ml with water; and the saponified material and remaining ester were extracted with five equal volumes of ethylacetate. The free and esterified compounds were separated by paper chromatography in appropriate systems (33).

e. Oxidation

The free steroids obtained by saponification were identified further by oxidation with 0.2 ml of a saturated solution of chromium trioxide(CrO_3) in glacial acetic acid. After thirty minutes of reaction

5.0 ml of deionized water were added to tubes and the aqueous solution was extracted immediately with 5.0 ml ethyl acetate. The extraction was repeated three times. The extraction volumes were combined and evaporated to dryness (33).

f. Column chromatography

Phenolic metabolites were purified by chromatography on silica gel columns, eluted sequentially with dichloromethane (CH_2Cl_2) containing 0%, 2%, 5%, 10% and 20% acetone. A total of 15 ml of each solvent mixture was added to the column and the effluent collected in three 5.0 ml aliquots, labeled as A, B and C. The standard estrogens eluted from the column were determined by Kober's reaction, and absorbance values were determined by using Allen's correction (3,102,17).

g. Methylation

Methylation of steroids was performed according to Attal's method (4). Six ml of 1 N NaOH were added to samples, followed by the addition of 5.0 ml of 4.5% (w/v) boric acid in water. The mixture was kept at $40^\circ C$ for 5 minutes; 0.5 ml of dimethyl sulfate was added and the mixture shaken vigorously and left overnight at $40^\circ C$. Two and half ml of 20% (w/v) aqueous sodium hydroxide was added and the mixture extracted with 5 ml n-hexane three times. The combined hexane extracts were washed with 2 ml distilled water and evaporated to dryness.

h. Thin-layer chromatography

Thin-layer chromatography was performed according to the method of Neher (79). The silica gel thin-layer chromatography plates were heated at $110^\circ C$ for one hour prior to use. After the samples were

loaded, the plates were placed in a sealed glass tank containing the appropriate developing solvents and ascending developing technique was employed (64,97,4).

The radioactive steroids on the plates were located by counting radioactivity on a thin-layer counter. The non-radioactive estrogens on the plates were detected by exposing the plates to iodine vapor.

i. Elution technique from thin-layer plates

The silica gel containing the steroids of interest was scraped off the plate 0.5 cm below and 0.5 cm above the area corresponding to radioactive area. The removed silica gel was extracted with proper solvents (4).

j. Gas chromatography

Suitable derivatives of steroids were made for gas chromatographic analysis. Bis-trimethyl silyl ether derivative of unknowns was formed by adding 25 μl of reagent mixture (1.6 ml N,N-dimethyl formamide, 0.1 ml trimethyl-chlorosilane and 0.3 ml Bis-(trimethylsilyl)-acetamide to the unknowns containing 10 microgram of cholestane (59).

Analysis of the samples and the blanks were performed by using either a 3' mm I.D. x 4' glass column coated with 3.8% SE-30 or a 3' mm I.D. x 6' glass column coated with 2.0% XE-60 on Model 402 High Efficiency Gas Chromatograph.

IV. RESULTS

A. Identification of metabolites X_1 and X_2

Figure 5 is the tracing of the initial paper chromatogram of neutral metabolites isolated from the incubation media. X_1 and X_2 were isolated from the most polar fraction of the non-phenolic metabolites (Figure 5 area #1). Further purification and identification of unknowns are presented in Figures 6 and 7. The chromatographic evidence supporting the identities of X_1 and X_2 is shown in Tables 2 and 3.

Both X_1 and X_2 have an ultra-violet absorption maximum around 240 mμ. Acetylation of X_1 produced a monoacetate derivative of lower polarity. The monoacetate derivative of X_1 had the same mobility as the authentic 20α-acetate derivative of pregn-4-ene-17α,20α-dihydroxy-3-one (17α,20α-dihydroxyprogesterone) in three different paper chromatographic systems (Table 2). The mobility of X_2 was slightly less polar than X_1. It also formed a monoacetate derivative on acetylation and had the same chromatographic R_f values as those of the authentic 20β-acetate derivative of pregn-4-ene-17α,20β-diol-3-one (17α,20β-dihydroxyprogesterone) in several different paper chromatographic systems (Table 3).

Oxidation of X_1 and X_2, obtained from the saponified acetate derivatives, yielded from each a major product with a chromatographic mobility identical to standard androstenedione and a minor product of R_f value identical to that of 17α-hydroxyprogesterone (Tables 2 and 3).

$^3H/^{14}C$ Ratios of initial substrates and neutral metabolites X_1 and X_2 were listed in Table 4. Both unknowns contained tritium and carbon-14 label. However, the ratios were less than that of the substrates.
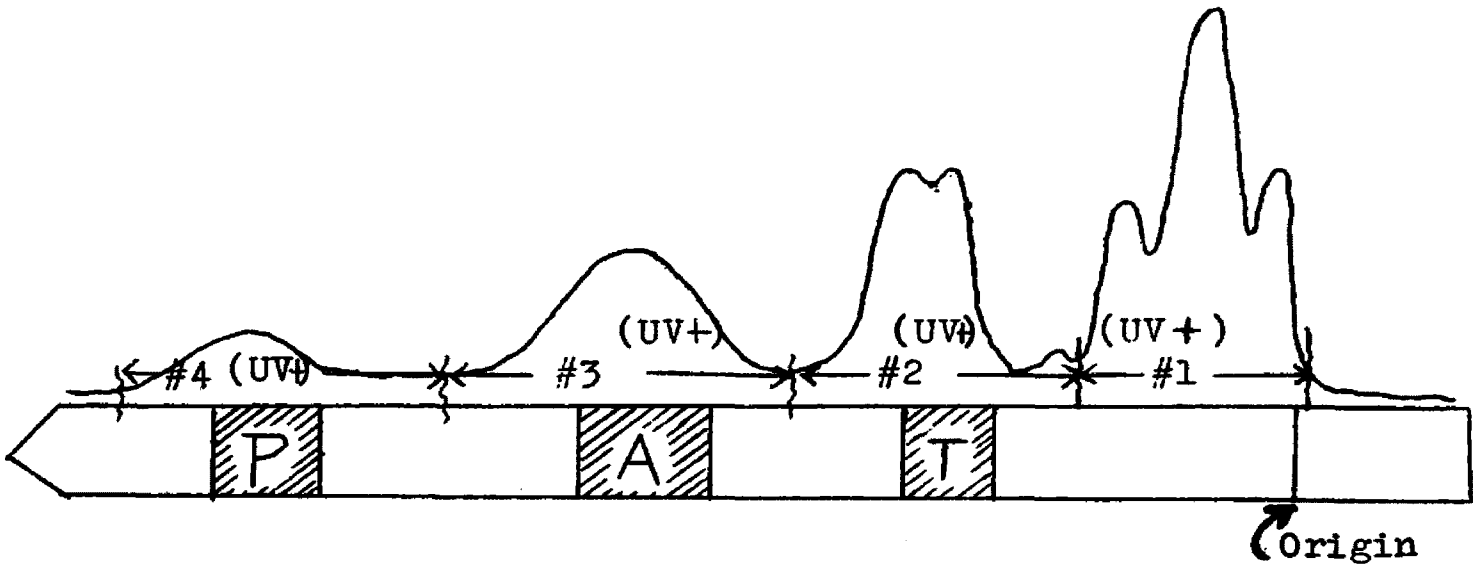
Figure 5. Tracing of the first chromatographic separation of radioactive neutral metabolites isolated from incubations of phalarope ovarian tissues cell-free homogenate with 17α-hydroxypregnenolone-7α-^{3}H and 17α-hydroxyprogesterone-4-^{14}C substrates.

T = authentic testosterone

A = authentic androstenedione

P = authentic progesterone
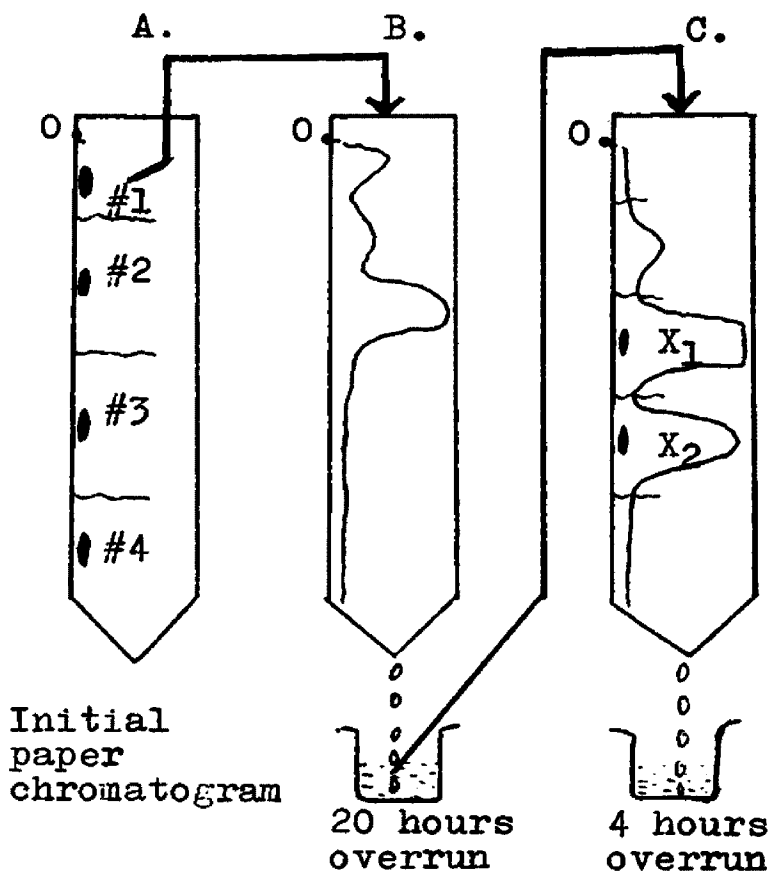
Figure 6. Purification of neutral steroids in the benzene/formamide system by paper chromatography.

O.= Origin

● = ultra-violet positive steroids

Figure 7. Procedure used for isolation and identification of X_1 and X_2

X_1 , X_2

heptane:benzene(1:1)/formamide 8 hrs.overrun(PC)

↓

acetylated with 0.2 ml Pyridine:acetic anhydride (4:1)

↓

heptane:benzene(1:1)/formamide to front(PC)

↓

heptane:benzene(1:1)/formamide 2 hrs overrun(PC)

↓

benzene/formamide to front(PC)

↓

heptane/formamide 4 hrs overrun(PC)

↓

saponified with 0.5 ml ethanol and 0.5 ml 2.5% sodium carbonate

↓

heptane:benzene(1:1)/formamide 2 hrs. overrun(PC)

↓

saponified acetate derivatives of X_1, X_2

½   ½

Chloroform:acetone   oxidized with 0.2 ml
(50:50 v/v) (TLC)   CrO_3/acetic acid

↓   ↓

Bis-trimethylsilyl   heptane:benzene(1:1)/
derivative   formamide to front(PC)

↓   ↓

gas chromatographic analysis   benzene/formamide to
on 3.8% SE-30 column   front(PC)

*

PC :   paper chromatography
TLC: thin layer chromatography

Table 2   Chromatographic identification of X_1

Paper Chromatographic System	Prior Treatment	R_f values	
		X_1	Authentic Standard Pregn-4-ene-17α,20α-diol-3-one
heptane:benzene (1:1)/formamide 8 hrs. overrun	none	4.7-12.5* (3.6)	5.0-12.9* (8.8)
heptane:benzene (1:1)/formamide	Ac	0.32	0.32
heptane:benzene (1:1)/formamide 2 hrs. overrun	Ac	23.2-30.0* (26.5)	24.0-27.2* (26.6)
benzene/formamide	Ac	0.73	0.73
heptane/formamide 4 hrs. overrun	Ac	1.5-6.8* (3.7)	3.0-4.2* (3.6)
heptane:benzene (1:1)/formamide 2 hrs. overrun	Sa	2.7-4.5* (3.6) 25.0-31.5* (23.1)	2.5-4.1* (3.6) 26.5-31.0* (28.3)
heptane:benzene (1:1)/formamide	Ox	0.14** 0.47***	0.15** 0.47***
benzene/formamide	Ox	0.53** 0.73***	0.54** 0.78***
chloroform:acetone (50:50 v/v)****	Sa	0.44	0.44

*=mobility from origin(cm)
**=mobility similar to 17α-hydroxyprogesterone in all two systems.
***=mobility similar to androstenedione in all two systems.
****=thin-layer chromatography.
Ac=acetylated.
Ox=oxidized.
Sa=saponified.

Table 3.   Chromatographic identification of X_2

Paper Chromatographic system	Prior Treatment	R_f values	
		X_2	Authentic Standard pregn-4-ene-17α,20β-diol-3-one
heptane:benzene (1:1)/formamide 8 hrs.overrun	none	9.6-15.0* (11.7)	10.0-12.9* (11.5)
heptane:benzene (1:1)/formamide	Ac	0.32	0.31
heptane:benzene (1:1)/formamide 2 hrs. overrun	Ac	24.6-32.0* (27.9)	25.8-29.3* (27.6)
benzene/formamide	Ac	0.73	0.73
heptane/formamide 4 hrs.overrun	Ac	2.4-6.6* (4.2)	2.9-4.9* (3.9)
heptane:benzene (1:1)/formamide 2 hrs.overrun	Sa	3.0-6.5* (4.2) 24.0-31.0* (27.0)	3.5-4.6* (4.1) 24.7-28.7 (26.8)
heptane:benzene (1:1)/formamide	Ox	0.17** 0.54***	0.17** 0.54***
benzene/formamide	Ox	0.55** 0.78****	0.54** 0.78***
chloroform.acetone (50:50 v/v)****	Sa	0.56	0.56

*=mobility from origin (cm)
Ac=acetylated.
Ox=oxidized.
Sa=saponified.
**=mobility similar to 17α-hydroxyprogesterone in both two
   systems.
***=mobility similar to androstenedione in both two systems.
****=thin layer chromatography.

-35-

This means that the formation of unknowns from 17α-hydroxypregnenolone-7-^{3}H was less than that from 17α-hydroxyprogesterone.

The results of paper chromatographic analysis of the parent compound, the acetate derivative and/oxidation products of X_1 and X_2 are
the
conclusive evidence that X_1 is pregn-4-ene-17α,20α-diol-3-one, whereas X_2 is pregn-4-ene-17α,20β-diol-3-one.

B. Partially characterized metabolites

Figure 8 shows the isolation and identification processes used for phenolic steroids. The radiochromatogram scanner tracing of the initial paper chromatographic separation of the phenolic metabolites is presented in Figure 9. Three metabolic fractions, #P_1, #P_2, #P_3 were eluted from the chromatograms. #P_2 corresponded to the area of standard 17β-estradiol and #P_3 corresponded to the area of standard estrone on the chromatogram. The P_2 and P_3 fractions were further purified through silica gel columns. The effluent patterns of estrone and 17β-estradiol through silica gel columns are indicated in Figure 11. Estrone was eluted from the column in fractions 2%B through 5%C acetone in CH_2Cl_2 whereas 17β-estradiol was eluted from 5%C to 20% A acetone in CH_2Cl_2 fractions. After collecting the eluted estrone and 17β-estradiol fractions from the column chromatography of fractions P_2 and P_3, the methoxy-derivatives were formed.

The thin-layer chromatogram of the methylated products of P_2 and P_3 is shown in Figure 12. The mobility of 3-methoxy-17β-estradiol is similar to that of some impurity in the methylation blank. No spot corresponding to authentic 3-methoxy-estrone was found in either P_2 or

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Table 4. $^3H/^{14}C$ ratios of initial substrates and neutral metabolites X_1, X_2

	$^3H/^{14}C$
Substrates 17α-hydroxypregnenolone-7-α-^3H 17α-hydroxyprogesterone-4-^{14}C	2.535
Metabolites X_1	0.727
X_2	0.456

Table 5. Percent recovery of estrogens in the isolation processes.

Standard Estrogens	Recovery (Initial Extraction)	Recovery (Silica gel Column)	Recovery (Total)
Estrone-6,7-α-^3H	59.20%	85.40%	50.10%
17β-Estradiol-4-^{14}C	39.15%	58.90%	23.03%

Figure 8. Procedure used for isolation of phenolic metabolites from

phalarope ovarian tissue homogenate incubations.

alkali-soluble phenolic metabolites

adjusted to pH 9.0±0.5

ether soluble

water soluble
(discard)

benzene/formamide to front(PC)
(Fig 9.)

#P$_1$

#P$_2$ #P$_3$

chloroform/formamide
to front(PC)(Fig.10)

passed through separate silica gel column

#P$_1$-A   #P$_1$-B   #P$_1$-C

eluted sequentially with 15 ml of each solvent
of CH$_2$Cl$_2$ containing 0%, 2%, 5%, 10% and
20% acetone.

appropriate fractions were combined.
(Fig. 11)

methylated with 0.5 ml dimethyl sulfate
and 5.0 ml 4.5%(w/v) boric acid in
water.

benzene:methanol(99:1 v/v) to front
(TLC) (Fig. 12)

eluted

gas chromatographic analysis on 3.8%
SE-30 column (Table 6)

PC = Paper chromatography

TLC = Thin layer chromatography

Figure 9   Tracing of the first paper chromatogram of phenolic metabolites isolated from incubation of phalarope ovarian tissue cell-free homogenate with 17α-hydroxypregnenolone-7-α-^{3}H and 17α-hydroxyprogesterone-4-^{14}C in the benzene/formamide system.

E_1=estrone , E_2=17β-estradiol
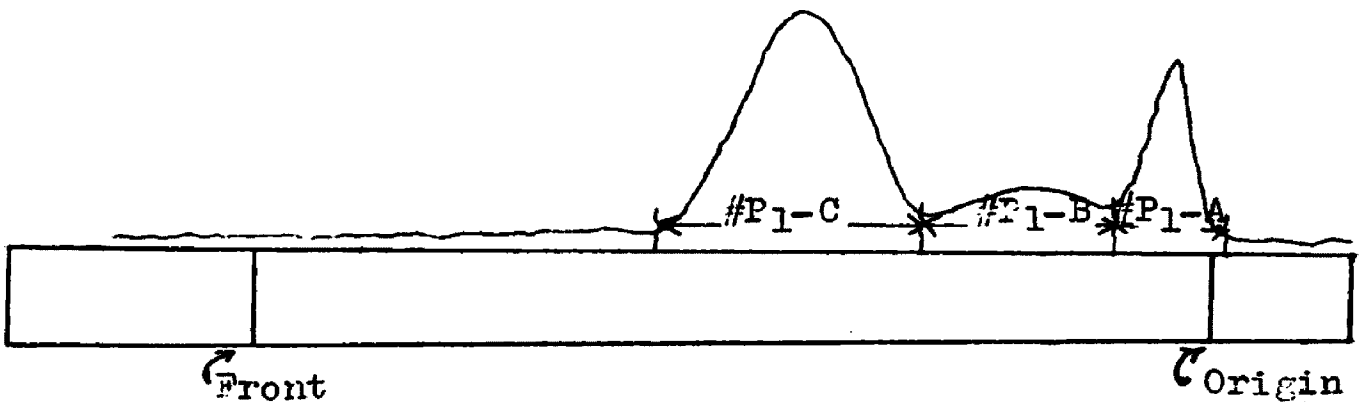
Figure 10   Tracing of paper chromatogram of phenolic metabolite #P$_1$ fraction in the chloroform/formamide system.
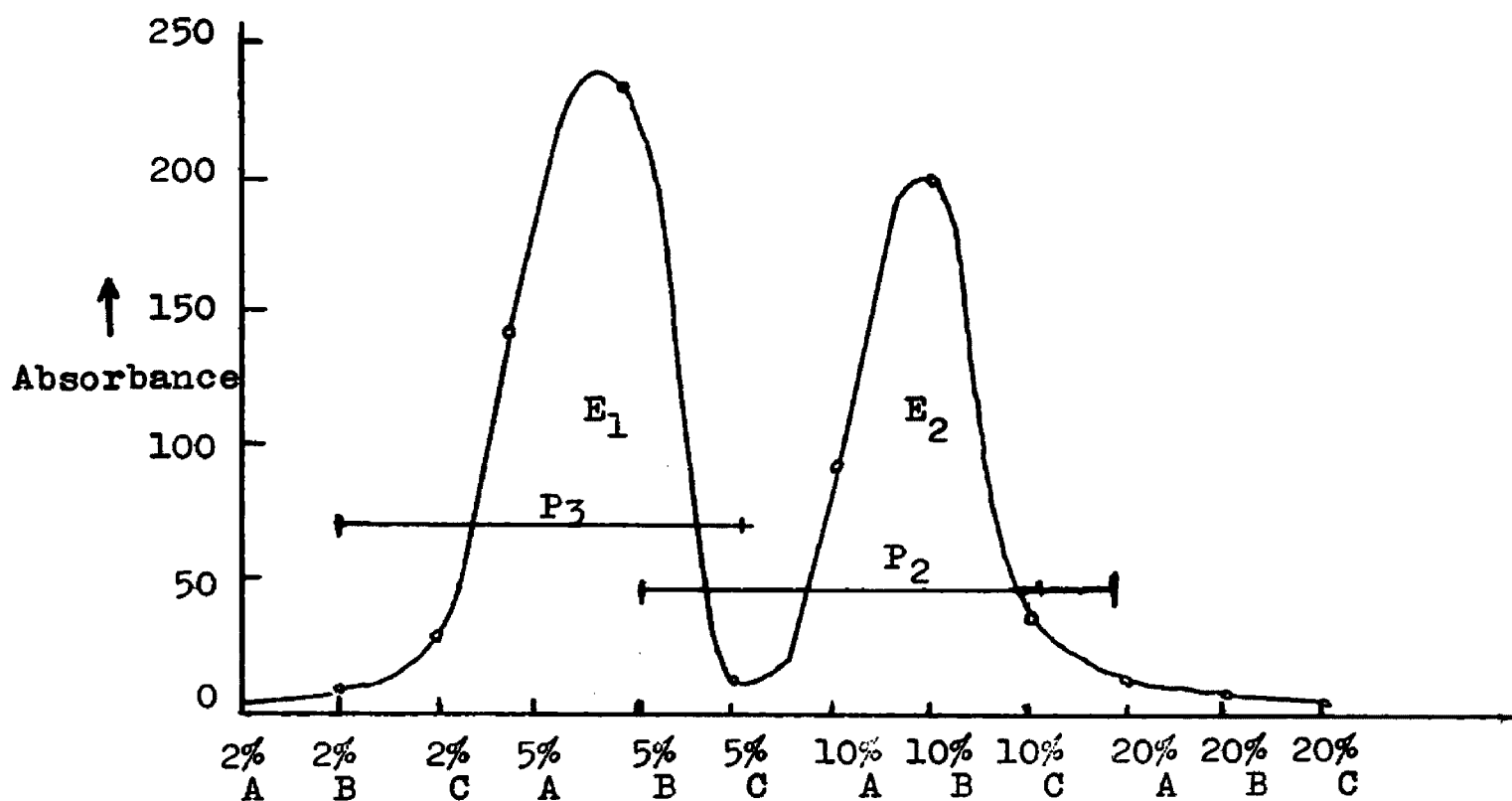
Figure 11. Effluent patterns of estrone and 17β-estradiol in silica gel column with different polarity of eluting solvents.

Remark: 2% A = 2% acetone in CH_2Cl_2 fraction A.

E_1 = Estrone

E_2 = 17β-estradiol
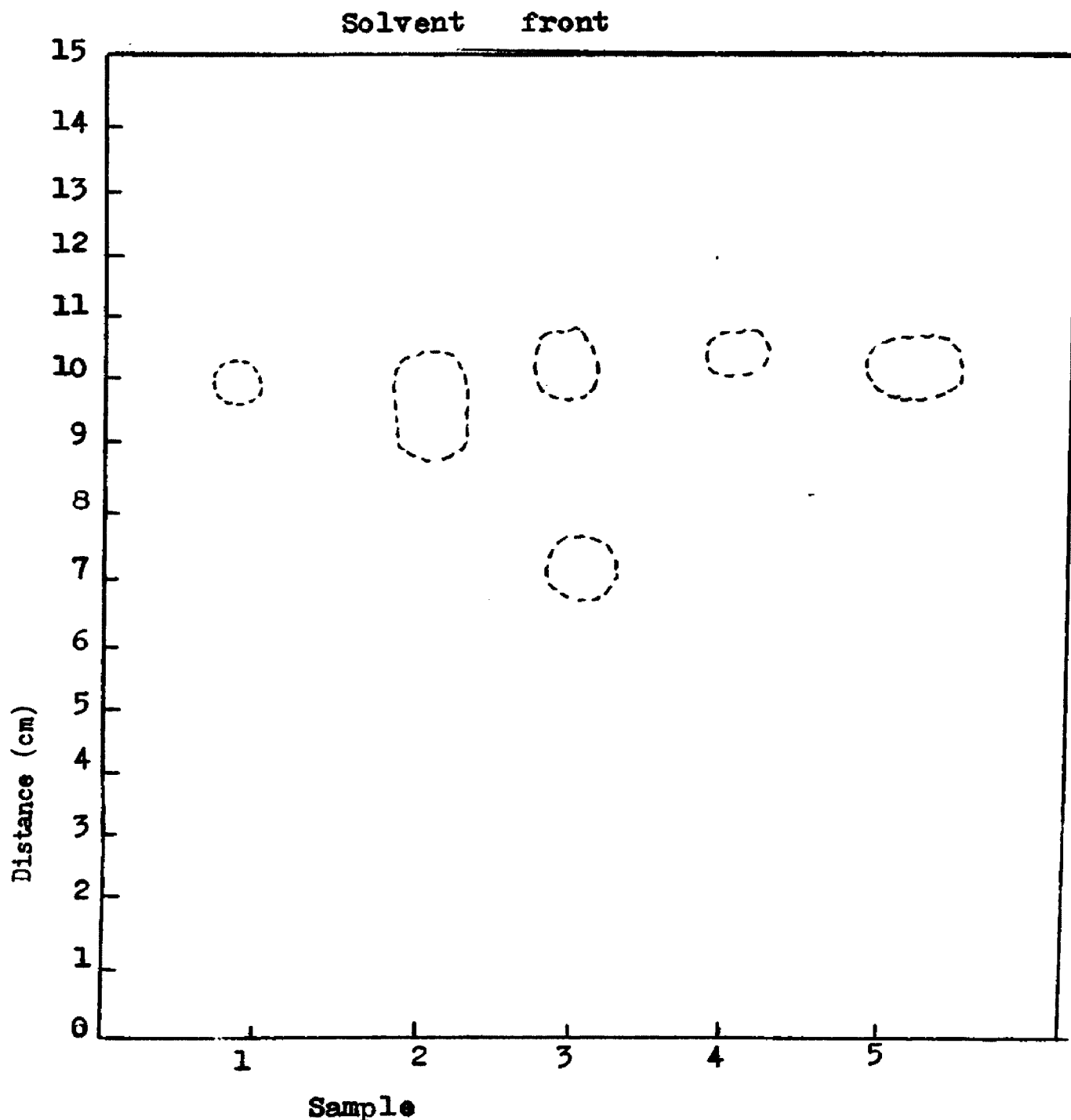
Figure 12   Thin layer chromatogram of methylated estrogen
             samples in the benzene:methanol(99:1 v/v)
             system.
             Sample 1 : methylation blank
               "   2 : methoxy-#P$_3$
               "   3 : authentic methoxy-estrone
               "   4 : authentic methoxy-17β-estradiol
               "   5 : methoxy-#P$_2$

Table 6. Retention time of gas chromatographic analysis of methoxy

derivatives of #P$_2$ and #P$_3$

Sample	Temperature °C			R$_T$*
	Flash Heater	Flame Detector	Colum Oven	
Methylation TLC Blank	283	263	239	0.417
Standard 3-MeO-E$_1$	"	"	"	0.409
Standard 3-MeO-E$_2$	"	"	"	0.442
Methoxy- #P$_2$	281	259	240	0.405
Methoxy- #P$_3$	282	261	240	0.410
Methylation Column Blank	281	259	240	0.407

Colum operating conditions:

H$_2$ 38.0 ml/min.   Range 10 Dual

H$_e$ 45.0 ml/min.   Flame Detector

Air 260 ml/min.   3.8% SE-30 Glass Column 3 mm I.D.x4'

R$_T$ = Relative retention time to cholestane.

Retention time of cholestane is 13.8 ± 0.4 minutes.

repeated. It was found that none of the neutral steroids present in the alkali-insoluble fraction, separated from original P_1-C, had a mobility similar to that of alkali-soluble P_1-C fraction. The polarity of the P_1-C fraction was between 17β-estradiol and 16-oxo-estrone (Table 9). It might be suspected that both P_1-B and P_1-C contain some phenolic compounds transformed from estrone or estradiol via hydroxylation or oxidation reaction. Insufficient amount of metabolites precluded the further identification of these compounds.

Table 7. R_f values of phenolic metabolite #P_1-A

Chromatographic system	Steroids	R_f
Chloroform/formamide (PC)*	#P_1-A	0.014
	estriol	0.015
	16-epiestriol	0.064
	17-epiestriol	0.050
	17β-estradiol	0.287
	17α-estradiol	0.291
	16α-OH-estrone	0.126
	estrone	0.501
Chloroform:ethanol(90:10) (TLC)*	#P_1-A	5.47**
	estriol	5.85**
	16-epiestriol $\Big\}$ 17-epiestriol	8.0-9.7**

PC* = paper chromatography
TLC* = thin layer chromatography (rechromatographed three times to the solvent front)
**   = migration distance from origin(cm)

Table 8. R_f values of phenolic metabolite #P_1-B

Paper Chromatographic System	Steroids	R_f
Chloroform/formamide	#P_1-B	0.501
	estriol	0.015
	16α-OH-estrone	0.126
	17β-estradiol	0.287
	17α-estradiol	0.291
Chloroform/formamide 4 hrs. overrun	#P_1-B	4.5-12.5* (8.7)
	16α-OH-estrone	8.5-12.5* (11.0)
Chloroform/formamide 6 hrs. overrun	#P_1-B	0.503**
	16α-OH-estrone	0.604**
	16-oxo-17β-estradiol	0.522**

* = mobility from origin (cm)
** = R_s value relative to internal standard pregn-4-ene-17α,20α-diol-3-one

Table 9. R_f values of phenolic metabolite #P_1-C

Paper Chromatography System	Steroids	R_f
Chloroform/formamide	#P_1-C	0.505
	17ß-estradiol	0.186
Benzene/formamide 4 hrs. overrun	#P_1-C	0.7-5.0* (2.9)
	estrone	28.7-34.8* (31.7)
	17ß-estradiol	10.6-14.1* (12.3)
Chloroform:benzene (1:1)/formamide	#P_1-C	0.108
	17ß-estradiol	0.208
	16∝-OH-estrone	0.054
Chloroform/formamide	#P_1-C	0.140** 0.276***
	17ß-estradiol	0.233
	16-oxo-estradiol	0.049
	16-oxo-estrone	0.116

* = mobility from origin (cm)
** = R_f value of alkali-insoluble fraction
*** = R_f value of alkali-soluble fraction

V. DISCUSSION

The neutral metabolite X_1 was identified as $17\alpha,20\alpha$-dihydroxy-progesterone while X_2 was $17\alpha,20\beta$-dihydroxyprogesterone from chromatographic evidence, but the mobility of X_1 also corresponded to that of pregn-5-ene-$3\beta,17\alpha,20\beta$-triol ($17\alpha,20\beta$-dihydroxypregnenolone) in the heptane:benzene(1:1)/formamide paper chromatographic system. Since X_1 is an ultraviolet positive steroid and forms monoacetate upon acetylation, the possibility of X_1 being $17\alpha,20\beta$-dihydroxypregnenolone was ruled out.

20α and 20β reduced isomers of pregn-4-ene-11β-ol-3,20-dione (11β-hydroxyprogesterone) cannot be separated from 20 reduced epimers of 17α-hydroxyprogesterone in several paper chromatographic systems (79). Since X_1 and X_2 yielded androstenedione and 17α-hydroxyprogesterone upon oxidation with CrO_3/acetic acid, products which would not be formed from $11\beta,20\alpha(20\beta)$-dihydroxyprogesterone, this possibility is eliminated. Also since the starting substrates contained 17α-hydroxyl groups the products must either still contain this group or be C-19 or C-18 steroids.

$^3H/^{14}C$ Ratios of X_1 and X_2 show that both unknowns can be formed from 17α-hydroxypregnenolone-7-α-^3H and 17α-hydroxyprogesterone-4-^{14}C. However, the $^3H/^{14}C$ ratios of X_1 and X_2 are lower than that of the initial substrates (Table 2). This indicates that the transformation of 17α-hydroxypregnenolone into X_1 and X_2 was less than that of 17α-hydroxy-progesterone. This supports the identification of these unknowns.

The biosynthesis of 20α or 20β or both reduced epimers of 17α-hydroxyprogesterone has been reported in a variety of tissues including

human testes (1,66); mouse testes (29); bovine adrenal gland (63,76); human ovarian tissue (90,107) and testes of English sparrow (Passer domesticus) (34). These steroid glycols were also isolated from blood of Pacific salmon (Oncorhynchus nerka) (52). Therefore, 20-hydroxyde-hydrogenase is generally present in the vertebrate organism. It appears that the present report is the first finding of the formation of these steroid glycols by ovarian tissue. However, it does not provide infor-mation as to the quantitative transformation rate of these two compounds from either substrates nor their biological function in the avian sys-tem.

There are two possibilities of transformation of 17α-hydroxy-pregnenolone into 17α,20α(20β)-dihydroxyprogesterone. Either oxidation of 17α-hydroxypregnenolone ($\triangle^5$-3-ketosteroid) by $\triangle^5$-3β-hydroxysteroid dehydrogenase occurs prior to 20 reduction or vice versa. A kinetic study might provide information concerning which pathway is predominant.

The fact that the 17α,20α(20β)-dihydroxypregnenolone could not be isolated in these experiments does not mean that the compounds are not possible metabolites of 17α-hydroxypregnenolone.

It has been suggested by Axelrod et al. (5) and Lynn and Brown (66) that the pregn-4-ene-17α,20α-diol-3-one isolated by them from ovarian and testicular incubations could be an intermediate precursor of androstenedione. Evidence against this concept was provided by the investigations of Fevold and Eik-Nes (36), Ellis (29) and Dominguez (26). Fevold and Eik-Nes (36) demonstrated that the 20α-isomer was not metabolized significantly to other products by English sparrow testicular tissue, and indicated that the reduction of the 20-ketone of

17α-hydroxyprogesterone appeared to be a physiological irreversible reaction.

Ellis and Berliner (29) reported that 17α,20α-dihydroxyprogesterone was biotransformed into testosterone very slowly in mouse testes. This statement was reinforced by the work of Dominguez (26). He tested the role of 17α,20β-dihydroxyprogesterone in the biosynthesis of androstenedione and testosterone in normal testes and observed that 17α-hydroxyprogesterone was readily converted to androstenedione and testosterone while pregn-4-ene-17α,20β-diol-3-one did not seem to be directly transformed to androstenedione and testosterone in appreciable or measurable amounts without previous conversion to 17α-hydroxyprogesterone. However, the conversion of 20β-glycol to 17α-hydroxyprogesterone was limited at all times unless 17α-hydroxyprogesterone had been almost completely metabolized. It appears that reduction of the 20-keto group of 17α-hydroxyprogesterone by 20β-hydroxy-dehydrogenase is not a readily reversible reaction.

Fevold and Eik-Nes (36) also reported that 20β-isomer was androgenically inactive in the sparrow beak assay and inhibited side chain splitting enzyme(C-17,20,21-desmolase). However, the significant function of these two steroids in the living organism needs further investigation.

In the course of studies on the biosynthetic pathways of estrogens, the functions of pregnenolone, progesterone and androgens acting as precusors of estrogens synthesis in the ovarian tissue have been extensively investigated (96,6,112,50,86,107,5,40).

Estrogens can arise from pregnenolone via androgens by two different pathways. The classical Δ^4-pathway which involves the formation of testosterone via progesterone, 17α-hydroxyprogesterone and androstenedione. It was first elucidated by Slaunwhite and Samuels (93) in the testicular tissue and later Solmon et al. (96) reported a Δ^4-pathway exists from progesterone to estrogens in bovine ovarian tissue. This pathway has been verified by several other workers (86,28,29). An alternative Δ^5-pathway has been first suggested in testicular tissue by Neher and Wettstein (78). This Δ^5-route of synthesis of testosterone involves 17α-hydroxypregnenolone, dehydroepiandrosterone and androstenedione as intermediates. Ryan and Smith (87) demonstrated that Δ^5-route is a more active pathway of estrogen biosynthesis in the human ovary. This alternative pathway in gonadal tissue has been confirmed by several other workers (57,38,39,109,37).

Fevold and Pfeiffer (37) investigated androgen biosynthesis in phalarope ovary incubated with prenenolone and found that Δ^5-route is the predominant pathway and androstenedione is an intermediate in testosterone biosynthesis. This finding is contrary to some of the evidence in the mammalian gonadal tissue concerning the metabolic pathways of testosterone formation which involves androstenediol as an intermediate, thus by passing androstenedione (9,38,82,29,94).

The conversion of C-19 steroids to estrogens has been amply demonstrated (75,6,109). The fact that C-19-hydroxyl compound is an intermediate in estrogen biosynthesis is strongly indicated in ovaries and placenta (73,84,74,110). Wilcox and Engel (110) demonstrated that 19-hydroxyandrostenedione is an obligatory intermediate and 19-hydroxy-

lation is the rate limiting step in the formation of estrogens in placenta. However, whether 19-hydroxyandrostenedione or 19-hydroxytestosterone would be the obligatory intermediate of estrogen biosynthesis in phalarope ovary needs further investigation.

Since the ratio of radioactive androstenedione to radioactive testosterone in phalarope ovaries incubated with pregnenolone-7-α-^{3}H was markedly less than one in the small ovaries and one or greater in the larger ovaries, it was suggested that the relative increase in the androstenedione formation during the period of ovarian development might favor estrogen biosynthesis, if 19-hydroxyandrostenedione is an obligatory intermediate and 19-hydroxylation is the rate limiting step as in the case of placenta (37). However, since this ratio depends mainly on the 17β-hydroxysteroid dehydrogenase present in the gland, the biosynthesis of estrogens from androgens in the phalarope ovary can not be solved unless further knowledge concerning the aromatization steps, level of aromatizing enzyme, rate limiting step, obligatory intermediate and activity of 17β-hydroxysteroid dehydrogenase is available.

The classical idea of estriol biosynthesis was thought to be transformation from estrone and 17β-estradiol. It has been well demonstrated in the human ovary (112,95), in human fetal liver (32) and rat liver (42). It was also isolated from human urine after administration of 17β-estradiol (11,22). Recently, Ryan (85) demonstrated that estriol can be produced from aromatization of androst-5-ene-3β,16α,17β-triol and via 16α-hydroxyestrone as an intermediate in human placenta. Furthermore, 16α-hydroxyprogesterone has also been reported to be a precursor

of estriol biosynthesis by sow ovary (56). Which pathway is the predominant route in the phalarope ovary is not known. It might depend on the substrate sepcificity of the 16-hydroxylase, activity of the 16-hydroxylase present in the gland and the rate limiting step and aromatization rate of 16-hydroxyl C-19 steroids compared to C-19 steroids.

Since the attempt to isolate 16α,17α-dihydroxyprogesterone from 17α-hydroxyprogesterone failed in the sow ovary (56), this might indicate that the 17α-hydroxyl group could interfere with the 16α-hydroxylase enzyme system. It is thought that if estriol could be formed from 17α-hydroxypregnenolone or 17α-hydroxyprogesterone in the present experiments, possibly the classical pathway would be preferred. However, this problem needs further investigation.

Another partially identified phenolic compound is 16-oxo-17β-estradiol. Since 16α-hydroxyestrone readily undergoes transformation to 16-oxo-17β-estradiol in alkali solution during the extraction of the phenolic fraction by aqueous alkali from ether (72), it is suspected that the partially identified 16-oxo-17β-estradiol might be an artifact produced from 16α-hydroxyestrone.

The attempt to isolate estrogens from phalarope ovarian tissue incubated with 17α-hydroxypregnenolone and 17α-hydroxyprogesterone was unsuccessful in the present experiment. Only partial characterization was achieved. Possible reasons for this lack of success could be the low recovery of estrogens from the incubation medium, insufficient amount of estrogens synthesized, and also the possible deleterious effect of the prolonged storage of the tissue in the frozen state on its ability to synthesize estrogens.

VI. SUMMARY

17α-Hydroxypregnenolone-7^{3}H and 17α-hydroxyprogesterone-4-^{14}C have been incubated with homogenized Wilson's phalarope ovarian tissue in Krebs-Ringer phosphate buffer (pH 7.35) containing ATP, DPN, TPN, sodium fumarate, nicotinamide, glucose, glucose-6-phosphate and glucose-6-phosphate dehydrogenase as cofactors and additives. The incubations were performed in a shaking incubator at 41°C for three hours. Following extraction and paper chromatography, two polar non-phenolic compounds were identified by comparing the chromatographic mobilities of the parent steroids, the acetate derivatives and the oxidation products with authentic standards. It was shown that X_1 is pregn-4-ene-17α,20α-diol-3-one and X_2 is pregn-4-ene-17α,20β-diol-3-one. Some phenolic steroids were partially characterized as estrone, estriol and 16-oxo-17β-estradiol. However, due to the limited amount of these metabolites, no further investigation could be carried out.

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PART B

IN VITRO STUDIES OF 17β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN

AVIAN OVARIAN AND TESTICULAR TISSUES

I. INTRODUCTION

17β-Hydroxysteroid dehydrogenase (17β-HSD) is a key enzyme

required for testosterone biosynthesis in phalarope ovarian and tes-

ticular tissue. The enzyme possesses high affinity and structural

specificity for its substrate. It is thought that a correlation between

testosterone biosynthetic capacity and enzyme level may exist in these

tissues. The purpose of these experiments is to measure in vitro activ-

ity of 17β-hydroxysteroid dehydrogenase in phalarope gonadal tissues.

During the earlier phase of the breeding cycle, the female

phalaropes apparently secrete more androgen than the males (26), and

testosterone but not estradiol induces nuptial feathers in phalaropes

(29). It appears that the high ovarian content of testosterone is

related to the brighter plumage and more aggressive behavior of females

than males in this species. Furthermore, Fevold and Pfeiffer (23)

reported that there was a marked shift favoring androstenedione produc-

tion over testosterone production in the larger phalarope ovaries incu-

bated with 17α-hydroxyprogesterone and 17α-hydroxypregnenolone as sub-

strates. It was suggested that testosterone production would decrease

as the follicles approached maturity. On the other hand, since the male

phalaropes could not produce sufficient androgen in early spring to

stimulate development of the bright female type breeding plumage and

since testosterone and prolactin are necessary for the development of
the brood patch found only in the male of the species (28), it would be
anticipated that the male would increase the production of testosterone
in the late spring of the breeding cycle. Therefore, the secretion of
testosterone by the phalarope gonads might follow the curve presented in
Figure 1 (Part A, page 3), as mentioned in the previous hypothesis. The
present experiments were made to investigate this hypothesis by measuring
the activity of 17β-hydroxysteroid dehydrogenase in the phalarope
gonadal tissues.

In vitro 17β-hydroxysteroid dehydrogenase activity in ovarian and
testicular homogenates from phalaropes was measured by the conversion
rate of androstenedione to testosterone as described by Schoen (48).

II. HISTORICAL REVIEW

A. Avian gonadal cycles

The seasonal character of reproductive periodicity in the wild birds has been extensively investigated since the work of Schäfer (47). Although such cycles occur in both male and female birds, the testicular cycle has been studied more thoroughly while the ovarian cycle has received little attention.

In general, during the sexually inactive period, the sex glands of most wild birds regress to tiny rudiments. In the testis at this time, one finds only inactive spermatogonia, in the ovary only small ovocytes. At the approach of the breeding season, these glands enlarge very rapidly. Spermatogenesis proceeds quickly to the production of millions of ripe spermatozoa. Epididymis and vas deferens enlarge correspondingly and become filled with seminal fluid. In a similar way, the female genital organs develop. The thin and straight oviduct becomes convoluted and very corpus, due to the enormous development of its glanular epithelium.

Very few reports published discuss the annual gonadal cycles of Wilson's phalarope. However, the gonadal cycles of phalarope is similar to that of other temperature zone birds. Bent (7) reported that young birds of the Wilson's phalarope are in winter plumage by September. A partial prenuptial molt in April and May, involving the tail, the wing coverts and all the body feathers, produces the brilliant plumage of the female and duller plumage of the male. The growth of this plumage is correlated with the onset of aggressive courtship behavior at the start

of the breeding season. The date of egg laying varies from May 15 to June 24 in North America. In summer, the complete post nuptial molt produces the gray winter plumage in both sexes. Therefore, it is suggested that the gonadal cycles of phalarope might be correlated with that of the English sparrow (58,55).

In general, gonadal size is minimal during winter; begins to increase in late winter and early spring; reaches its maximum in late spring and early summer; decreases in summer and early fall; and remains at this stage until the start of another cycle (22). Witschi (58) found that the testis of the English sparrow increases in weight 500 fold or more and the ovary in the English sparrow increases from less than 10 mg to 500 and more milligrams from winter to spring or summer. A typical annual cycle is illustrated in Figure 1. It is believed that some environmental and physiological factors control the seasonal sexual cycle of birds. These factors include the length of daily photoperiods, temperature, food supply, gonadotrophins, sex hormones secretion and perhaps others.

Schäfer (48) was one of the first to suggest that the constant change in the length of daily photoperiod might be considered as a factor in the avian sexual cycle. Rowan (44) found that precocious spermatogenesis could be stimulated in sexually inactive male Juncos by adding several hours of artifical illumination to natural winter day lengths and thereby determined that the incidence of daily light is actually a factor in seasonal sexual activity. Further investigations of the relationship of photoperiod to seasonal sexual activity of birds were reported by
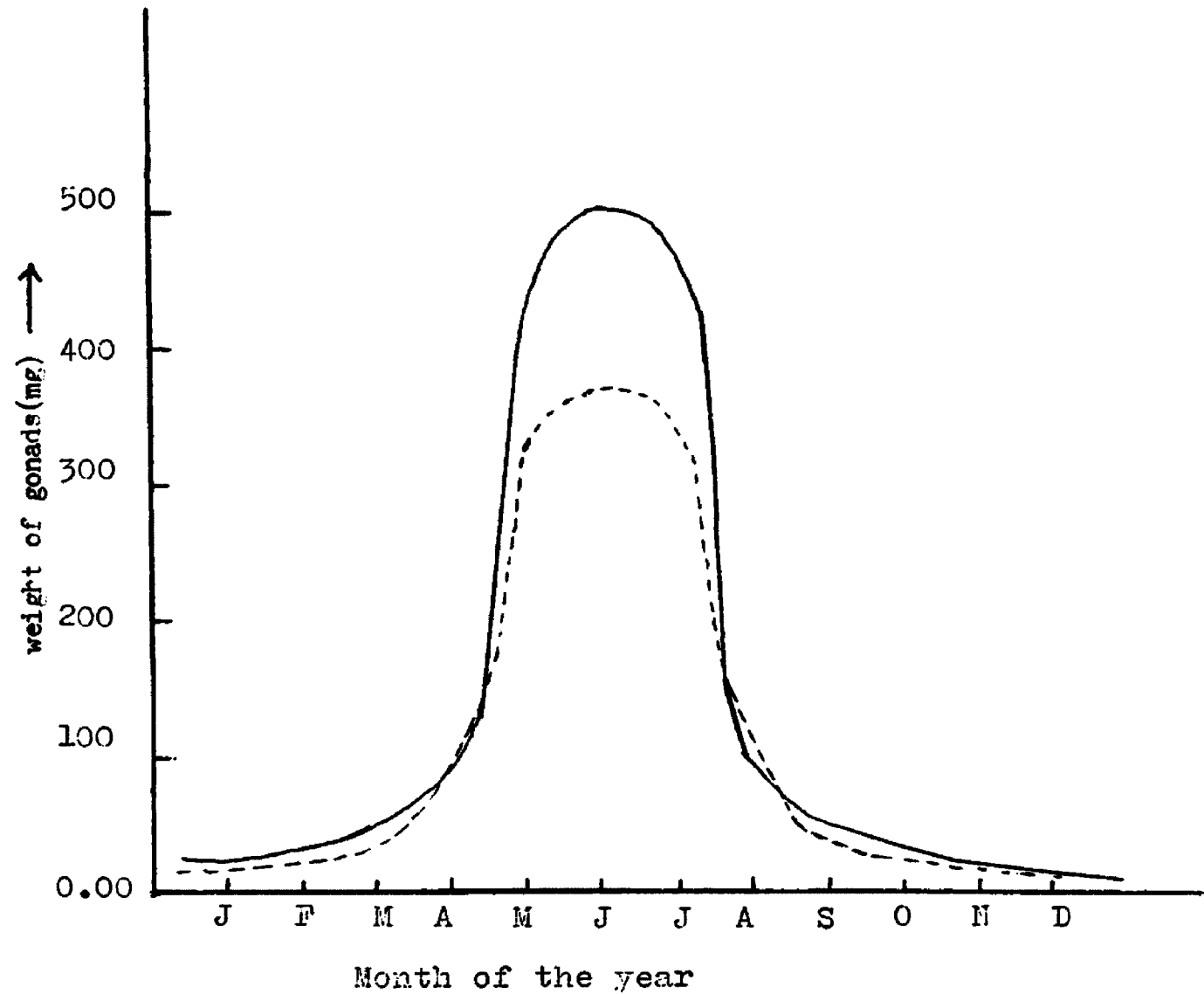
Figure 1. Typical annual gonadal cycle of English Sparrow. Modified from Witschi(1935)

———————— Ovarian cycle

— — — — — — Testicular cycle

Rowan (45, 46) and Bissonnette (8,9,10,11).

Bissonnette (10) demonstrated that red light stimulates testicular development to a greater degree than white light and green light inhibits development of the testis. Ringeon (42), Burger (16) and Benoit (6) agree that the far infrared and near infrared are not stimulatory, while the most effective wave lengths lie in the yellow-red. The minimum daily photoperiod required for gonadal stimulation is about ten hours for several species of birds (3,15). Some workers observed that the gonadal growth of birds stimulated by light was due to the stimulation of pituitary gonadotrophin secretion (4,5,38). Gonadotrophins secreted by the pituitary are believed to stimulate the gonads to produce sex hormones and to cause gametogenesis. The release of sex hormones is closely correlated with the development of the reproductive organs, secondary sexual characters, some sexually dimorphic characters and behavioral patterns.

The demonstration of a refractory period (38,40,60) indicates that a mechanism is incorporated which prevents the birds from responding to light and thus permits a "rest" before the bird returns to breeding condition. The duration of the refractory period in conjunction with the annual light cycle seems to control the general form of the annual reproductive cycle of many birds tested. Benoit (5) presented strong evidence that the anterior pituitary in domestic drakes has a refractory phase that may be independent of the gonads. First they showed that bilateral castration did not induce pituitary hypertrophy during the period while the testes were inactive. Secondly,

there is no compensatory testicular hypertrophy after unilateral castra-
tion. Müller (38) demonstrated that the inactive testes of adult and
immature golden-crowned sparrow could not be activated by injection of
pregnant mare serum. This suggests that the failure of testicular
response is not due to a refractoriness of the testis. Lofts and Marshall
(36) also give the evidence that it is the anterior pituitary and not
the testis that becomes seasonally refractory.

Riley and Witschi (41) found that male English sparrows responded
equally well at all seasons to injected gonadotrophin. Females, how-
ever, showed a seasonal response to the same treatment. The ovarian
response to gonadotrophin was found to be poor in immature birds, but
quite good immediately before ovulation. Breneman (13) also observed
that ovaries of chicks are less responsive to pituitary extracts than
the testes.

Breneman (14) reported that little effect on gonads and pituitary
of the pullet is produced when either estriol or testosterone is admin-
istered alone, but when estrogen and androgen are administered simul-
taneously maximal stimulation of the reproductive system and pituitary
occurs.

Romanoff and Romanoff (43) observed that oviducts in Leghorn
pullets of 4 months averaged 1.1g but after the first ovulation the
oviduct weight increases to 77.2 grams. Breneman (14) demonstrated that
the ovarian growth of pullets does not occur until the interval between
115 and 126 days of post-hatching and the weight increases almost twelve
times from 495.7 to 5781.1 mg. Therefore, pullets must have considerable

amounts of both androgen and estrogen present coincidental with the beginning of maximal ovarian growth. He also reported that maximal pituitary growth of pullets, from 5.56 to 8.23 mg, occurs between 115 and 126 days of post-hatching, while potency increases from 7.3 to 14.4 c.u.* Obviously, gonadotrophin content of the pituitary is not inhibited by the gonadal hormones.

External temperature has no prohibitive effect in most birds tested. Rowan's original work was done at sub-zero temperature. Bissonnette and Csech induced pheasants to lay eggs in snow banks. Kendeigh compared temperature of 72°F with 36°F, for their effect on the English sparrow and noticed no difference in response due to temperature. However, some other reports indicate that the reproductive activity is depressed by cold (17).

It would seem that the neurohumoral mechanism stimulating the anterior pituitary will operate fully in the seasonal wild birds only if a combination of environmental events to which the species has envolved its reproductive response take place. If the environment is too dark, too cold or too hot, if there is a lack of water, food, a mate or of any other appropriate stimuli, the neural mechanism that governs the anterior pituitary will not operate or will operate partially, with resulting failure of spermatogenesis, ovulation or reproduction.

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* c.u. = a net increase of 35% in the weight of the testes of assay chicks over those of the control chicks.

B. 17β-Hydroxysteroid dehydrogenase

17β-Hydroxysteroid dehydrogenases are widely distributed in the living organisms. They are pyridine-nucleotide linked enzymes which catalyze reversible oxidation of hydroxyl groups on the steroid nucleus (53). Either NAD or NADP or both may act as coenzymes for a specific 17β-HSD from different tissues.

One of these enzymes was first prepared by Talalay and Marcus (52) from Pseudomonas testosteroni. A placental 17β-HSD has been studied by Langer et al. (35) and Jarabak (27). The placental enzyme can utilize both NAD and NADP as coenzymes, the latter giving about one half of the initial rate of the former (34). However, the enzyme in the rabbit ovaries was found to be NADP predominant (19). All of the 17β-hydroxysteroid dehydrogenases investigated possess essential sulfhydryl groups and are inactivated by cupric, mercuric and ferric ions and activated by zinic (34).

The placental enzymes have an absolute steric specificity for the 17β-hydroxyl group, but the phenolic hydroxyl group is not absolutely necessary for the reaction between enzyme and substrate (35). Hagerman et al. (25) have demonstrated that the NAD and NADP dehydrogenations of estradiol are catalyzed by different enzymes. The reaction catalyzed by these enzymes are represented as follows:

$$\text{steroid alcohol} + \text{NAD(NADP)} \rightleftharpoons \text{steroid ketone} + \text{NADH+H}^+$$
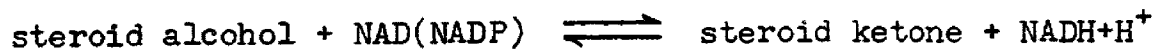
Langer and Engel (34) suggested that 17β-HSD is identical to the 17β-estradiol mediated transhydrogenase in placental tissue and 17β-estradiol functions as a coenzyme or cosubstrate in a transhydrogenation

reaction catalyzed by the 17β-hydroxysteroid dehydrogenase. The coenzyme function of 17β-estradiol was also supported by Jarabak _et al_. (27) and Crist and Warren (18).

The reaction of conversion of testosterone to androstenedione has been studied in various organisms. Namely, in liver (50); in _Penicillium lilacinum_ and _Pseudomonas testosteroni_ (52), (54); in the ovary of rat, guinea, pig, human (20) and rabbit (19,21); in the testes of rat, rabbit (20,21) and human (59, 48). The K_{eq} values of these enzymes vary in the range of $1-40 \times 10^{-9}$ at 25°C (37). Therefore, the enzymes favor the formation of the steroid alcohol. Most of the 17β-HSD studied not only catalyze dehydrogenation reaction of testosterone but also some other steroids with 17β-hydroxyl group, especially 17β-estradiol. However, some rate difference exists in the dehydrogenation reactions of these steroids in various tissue (52,35,27,21). Since the enzymes examined for their substrate specificity are only partially purified, it is questionable whether the enzyme catalyzing the oxidation of C-17-hydroxyl function of testosterone and 17β-estradiol is the same enzyme acting on different substrates or discrete enzymes for each substrate in the tissue. This problem still needs further investigation.

III. EXPERIMENTAL MATERIALS AND PROCEDURES

A. Experimental animals

Source of birds and tissues was the same as Part A, p. 17, except testes of Wilson's phalarope and ovaries of northern phalarope in the first group of experiment were used.

B. **Chemicals**

    1. **Androstenedione-4-^{14}C** (Lot number CFA 248, Batch 3) with specific activity 34.8 mc/mmole was obtained from the Nuclear Chicago Corporation. It was purified by paper chromatography in the heptane: benzene(1:1)/formamide system prior to use.

    2. **Testosterone-1,2-^{3}H** (Lot number 984-1 -41, Nuclear Chicago) had a specific activity of 38.8 mc/mmole and was used as an internal standard after being purified by paper chromatography in the heptane: benzene(1:1)/formamide system.

    3. **Instant thin-layer chromatographic media** was obtained from Gelman Instrument Company (Lot No. 28313), size 20x20 cm.

    4. Other items were the same as in Part A, p. 18-19.

C. **Equipment** - See Part A, p. 20, 21.

D. **Procedures**

    1. **Substrate preparation**

    10.7 Microcuries of purified androstenedione-4-^{14}C and 4.696 micromoles of non-radioactive androstenedione were added to the same tube and the solution was evaporated to dryness. The reside was redissolved in 10 ml solution of absolute ethanol:propylene glycol(1:1) prior to use.

    2. **Tissue preparation**

    Ovaries and testes of different weights were divided into twelve groups (Table 1), thawed, and homogenized in twelve separate Ten-Breock tissue grinders. The Krebs-Ringer phosphate buffer in which the tissues were homogenized had a pH of 7.35 and contained 0.04M nicotinamide.

Tissue to buffer ratio was 1:40. All tissue preparation was carried out at 0°C.

3. Preparation of incubation flask

All glassware was sterilized prior to use. Flasks used for the incubations were the same as in Part A, p. 22. One-tenth microcurie and 0.05 μmole androstenedione-4-^{14}C in 0.1ml of ethanol:propylene glycol (1:1 v/v) were generally added to the flasks. In some cases half or double this amount of substrate was used (Tables 3 and 4). The ethanol was evaporated. An aliquot of the substrate was taken during the initial and final period of substrate addition to the flasks. These aliquots were assayed for ^{14}C content by liquid scintillation spectrometry and the values averaged to calculate the amount of ^{14}C-androstenedione added to the flasks (Table 2).

After the ethanol was evaporated, 5.0 microliters(10 μg) of Sigma Type VU glucose-6-phosphate dehydrogenase was added to each flask, followed by the addition of one ml of buffer containing all other cofactors. The final cofactors concentrations were the same as indicated in Part A, p. 22. Finally, one ml of the whole homogenated in buffer containing nicotinamide was added to each of flask. The flasks were gassed for one minute with a 95%O_2-5%CO_2 immediately after the addition of the homogenate and tightly stoppered. Zero time control incubations were terminated immediately after the one minute gassing period by adding 5.0 ml diethyl ether:ethyl acetate(4:1v/v) to the incubation flask and placing in the freezer at -20°C.

4. Incubation

Table 1

Phalarope tissue used for incubation

Homog. No.	Sex	Bird No.	Gonad Wts(mg)	Homog. Vol.(ml)	Amt.Tissue mg/flask	No. of Flasks
1	♀	66-35*	37.0	2.5	14.8	2
2	♀	66-37	61.6	6.5	20.6	6
		66-36	70.4			
3	♀	66-20	147.4	6.5	22.7	6
4	♀	66-27	166.6	13.4	25.0	12
		66-18	168.8			
5	♀	66-6	219.0	7.8	25.0	6
6	♀	66-7	224.0	9.0	25.0	6
7	♀	66-19	302.6	12.1	25.0	6
8	♀	66-8	500.8	10.0	50.0	6
9	♀	66-29	2323.8	23.2	100.0	6
10	♂	66-33	443.0	8.9	50.0	6
11	♂	66-15	325.0	13.0	25.0	12
12	♂	66-16	217.0	8.7	25.0	6

* Northern phalarope

After gassing the incubation flasks were placed immediately in a constant temperature, shaking water bath and incubated at 41°C for ten or twenty minutes. The reactions were stopped by the addition of the same organic solvent as mentioned above.

5. Extraction

Testosterone-1,2-^{3H} ($\sim$0.08 μc) was added to each incubation flask before initial extraction. The amount added was checked with each group of extraction flasks (Table 2). This added steroid was used as a chromatographic standard as well as for the calculation of estimated percent recovery figures. The extraction method was the same as stated in Part A, p. 24.

6. Product purification, isolation and identification

a. The first chromatographic separation of extracted media was performed in the same way as for the separation of netural steroids as indicated in Part A, p. 25. The typical tracing of steroid metabolites on the paper chromatogram is shown in Figure 2. The chromatogram was divided into three parts. The first fraction corresponded the area of authentic testosterone and the third fraction was the unmetabolized androstenedione. The second fraction was the area between authentic testosterone and andostenedione. The steroids on the chromatogram strips were cut and eluted with 15 ml absolute ethanol and the solvent evaporated to dryness.

b. Further identification of product

Like fraction of the number one area from the first chromatograms were pooled and rechromatographed on silica gel thin-layer

Table 2

Total radioactivity of substrate and internal extraction standard added

to the incubation flasks before and after incubation, respectively

Compound	DPM
Androstenedione-4-^{14}C	
initial	229309
final	240989
Testosterone-1,2-^{3}H	
flasks 1-32	149959
flasks 33-48	172844
flasks 49-60	184421
flasks 61-72	199130
flasks 73-80	37203
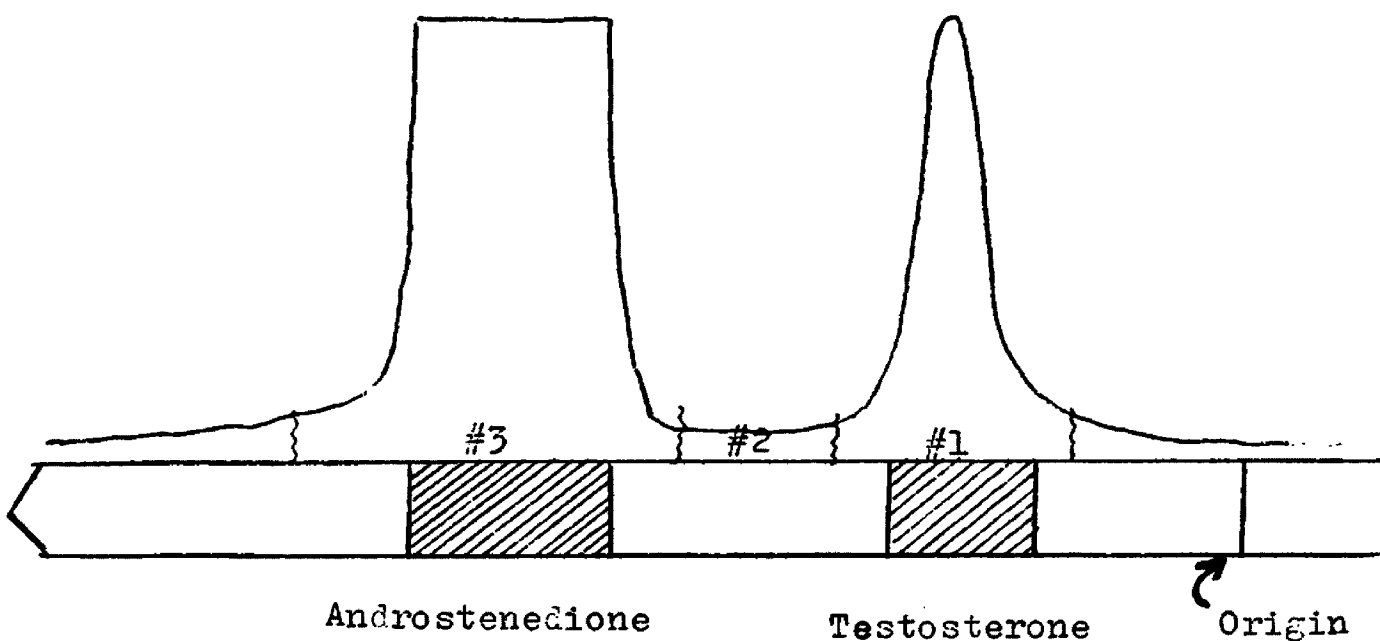
Figure 2. Tracing of the first chromatographic separation of radioactive metabolites isolated from incubations of phalarope gonadal tissues cell-free homogenate with androstenedione-4-^{14}C substrate.

plates in the cyclohexane:ethyl acetate (50:50 v/v) system. Further chromatographic identification of metabolite was performed by comparing the mobilities of the parent compound and the products of acetylation, saponification and oxidation with authentic standards as described in Part A, pp. 26-27.

7. Quantification of metabolites

The residue of each fraction eluted from the initial chromatogram was redissolved in 1.0 ml absolute ethanol and 0.1 ml duplicate were placed in liquid scintillation counting vials. After evaporation of the ethanol, ten ml of counting fluid were added to each vial. Radioactivity in all samples was quantified by counting the aliquots in the liquid scintillation spectrometer. The calculation of the amount of tritium and ^{14}C present in each sample was carried out on an IBM 1620 data processing system using standard equations* (Nuclear Chicago Liquid Scintillation Manual).

The activity of 17β-hydroxysteroid dehydrogenase was calculated by the percent conversion rate of androstenedione to testosterone. The original values of percent conversion were adjusted by the percent recovery of testosterone. The zero time control values were then substrated from this figure to obtain the true values per unit time.

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* I wish to thank Professor John Peterson for writing the program for these equations.

IV. RESULTS

A. Effect of substrate concentration on the velocity of the 17β-hydroxy-
steroid dehydrogenase reaction in ovarian and testicular tissues.

When 25.0 mg of ovarian tissue were used with amounts of sub-
strate varying from 25.0-50.0 μM, there was no significant variation
in the velocity of the 17β-hydroxysteroid dehydrogenase reaction (Fig-
ure 3). Likewise, a similar curve was obtained with increasing substrate
concentration from 25.5-50.0 μM with 25.0 mg of testicular tissue (Fig-
ure 3). These results indicate that the enzyme is saturated with the sub-
strate in the present experiments at the 25 μM substrate concentration used.

B. Rate of formation of testosterone from androstenedione in phalarope
ovarian and testicular tissues.

The original data of the conversion rate of testosterone from andro-
stenedione in various phalarope ovarian and testicular tissues were
listed in Tables 3 and 4. These data have been corrected for the per-
cent recovery of testosterone. The average percent conversion of
androstenedione to testosterone was obtained by using the method of
least squares. The values after correcting to 100 mg tissue weight
basis were plotted against incubation times and shown in Figures 4 and
5. The slope of the lines in Figure 4 varies with ovarian weight but
is not directly proportional to the weight. In Figure 5 the slope of
the lines increases with increasing testicular weight. This indicates
that enzyme specific activity increases as the testicular size increases.

The specific enzyme activity is expressed as percent conversion
per hour per mg of tissue. These data were plotted versus gonadal
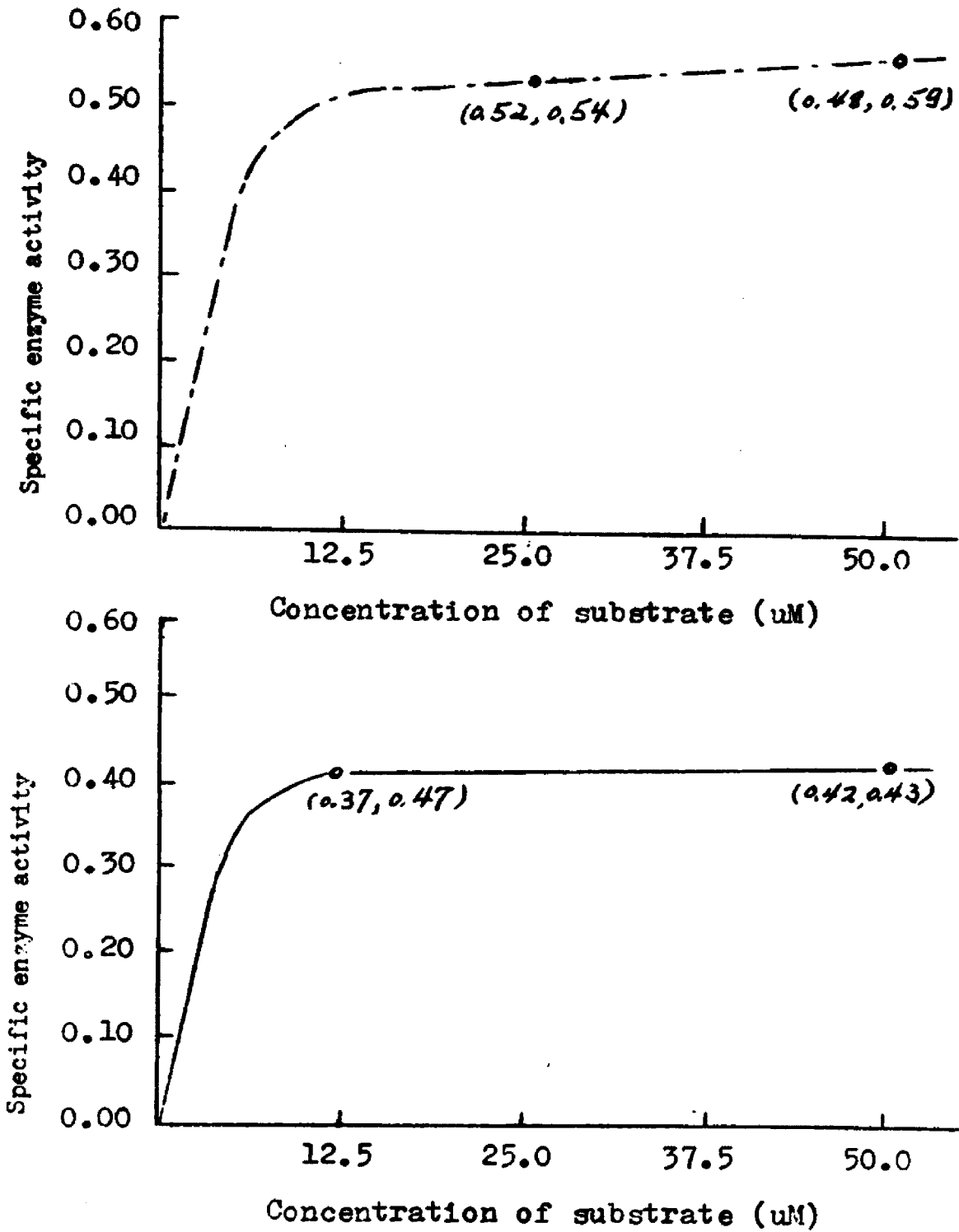
Figure 3   Effect of substrate concentration on the
velocity of the 17β-hydroxysteroid
dehydrogenase reaction in phalarope
gonadal tissues.

— - — - — — ovarian tissue

——————— testicular tissue

Table 3. Effect of varying ovarian weight on conversion of andro-
stenedione substrate to testosterone

Flask No.	Ave. ovarian wt. (mg)	Amt. tissue/ flask (mg)	Incub. time (min.)	% recov. of Test.	% initial A* radioact.		
					T** formed	Others*	A* remaining
1	37.0	14.8	20	91.95	2.54	0.14	78.69
2	"	"	20	88.24	2.18	0.28	84.76
3	66.0	20.6	0	69.25	1.56	0.21	83.71
4	"	"	10	78.29	2.98	0.20	84.51
5	"	"	20	82.02	5.95	0.49	82.84
6	"	"	0	97.15	1.91	0.13	88.60
7	"	"	10	94.28	3.16	0.22	87.46
8	"	"	20	95.13	6.52	0.46	84.39
9	147.4	22.7	0	88.19	0.54	0.09	92.08
10	"	"	10	81.50	3.06	0.55	81.78
11	"	"	20	93.66	6.19	2.24	82.38
12	"	"	0	80.70	0.38	0.12	89.22
13	"	"	10	88.07	3.20	0.96	87.34
14	"	"	20	89.33	10.02	2.72	77.42
15*	167.7	25.0	0	80.52	4.89	0.45	80.90
16*	"	"	10	84.78	4.86	0.13	84.69
17*	"	"	20	100.83	5.96	0.26	83.14
18	"	"	0	103.33	0.39	0.07	91.11
19	"	"	10	101.32	2.84	0.18	95.05
20	"	"	20	102.00	8.36	0.56	79.31
21*	"	"	0	95.50	1.67	0.13	85.92
22*	"	"	10	101.93	3.10	0.09	89.18
23*	"	"	20	86.94	6.15	0.16	83.63
24	"	"	0	106.51	0.31	0.05	89.50
25	"	"	10	102.79	2.61	0.09	92.13
26	"	"	20	109.65	4.57	0.14	88.84
27	219.0	25.0	0	97.53	0.25	0.08	92.18
28	"	"	10	104.56	1.90	0.28	91.51
29	"	"	20	107.33	3.40	0.22	93.81
30	"	"	0	102.38	0.21	0.04	95.11
31	"	"	10	111.76	1.64	0.08	89.96
32	"	"	20	107.73	3.49	0.23	86.98

A* = Androstenedione.

T** = Testosterone (data corrected for % of recovery).

Others* = Area lying between testosterone and androstenedione on the
initial paper chromatogram of incubation media extracts.
Both (Others) and (Androstenedione remaining) are raw data.

* = Incubation flask contained double amount of substrate.

Table 3. Effect of varying ovarian weight on conversion of androstenedione substrate to testosterone (cont.)

Flask No.	Ave. ovarian wt. (mg)	Amt. tissue/ flask (mg)	Incub. time (min.)	% recov. of Test.	% initial A* radioact.		
					T** formed	Others*	A* remaining
33	224.0	25.0	0	97.67	0.35	0.07	95.98
34	"	"	10	106.51	3.72	0.16	87.74
35	"	"	20	98.06	8.12	0.14	85.42
36	"	"	0	99.99	0.30	0.06	91.04
37	"	"	10	95.73	4.56	0.23	89.07
38	"	"	20	110.91	8.26	0.20	79.63
39	302.6	25.0	0	101.91	0.42	0.09	94.01
40	"	"	10	96.72	1.91	0.20	87.97
41	"	"	20	103.15	4.33	0.35	80.27
42	"	"	0	100.15	0.23	0.04	94.13
43	"	"	10	95.84	1.72	0.12	92.85
44	"	"	20	100.65	3.66	0.26	86.46
45	500.8	50.0	0	93.44	0.49	0.07	93.92
46	"	"	10	97.12	4.67	0.30	89.20
47	"	"	20	96.68	9.76	0.36	76.81
48	"	"	0	92.63	0.41	0.05	90.29
49	"	"	10	91.36	4.13	0.30	91.45
50	"	"	20	93.24	10.74	0.29	81.45
51	2323.8	100.0	0	92.14	0.18	0.05	92.85
52	"	"	10	89.45	2.71	0.15	91.34
53	"	"	20	99.62	9.34	0.44	81.87
54	"	"	0	95.63	0.21	0.04	91.18
55	"	"	10	81.29	2.72	0.22	88.49
56	"	"	20	88.68	5.64	0.28	84.31

A*   = Androstenedione.
T**   = Testosterone (data corrected for % of recovery).
Others* = Area lying between testosterone and androstenedione on the initial paper chromatogram of incubation media extracts. Both (Others) and (Androstenedione remaining) are raw data.

Table 4. Effect of varying testicular weight on conversion of androstenedione substrate to testosterone

Flask No.	Ave. testes wt. (mg)	Amt. tissue/ flask (mg)	Incub. time (min.)	% recov. of Test.	% initial A radioact.		
					T*** formed	Others‡	A remaining
57	443.0	50.0	0	87.16	0.06	0.07	91.43
58	"	"	10	94.25	8.05	0.38	81.09
59	"	"	20	108.48	21.10	0.30	69.42
60	"	"	0	84.90	0.06	0.02	89.28
61	"	"	10	90.66	8.62	0.13	84.27
62	"	"	20	96.22	21.30	0.18	73.72
63*	325.0	25.0	0	76.82	0.12	0.08	87.80
64*	"	"	10	85.51	1.50	0.07	80.16
65*	"	"	20	83.21	3.94	0.22	80.37
66**	"	"	0	91.47	0.01	0.04	94.48
67**	"	"	10	88.91	1.16	0.05	88.48
68**	"	"	20	93.30	4.66	0.07	73.36
69*	"	"	0	87.93	0.14	0.05	85.48
70*	"	"	10	89.21	1.28	0.49	82.56
71*	"	"	20	82.13	4.30	0.47	81.50
72**	"	"	0	84.24	0.01	0.03	88.10
73**	"	"	10	101.01	1.25	0.20	85.94
74**	"	"	20	102.18	3.41	0.14	84.22
75**	217.0	25.0	0	102.27	0.06	0.04	92.58
76**	"	"	10	111.40	2.35	0.15	84.14
77**	"	"	20	106.13	3.21	0.20	84.02
78**	"	"	0	101.36	0.06	0.02	93.16
79**	"	"	10	108.11	0.71	0.06	89.04
80**	"	"	20	111.76	2.42	0.13	90.82

* = double amount of substrate
** = half amount of substrate
A = Androstenedione
T*** = Testosterone (data corrected for % of recovery)
Others‡ = Area lying between testosterone and androstenedione on the initial paper chromatogram of incubation media extracts. Both (Others) and (androstenedione remaining) are raw data.

Table 5. Effect of gonadal weight on 17β-hydroxysteroid dehydrogenase activity

Homog. No.	Ave. gonadal wt. (mg)	Amount substrate/ flask (uM)	Ave.** specific enzyme activity	Ave.*** total enzyme activity
1	37.0	25.0	0.51	18.86
2	66.0	"	0.59	38.63
3	147.4	"	0.75	109.70
4	167.7	"	0.53	88.85
4	"	50.0	0.54	89.70
5	219.0	25.0	0.38	83.25
6	224.0	"	0.92	205.50
7	302.6	"	0.42	125.70
8	500.8	"	0.57	286.85
9	2323.8	"	0.16	370.20
10*	443.0	"	1.18	520.70
11*	325.0	12.5	0.42	136.25
11*	"	50.0	0.43	136.50
12*	217.0	12.5	0.32	67.85

* = testicular tissue
** = Average % conversion/hr./mg of gonadal tissue used for incubation
*** = Specific enzyme activity x total gonadal weight
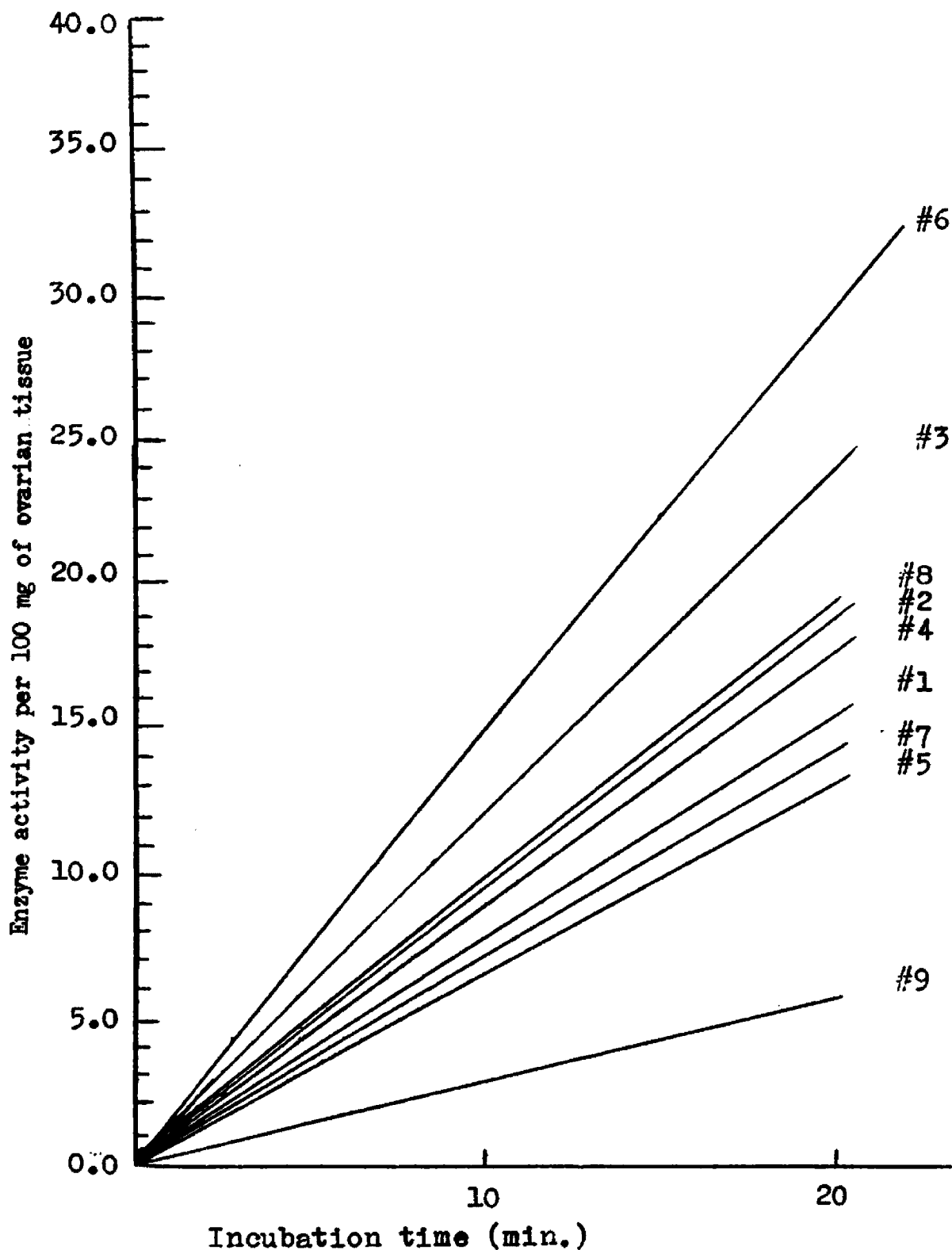
Figure 4   17 β-hydroxysteroid dehydrogenase activity per
100 mg of incubation tissue of different
ovarian weight.
1,2,3,4,5,6,7,8,9 = Homogenate No. of
different ovaries in order of increasing
ovarian weight(see Table 1, p. 79).
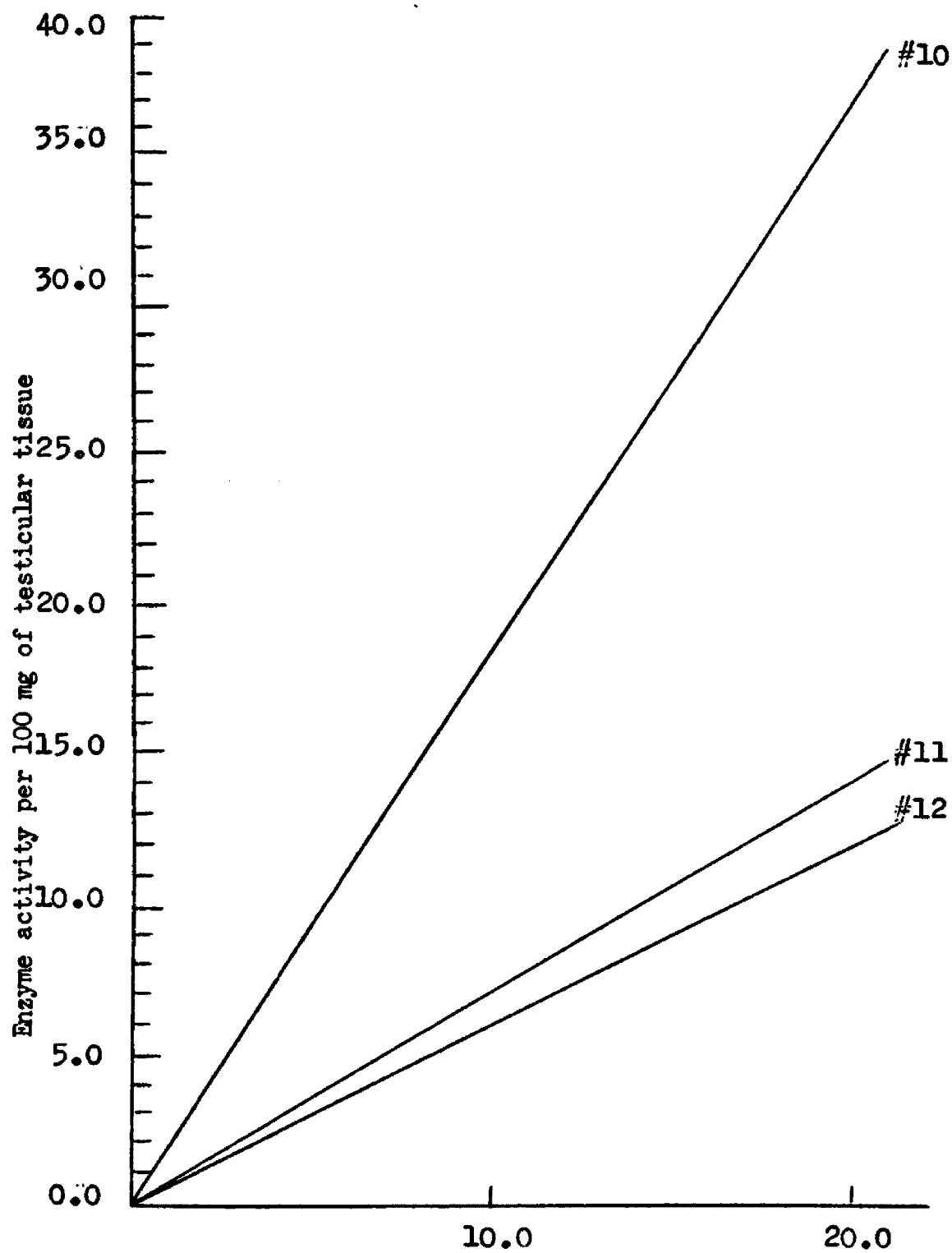
Figure 5   17β-hydroxysteroid dehydrogenase activity per
            100 mg of incubation tissue of different
            testicular weight.
            #10,11,12 = Homogenate No. of different paired
            testes in order of decreasing testicular weight
            (see Table 1, p.79)

weight. In general, the specific enzyme activity in ovarian tissue tended to increase then decrease as the ovarian weight increased from 37.0 mg to 2323.8 mg (Figure 6). However, there is a considerable variation of the specific activity in the 219 mg to 500.8 mg tissue weight range. Furthermore, a marked decrease of enzyme activity is observed in the largest ovary. In the testes incubations, the specific enzyme activity increases with testicular weight (Figure 7). This result corresponds to the hypothesis.

The total enzyme activity is expressed as average percent conversion per hour per gonadal weight, i.e., specific enzyme activity x total gonadal weight. Figure 8 shows that the total enzyme activity increases as ovarian size increases from 37.0 mg to 2323.8 mg. There is a variation in activity in the 140-300 mg tissue weight range. The total testicular enzyme activity as shown in Figure 9 increases as the gonad size increases. There is a remarkable increasing in the total testicular enzyme activity as the testes approach maximal weight. It is interesting to observe that the curve of total enzyme activity in the testes does not follow the same pattern as that in the ovaries. The former shows a concave upward curve, whereas the latter follows a partial concave downward pattern. It appears that the total enzyme activity tended to increase more rapidly than that in ovaries in the latter stages of gonadal development (Figures 8 and 9). This finding confirms part of original hypothesis.

C. Production identification

Figure 2 shows the tracing of the first paper chromatographic

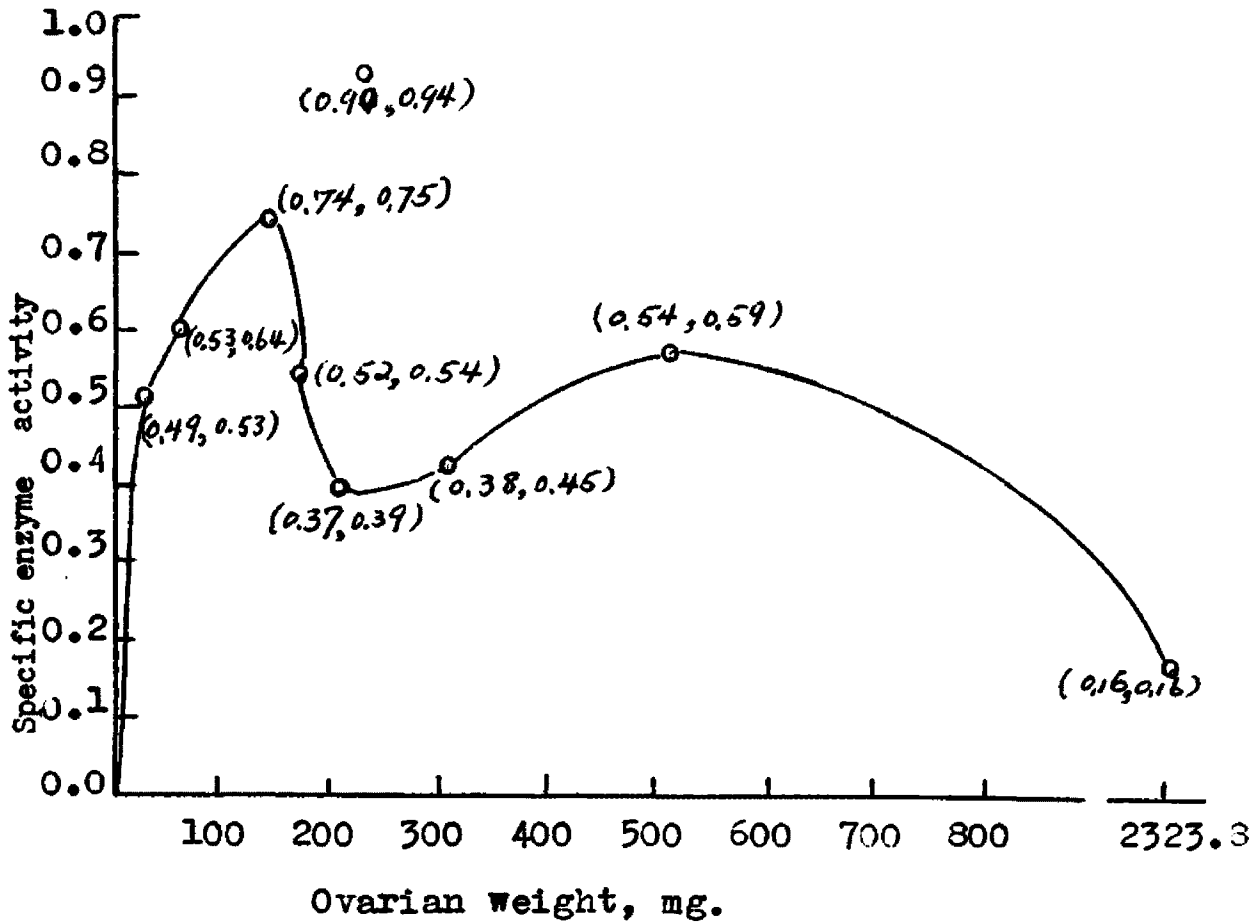Figure 6   Effect of ovarian weight on the specifc activity
of 17β -hydroxysteroid dehydrogenase
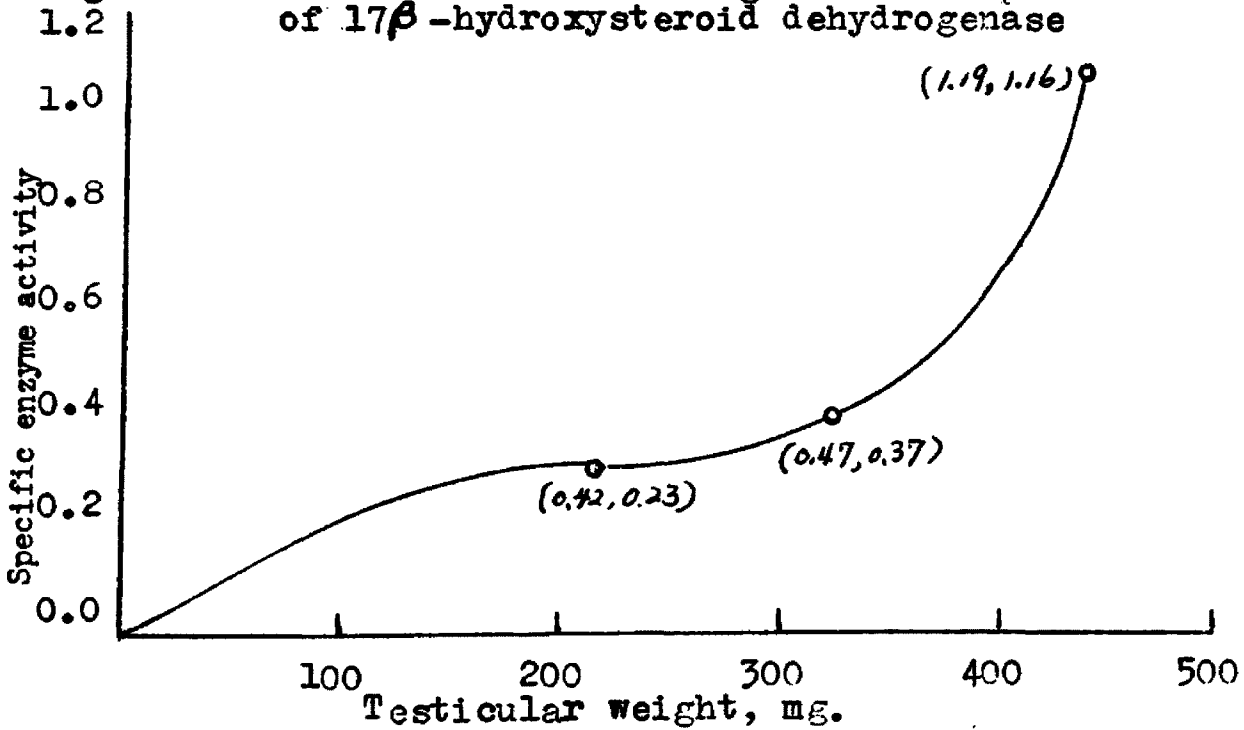

Figure 7   Effect of testicular weight on the specific
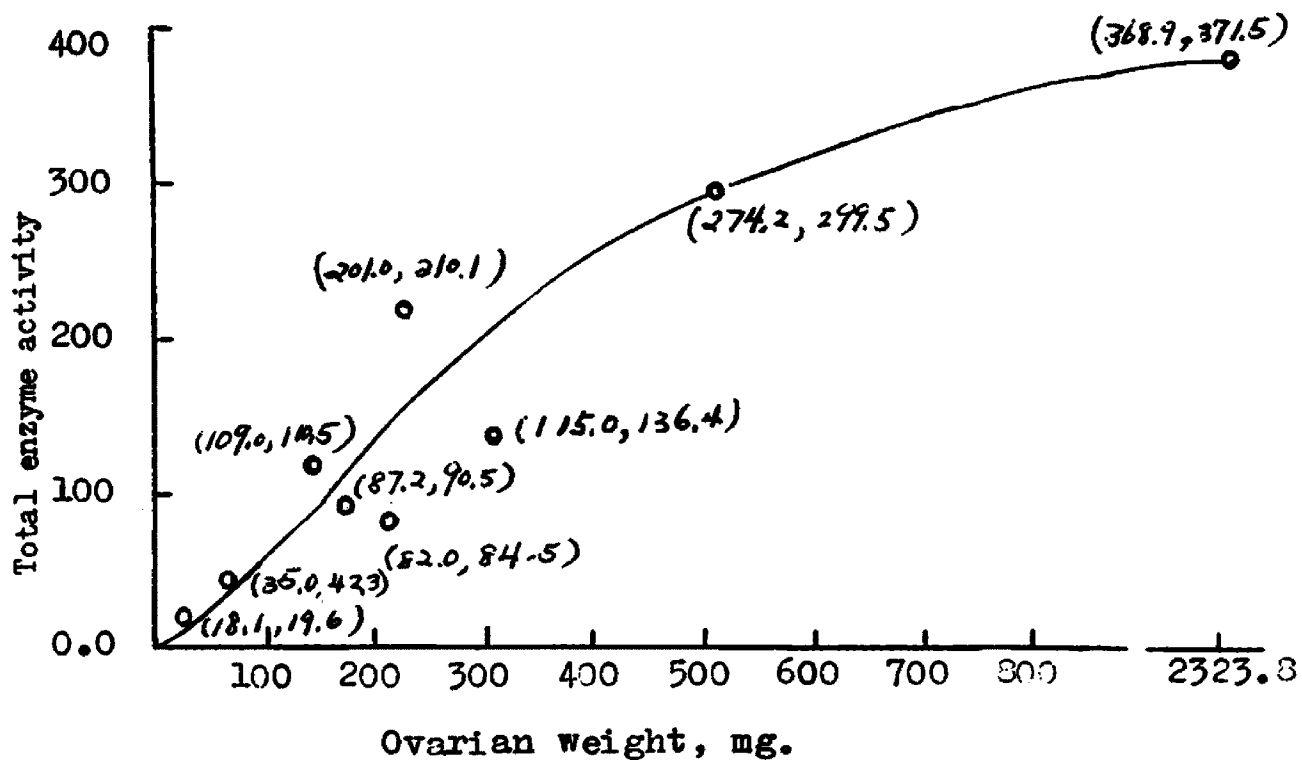activity of 17β-hydroxysteroid dehydrogenase

Figure 8   Effect of ovarian weight on the total activity
of 17 β-hydroxysteroid dehydrogenase
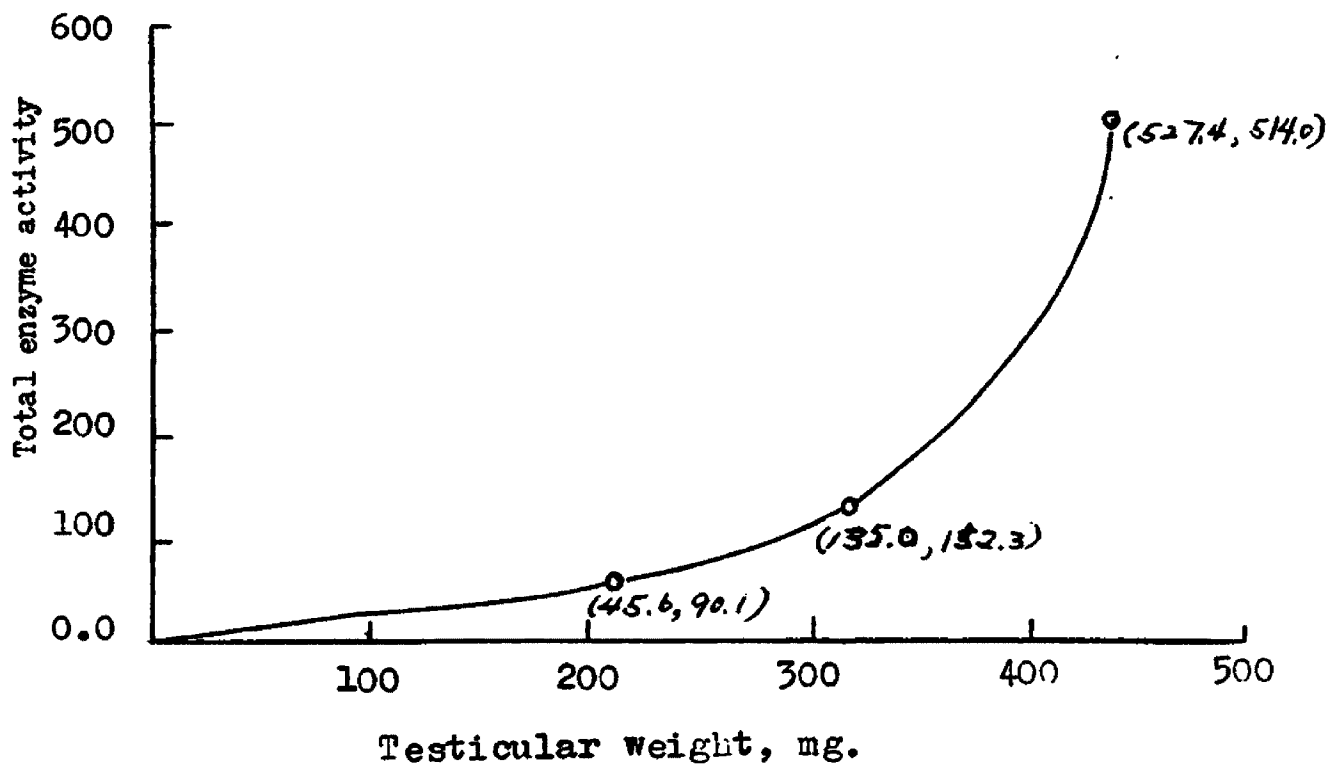
Figure 9   Effect of testosterone weight on the total
activity of 17 β-hydroxysteroid dehydrogenase

separation of radioactive metabolites isolated from incubations media. Since the mobility of fraction 1 corresponded to that of authentic testosterone, this area on the paper chromatogram was eluted and further identification of this unknown steroid was performed. The procedures of isolation and identification of this metabolite are indicated in Figure 10. The R_f values of metabolic product No. 1 and its derivatives are presented in Table 6. The ratios of $^3H/^{14}C$ found in this metabolite and its derivatives are shown in Table 7.

Fraction 1 showed a monoacetate derivative upon acetylation and was partially converted to the parent compound after saponification. Only androstenedione was obtained when the parent compound recovered after saponification was oxidized with CrO_3 in glacial acetic acid. The chromatographic mobilities of metabolite and its derivatives were identical to those of authentic testosterone, or testosterone with similar treatment, in several thin-layer and paper chromatographic systems.

There was no significant variation of the $^3H/^{14}C$ ratio of the parent compound recovered from saponification, of the acetate derivative, and of the oxidation product. However, the parent compound recovered after saponification had a slightly lower $^3H/^{14}C$ ratio than did the original acetate derivative (Table 7).

On the strength of the chromatographic evidence of the parent compound, the acetate derivative, the oxidation product and the $^3H/^{14}C$ ratio data, the metabolite 1 was identified as testosterone.

Figure 10.   Flow sheet for the isolation and identification of testosterone
             formed from androstenedione substrate as described in the text.

ORIGINAL CHROMATOGRAPHIC SEPARATION (FIG. 1)
↓
POOLED TESTOSTERONE AREA (#1 AREA IN FIG. 1)
↓
chromatographed in system 1 (TLC)
eluted with n-butanol:methanol(1:3)v/v,1 ml three times
↓
acetylated with pyridine/AC$_2$O(4:1)
chromatographed in system HB(PC)
↓
TESTOSTERONE ACETATE
↓
rechromatographed in system 2(ITLC)
↓
saponified and chromatographed in system HB(PC)
↓
TESTOSTERONE
↓
oxidized with CrO$_3$/HOAc; chromatographed in system
HB(PC); rechromatographed in system B
↓
ANDROSTENEDIONE

system 1 (TLC)   = cyclohexane:ethyl acetate (50:50)v/v, thin layer
                    chromatography

system 2 (ITLC)   = ethylacetate:benzene (1:4)v/v, instant thin layer
                    chromatography

system HB(PC)   = heptane:benzene (1:1)/formamide, paper chromatography

system B(PC)   = benzene/formamide, paper chromatography

AC$_2$O   = acetic anhydride

HOAc   = acetic acid

Table 6. Chromatographic identification of metabolite I.

R_f Values

	Standards**		Metabolite I			
	.c	Andro	Parent Cpd.	Acetate deriv.	Sapon. Acetate	Oxid. Prod.
TLC-1	0.178 ±0.003*	——	0.648 ±0.032	0.178 ±0.004	——	——
ITLC-2	——	0.598 ±0.003	——	0.581 ±0.048	——	——
PC-1	0.150 ±0.006	0.803 ±0.048	0.420 ±0.020	0.822 ±0.081	0.154 ±0.009	0.451 ±0.022
PC-2	——	——	0.671 ±0.074	——	——	0.723 ±0.053

Chromatographic system:

TLC-1 = thin layer chromatography; cyclohexane:ethyl acetate (50:50) v/v

ITLC-2 = instant thin layer chromatography; ethyl acetate:benzene (1:4)v/v

PC-1 = paper chromatography; heptane:benzene(1:1)/formamide

PC-2 = paper chromatography; benzene/formamide

* Standard deviation

**Abbreviations:
  T = Testosterone
  Andro = Androstenedione
  Ac = Acetate
  Sapon = Saponified
  Oxid = Oxidation
  Cpd = Compound
  deriv = derivative
  prod = product

Table 7. $3_H/14_C$ ratios of metabolite I

Group* No.	Acetate derivative	$3_H/14_C$ ratio Saponified Acetate	Oxidation product
1	24.92	22.74	23.07
2	13.76	12.67	12.89
3	11.82	8.33	8.21
4	15.24	13.49	13.59
5	27.25	18.37	18.38
6	13.31	12.07	12.13
7	29.32	26.98	27.30
8	11.67	10.34	10.55
9	17.03	15.48	15.72
10	6.29	5.07	5.21
11	20.04	17.31	17.54
12	8.34	7.22	7.42

* Homogenate No. (see Table 1, p. 75)

V. DISCUSSION

From the kinetic point of view, it is important to study the initial reaction velocity with a substrate concentration which is sufficient to saturate the enzyme, since the reaction proceeds at maximal velocity only under these conditions. To investigate whether the reduction of 17-ketone of androstenedione was proceeding at maximal rate with a 25 μM substrate concentration, an incubation was done using twice the amount of this substrate concentration. It was found that 25 μM androstenedione was enough for the reaction to proceed at a maximal rate under the experimental conditions used in the ovarian tissue. With testicular tissue, the reaction rates were the same with the substrate concentration of 12.5 and 50.0 μM (Figure 3). However, the ovarian tissue (167.7 mg ovary) used to determine if a 25 μM substrate concentration gave maximal reaction rates did not contain the highest total enzyme units per flask. The total arbitrary enzyme units of this enzyme were 13.5 u (0.54 u/mg x 25 mg) whereas the highest total enzyme units per flask were 28.5 u (0.57 u/mg x 50 mg). In the testicular tissue, the highest total number of enzyme units per flask, 59.0 u (1.18 u/mg x 50 mg), was about six times that of the flasks, 10.5 u (0.42 u/mg x 25 mg), incubated with two different substrate concentrations (Table 5). Therefore, it might be possible that in those incubation flasks containing more than 13.5 units in the ovarian tissue or 10.5 units in the testicular tissue, the reaction velocity was sub-maximal and the estimates of enzyme activity would be low. However, since the Km values of 17β-hydroxysteroid dehydrogenase from other tissue lies

between $1 \times 10^{-5} - 10^{-6}$ (27), and since the substrate concentration used in these experiments was 2.5×10^{-5} M, it appears that the substrate concentration is probably greater than the Km of the enzyme in these experiments.

It should be pointed out that since only conversion of exogenous substrate to product was measured, any marked difference in the concentration of endogenous androgens might alter the level of enzyme activity calculated in these experiments. However, since the substrate concentration of androstenedione used in these experiments was 0.05 μmole (14.3 μg) in homogenized gonadal tissues (25, 50 or 100 mg), it was about hundred to twenty-five times that of endogenous androgens reported in the phalarope ovarian and testicular tissues* (26). Therefore, the low levels of tissue androgens might not cause significant variation in the enzyme activity calculated in these experiments.

Talalay and Marcus (52), Langer et al. (35) and Jarabak et al. (27) studied substrate specificity of bacterial and human placental 17β-hydroxysteroid dehydrogenase and found that these enzymes could catalyze both the oxidation reaction of testosterone and 17β-estradiol to androstenedione and estrone, respectively. Some rate difference in these two reactions was observed. However, since the enzymes examined in their experiments were only partially purified, it is debatable whether these two reactions are mediated by the same enzyme.

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* The levels of endogenous androgens in phalarope gonadal tissues are as follows:
Ovary : A = 5.38×10^{-3} ug/mg; T = 1.16×10^{-3} ug/mg
Testis: A = 3.96×10^{-3} ug/mg; T = 1.26×10^{-3} ug/mg
A = Androstenedione; T = Testosterone
All these data are the highest values reported in the paper of Höhn and Cheng (26).

From a histochemical point of view, if only one protein exists, one would expect similar cellular localization of the enzyme. On the other hand, dissimilar histochemical localization of the staining reaction with various substrates catalyzed by the enzyme would indicate the existence of more than one enzyme. The difference demonstrated by Kellogg and Glenner (31) in the localization of NAD and NADP-linked dehydrogenases indicates that the enzymes are separate and distinct. This conclusion is supported by the report of Hagerman and Villee (25). They reported the separation of the placental NAD and NADP-linked 17β-hydroxysteroid dehydrogenases. Koide and Mitsudo (32) found that the staining reaction of the placental NAD and NADP-linked 17β-hydroxysteroid dehydrogenase with testosterone was restricted to the vessels which differed from the localization of 17β-estradiol with NAD. Botte et al. (12) also reported that the NADP-dependent placental 17β-hydroxysteroid dehydrogenase in mouse was present in the first trophoblastic generation giant cells and in the endodermal cells of the inverted yolk sac placenta only using 17β-estradiol as substrate and not with testosterone as substrate. These difference in the localization of the staining reaction with various 17β-hydroxyl steroids suggested that NAD and NADP-linked dehydrogenase for testosterone and 17β-estradiol are separate enzymes.

Davenport and Mallette (19) investigated the pH effect on the activity of rabbit ovarian 17β-hydroxysteroid dehydrogenase with either testosterone or 17β-estradiol as substrate and NADP as cofactor. The enzyme activity-pH curves with these two substrates were different.
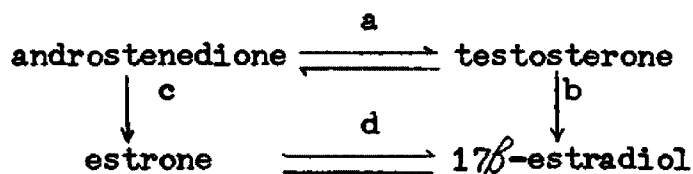
Furthermore, they also reported the different ratios of activity with NADP to those with NAD for testosterone (8.0) and 17β-estradiol (2.8). This might suggest that there are distinct enzymes catalyzing the dehydrogenation of these two 17β-hydroxyl steroids. Therefore, the total activity of 17β-hydroxysteroid dehydrogenase measured in the present experiments could reflect the level of secretion of testosterone in the avian gonadal tissue.

Figure 6 shows that the specific activity of 17β-hydroxysteroid dehydrogenase in the ovary appears to increase and then decrease with ovarian weight. Variation is shown in the 224 mg ovary. This might be due to individual variation. A remarkable decrease of specific activity is found in the largest ovary (Figure 6). The total enzyme activity shows the reverse result (Figure 8). This is probably due to quite a large portion of acellular material in the larger size of ovaries resulting from the development of the eggs. Therefore, the enzyme specific activity decreases whereas the total activity of enzyme increases with increasing ovarian weight.

In the original hypothesis, it was suggested that there might be a decrease in the total testosterone 17β-HSD activity as the size of ovary increases. Since the birds were shot in early May, ovarian weight was increasing or near maximum and it might be suspected that ovarian androgen production would be reduced to permit follicular development (2,30,56). However, the total enzyme activity in ovarian tissue obtained in the present experiments tended to increase with increasing size of ovaries. These results partially agree with the

findings of Höhn and Cheng (26). Theyreported the endogenous androgen
levels in phalarope ovarian tissue increased during the pre-laying, lay-
ing and post-laying period. On the contrary, the present experimental
results are at variance with the reports of Fevold and Pfeiffer (23)
and some other workers (30,51,1). These investigators used a substrate
prior to androstenedione in the metabolic pathway and found that the
androstenedione to testosterone ratio was greater than one in the
ovarian tissue. However, this ratio depends mainly on the activity of
17β-hydroxysteroid dehydrogenase present in the gland. The high ratio
might indicate low activity of 17β-HSD present in the gland.

Estrogens can be biosynthesized from either androstenedione or
testosterone as indicated in the following diagram:

$$\text{androstenedione} \underset{}{\overset{a}{\rightleftharpoons}} \text{testosterone}$$

$$\downarrow c \qquad\qquad\qquad \downarrow b$$

$$\text{estrone} \underset{}{\overset{d}{\rightleftharpoons}} 17\beta\text{-estradiol}$$

If pathway a and b are predominant in phalarope ovarian tissue, a high
level of testosterone could be used as the precusor for the synthesis
of estrogen. On the other hand, if pathway c is the dominant reaction
in estrone biosynthesis, a low activity of testosterone 17β-HSD would
be expected. In placental tissue (57), 19-hydroxyandrostenedione is an
obligatory intermediate and 19-hydroxylation is the rate limiting step
in the formation of estrogens from androstenedione. However, there is
no evidence whether this 19-hydroxyandrostenedione compound is also an
obligatory intermediate of estrogen synthesis in avian ovarian tissue.
Further knowledge concerning the aromatizing steps of estrogens bio-

synthesis from C-19-compound in the avian ovary is needed. Whether testosterone is the predominant precursor of estrogen biosynthesis in the phalarope ovary is unknown at this time, since what is true of one tissue may not be true of the other.

The increasing total enzyme activity with the size of phalarope ovary poses a problem concerning the function of testosterone in ovulation on the one hand and its effect on the total gonadotrophin secretion on the other.

Breneman (14) observed that there was a spurt in comb growth of pullet from 110 days through 126 days of post-patching. The comb achieves a size twelve times that recorded at 106 days. Besides, the ovarian growth between 115 and 126 days of age increases almost twelve times from 495.7 to 571.1 mg and potency of anterior pituitary increases twice amount between 115 to 126 days. Further investigation of testosterone propionate (TP) effect on birds disclosed that TP has a pronounced effect on pullet comb growth but its effect on ovarian and pituitary weight was not significant. However, the administration of combination of 17β-estradiol and testosterone at various dosage levels showed that little effect is produced when either of the two gonadal hormones is administered alone, but when estrogen and androgen are administered simultaneously, maximum stimulation of comb, ovarian weight, oviduct and pituitary occurs. Therefore, he concluded that ovarian secretion of androgen in pullets precedes ovarian growth. Androgen and estrogen might be present coincidentally with the beginning of maximal ovarian growth and the gonadotrophin content of pituitary

is not inhibited by the gonadal hormones. In examination of his experiments, it is found that he did not assay the relative potency of follicle stimulation hormone (FSH) and luteinizing hormones (LH) nor determine the time of estradiol and testosterone secretion. Therefore, whether testosterone could stimulate both gonadotrophins or might inhibit one or the other is doubtful since testosterone may differentially affect pituitary content of FSH and LH (49). It is also questionable whether estrogen and androgen will be secreted simultaneously at the beginning of maximum ovarian growth. Furthermore, the effect of gonadal hormones on the potency of gonadotrophin in his experiments is dose dependent. A combination of $1.0 \mu g$ 17β-estradiol and $1.0 \mu g$ testosterone decreased the potency of the pituitary. Therefore, the stimulation effect of testosterone on the pituitary gonadotrophin content in the presence of estrogens should be reconsidered.

It should be emphasized that some genetic and physiological differences exist between the phalarope and the pullet. First, there is no comb in the phalarope. Secondly, the phalarope only have four eggs per clutch instead of laying about 300 eggs per year as does the pullet. Thirdly, the phalarope shows sex reversal behavior. There might be considerable differences in the function of testosterone in the phalarope ovary as compared with that of the pullet. However, some conflicting reports have been demonstrated by other workers. Nelson and Stabler (39) administered TP to young female sparrow hawks and found that ovaries were not stimulated, although the oviducts were enlarged. Greep and Jones (24) reported that administration of testo-

sterone to rats results in a decrease in pituitary and ovarian weight

but that FSH content of the pituitary is increased. Testosterone has

been found to cause a decrease in estrogen secretion as well as a decrease

in ovarian weight in hypophysectomized prepuberal rats (33) and in

parabiotic rats (49). Since no estrogen determination was done in the

present experiments and no assay of gonadotrophin was performed either,

the function of testosterone in the phalarope ovary can not be solved

now. It is thought that it might augment with FSH and LH secretion to

stimulate ovarian development and estrogen secretion. However, further

knowledge of the pituitary-gonadal hormones relationship and the influ-

ence of gonadal hormones on avian ovarian development needs further

investigation.

Both the specific activity and total activity of 17β-hydroxy-

steroid dehydrogenase in the testicular tissue increased with the

increasing testicular size (Figures 7 and 9). There is considerably

more enzyme activity present in the largest testes as compared with

the other two groups of small testes. These results confirmed the

prediction of hypothesis concerning androgen formation by male

phalaropes during the reproductive seasons. Furthermore, the testes

appear to have a greater capacity than the ovary for synthesizing the

more active androgen (testosterone), in the later stages of gonadal

development (Figures 8 and 9), and agree with the work of Fevold and

Pfeiffer (23). During the breeding season the increase of testosterone

production will stimulate spermatogenesis of male phalaropes (28), and

since testosterone and prolactin are necessary for the development of

the brood patch, found only in the male of the species, it would be
expected that the male would increase the production of testosterone
as the testicular size increases.

The identity of testosterone was established by several criteria.
One of them was by measuring the $^3H/^{14}C$ ratio of the acetate derivative,
the parent compound and the oxidation product. It was found that the
$^3H/^{14}C$ ratio of the acetate derivative was constantly higher than that
of the parent steroid recovered from saponification (Table 7). The
most likely explanation is that the tritium label at the 1,2-position
of testosterone is unstable in the alkali saponification conditions,
due to the enolization of 3-ketone group. However, there was no sig-
nificant variation in the $^3H/^{14}C$ ratio of the parent compound, recovered
after saponification, and the oxidation product. The result confirms
the identity of the metabolite with testosterone.

VI. SUMMARY

Various weights of phalarope ovarian and testicular tissue homogenates were incubated with androstenedione-4-^{14}C in a Krebs-Ringer phosphate buffer (pH 7.35) with ATP, DPN, TPN, sodium furmarate, nicotinamide, glucose-6-phosphate, glucose and glucose-6-phosphate dehydrogenase as cofactors and additives. The incubations were carried out at 41°C for 0, 10 and 20 minutes. The radioactive steroids formed were extracted and isolated. The activity of 17β-hydroxysteroid dehydrogenase was calculated from the rate of conversion of androstenedione to testosterone.

The specific enzyme activity in the ovarian tissue first increases and then decreases with increasing size of ovary, whereas the total enzyme activity in the ovarian tissue tends to increase with ovarian weight. Both the specific activity and the total activity in the testicular tissue increase as the size of testes increases. The testes appear to have a greater capacity than the ovary for reducing androstenedione to testosterone in the later stages of gonadal development.

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