THE BIOLOGICAL AND CHEMICAL SYNTHESIS OF 16α 19-DIHYDROXY DEHYDROEPIANDROSTERONE

by

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(Professor in Charge of Thesis)

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TRIVAL AND SYSTEMATIC NAMES

DHA-acetate-3β acetoxyandrost-5-ene-17-one

DHA, dehydroepiandrosterone-3β hydroxyandrost-5-en-17-one

16α-OH-DHA-3β, 16α-dihydroxyandrost-5-en-17-one

19-OH-DHA-3β, 19-dihydroxyandrost-5-en-17-one

16α-19-diOH-DHA-3β, 16α, 19-trihydroxyandrost-5-en-17-one

A^4, androstenedione-androst-4-en-3, 17-dione

19-OH-A^4 - 19 hydroxyandrost-4-en-3, 17 dione

19-oxo-A^4 19 oxoandrost-4-en-3, 17 dione

DHA-S, dehydroepiandrosterone-3-sulfate

16α-OH-DHA-S-16α-hydroxyandrost-5-en-17-one-3 sulfate

A^4-S -androstene 3, 5 dienol sulfate -17 one

T, testosterone, 17β hydroxyandrost-4-en-3-one

Δ^5-triol, androstenetriol- androst-5-ene-3β, 16α, 17β-triol

19-OH-T, 19 hydroxytestosterone, 17β, 19 dihydroxyandrost-4-ene 3 one

Δ^5-triol-S 16α, 17β androst-5-ene-3 sulfate

E_1, estrone -3β-hydroxyestra 1, 3, 5 (10) trien-17-one

E_2, estradiol- estra 1, 3, 5 (10)-trien-3, 17β-diol

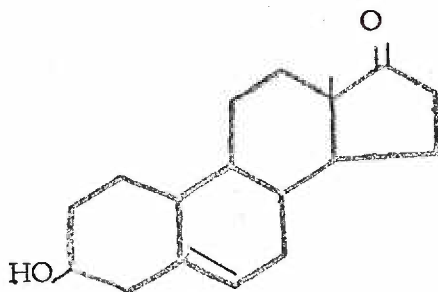
E_3, estriol-estriol- estra-1, 3, 5 (10)- trien-3, 16α, 17β-triol

SU-4885 (metapyrone)

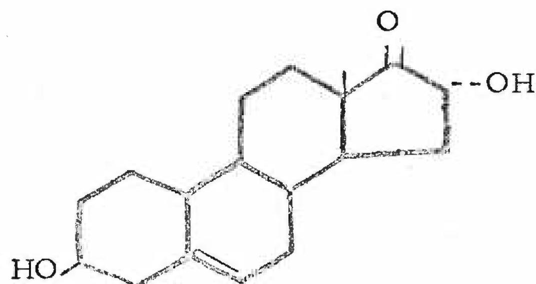
A^4-$4^{14}C$ androst-4 ene-17-one-4-^{14}C

DHA-S-7α ^3H 7α ^3H androst-5-ene-17-one-3-β-sulfate

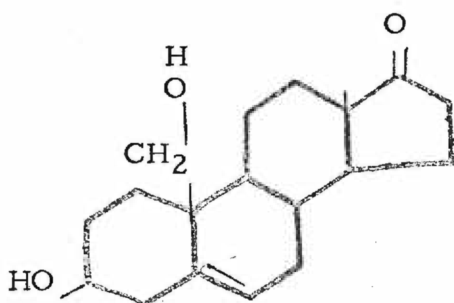
STRUCTURAL FORMULAS OF KEY STEROIDS



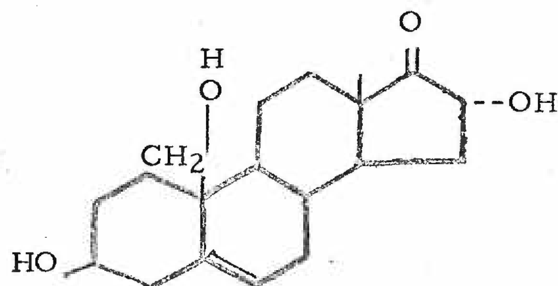
DHA
Dehydroepiandrosterone
3β, hydroxyandrost-5-ene-17-one



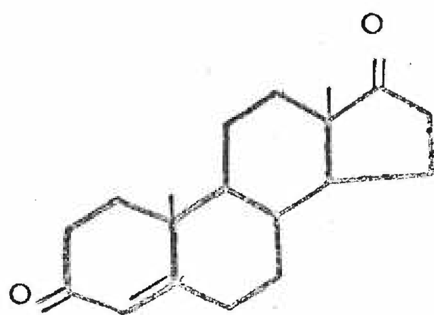
16α-OH-DHA
3β, 16α-dihydroxyandrost-
5-ene-17-one



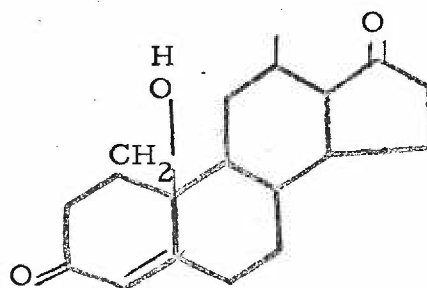
19-OH-DHA
3β, 19 dihydroxyandrost-
5-ene-17-one



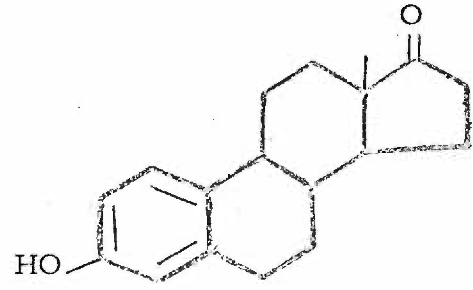
16 α, 19, Dihydroxy DHA
3β, 17α, 19 trihydroxyandrost
-5-ene-17-one



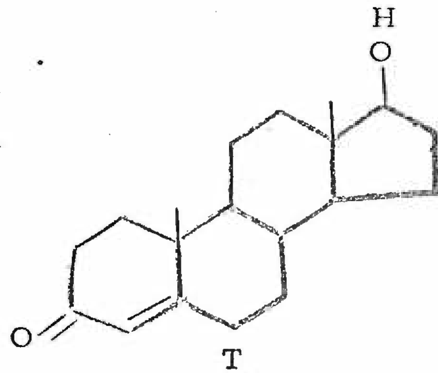
A^4
Androstenedione
Androst-4-ene 3, 17 dione



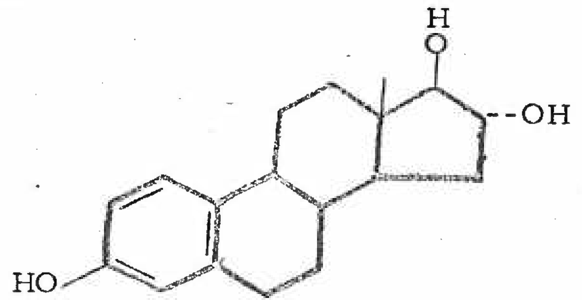
19-OH-A^4
19 Hydroxyandrost-
4-ene 3, 17 dione



E_1
Estrone

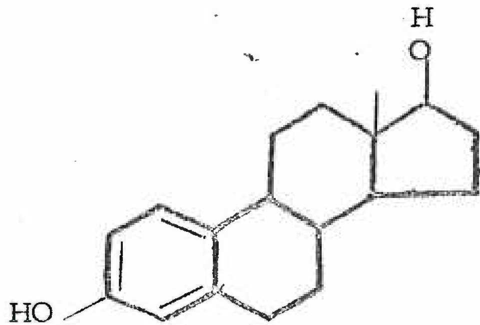


T
Testosterone
17βhydroxyandrost-4-ene-3-one



E_3

Estriol



E_2

Estradiol 17β

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1. INTRODUCTION

A. Statement of Problem

The mechanisms of the biosynthesis of large quantities of estriol during human pregnancy has proved an intriguing problem. The biochemists' search for the precursors and metabolic pathways of this important estrogen, and the clinicians' dilemma over the significance of urinary estriol excretion have culminated in intensive studies by many investigators. The present thesis work had as its objectives those problems which combined both biochemical and chemical interests.

It is generally accepted that estriol precursors are neutral steroids arising from the fetal adrenal which are 16α-hydroxylated in the fetal liver and aromatized by the placenta. Understanding the pathway of estriol biosynthesis thus necessarily requires availability of the neutral steroid intermediates which may be involved. The original intent of this work was to synthesize a new steroid which might be an intermediate, 3β, 16α, 19-trihydroxyandrost-5-ene-17-one (also known as 16α, 19-dihydroxy dehydroepiandrosterone) and to study its significance in the biosynthesis of estriol by a placental aromatizing system. The first approach to chemical synthesis was to combine known reactions in the synthesis of 19-OH-DHA and 16α-OH-DHA. An outline of this approach is given in Figure 1. The second approach to synthesis was to utilize microbiological 16α-hydroxylation of 19-OH-DHA. Since 19-OH-DHA was not commercially available, it also had to be synthesized from DHA-3 acetate. A third possible

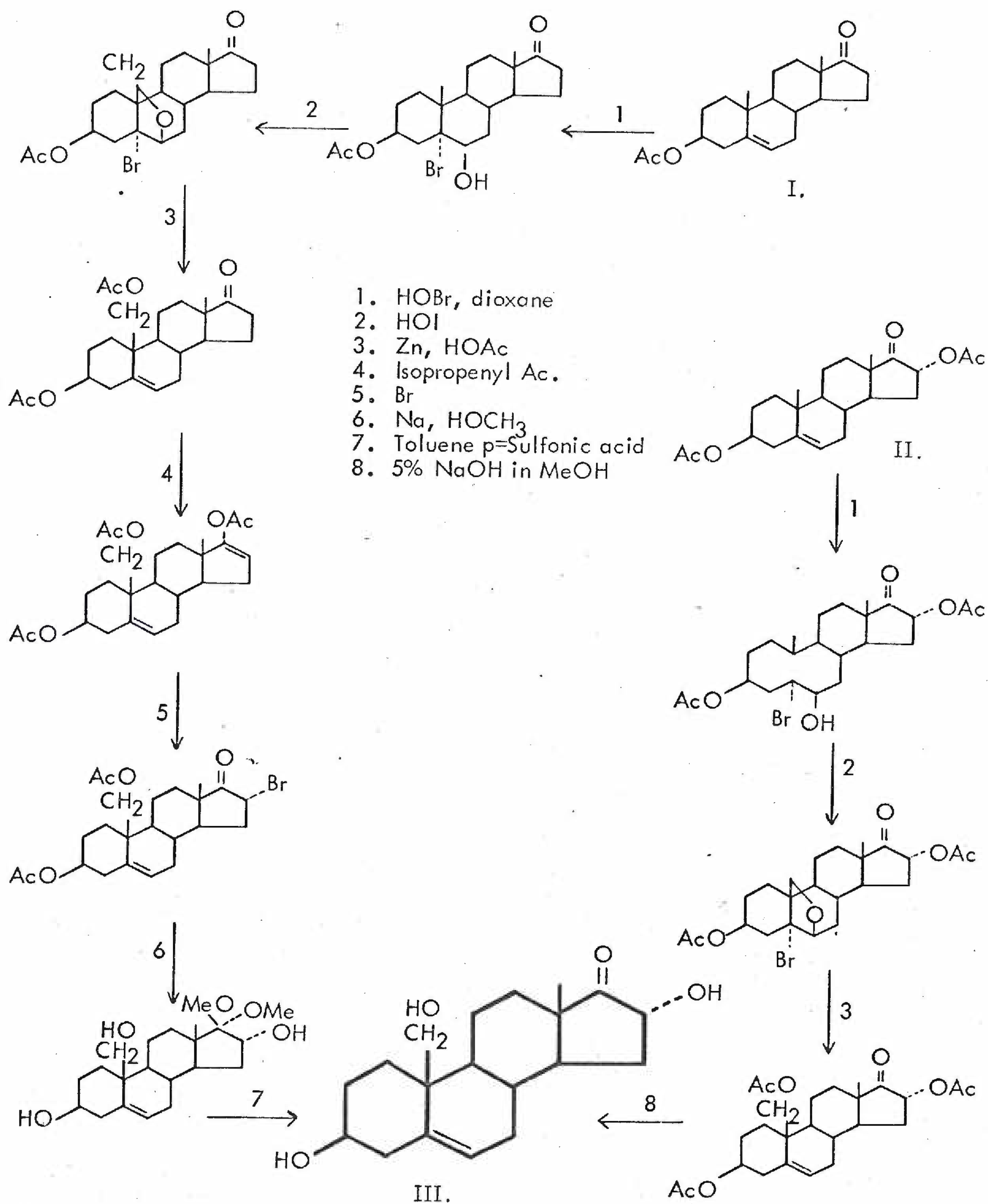
Figure No. 1.

Chemical synthesis of 16α, 19, dihydroxy DHA.

I. DHA-3-acetate.

II. Diacetoxy derivative of 16α-OH-DHA.

III. 16α, 19-dihydroxy DHA.



1. HOBr, dioxane
2. HOI
3. Zn, HOAc
4. Isopropenyl Ac.
5. Br
6. Na, HOCH3
7. Toluene p=Sulfonic acid
8. 5% NaOH in MeOH
I.
II.
III.
AcO
CH2
Br
OH
OAc
MeO
OMe
HO

route to synthesis of the desired compound was the incubation of 19-OH-DHA with male rat liver microsomes.

The second objective of this work was to attempt the isolation of 19-OH-DHA after incubation of DHA with placental microsomes. Such an isolation was reported by O'Kelly and Grant (62) while the synthetic and incubation experiments were in progress in our laboratory.

A third objective was to study alternative biosynthetic pathways of estrogens from neutral steroids using a placental microsomal system, utilizing radioactive precursors, and assessing the significance of 19-OH-DHA as an intermediate.

11. HISTORICAL ASPECTS OF STEROID INTERCONVERSION

A. Estriol Biosynthesis in the Maternal-Fetal-Placental Unit

Measurement of urinary estriol excretion during pregnancy has proven useful in evaluating fetal viability under certain clinical conditions. For example, a fifty percent reduction in urinary estriol excretion occurs with fetal death in utero (16, 9). Understanding this relationship between fetal viability and maternal urinary estriol excretion has been the source of considerable research effort; however, the precise details of the biosynthetic pathway of this important 16α-hydroxylated estrogen in the maternal-fetal-placental unit have not been fully established.

The three-decade search for the precursors of estriol began to be productive when Ryan (64) demonstrated the conversion of C_{19} steroids, having a Δ^4 -ene-3-one or a -Δ^5-3β-hydroxystructure, to estrogens by human placental microsomes. The possibility that C_{19} neutral steroids might arise in the fetus and be aromatized by the placenta led to a search

for these compounds in the term fetal circulation. DHA-S and 16α-OH-DHA-S have thus far been demonstrated in term fetal circulations in concentrations greater than 150 μg/ml of plasma and both compounds have been shown to have a significant arteriovenous difference indicating the fetal production of these two steroids (15, 20, 48).

Further evidence for the fetal production of neutral steroids has come from studying the neonate and the urinary estriol excretion in the anencephalic pregnancy. Isolation of 16α-hydroxydehydroepiandrosterone, 16-keto-androstenediol and Δ^5-androstenetriol from the urine of newborn infants has been carried out (5, 63). Confirmation of these findings, and the demonstration that infants born of mothers whose estriol excretion during pregnancy was reduced, have decreased rates of excretion of 16α-dihydroxydehydroepiandrosterone and 16-keto-androstenediol support the role of the fetus in supplying neutral steroid precursors of estriol — at least in the latter part of pregnancy. It should be noted that individuals with molar pregnancies also have decreased estriol excretion and decreased estriol production rates (3).

Study of urinary estriol excretion in pregnancies complicated by anencephaly has proven particularly useful in elucidating the source and precursors of estriol. Thus it has been known for a long time that the fetus has highly atrophic adrenal glands when it is without a hypothalamic center or a normal anterior lobe of the pituitary. Only recently was it recognized that such pregnancies are characterized by a rather low excretion of estrogens (27). In four such cases, data have been presented in whcih measurement of cord levels of DHA-S and 16α-

OH-DHA-S indicated a very low level of these compounds as compared to normal values (14). However, the levels of these two compounds in the maternal circulation were very similar to those previously reported for normal. The fetal adrenal gland has been shown capable of synthesis of DHA from acetate ^{14}-C by the age of 12-22 weeks. The order of magnitude of synthesis is similar to hyperplastic adult adrenal tissue. The maternal adrenal has less of a role as suggested by the observation of normal urinary estrogens in pregnant adrenalectomized women (18). The search for the estriol precursors during pregnancy is further complicated by the changing role of the fetus and placenta as endocrine organs throughout pregnancy. For example, homogenates of placenta at midtrimester are capable of 16α-and 16β-hydroxylation of estrone while the term placenta is incapable of 16α-hydroxylation of estrone, estradiol, DHA, androstenedione or testosterone (6). On the other hand, homogenates of fetal liver are capable of 16α-and 16β-hydroxylation of estrone at all periods of gestation including the neonatal period (66). Perfusion studies of midterm fetuses have demonstrated the conversion of both DHA and DHA-S to 16α-OH-DHA and Δ^5-triol -S[6], however, perfusion studies with previable fetuses, as well as incubations with fetal liver and adrenal homogenates, gave no hydroxylation of androstenedione (49). Fetal liver homogenates have been shown to be capable of aromatizing androstenedione and testosterone to estrone and estriol, but only slight aromatization occurred with DHA (66, 50, 28). A summary of the sources of the estriol excreted during pregnancy is given in Figure II.

MATERNAL URINARY ESTRIOL EXCRETION

Role of Maternal, Fetal, and Placental Organisms
Modified from Gold "Gynecological Endocrinology"

MATERNAL

Acetate
↓
Cholesterol
↓
Δ^5 pregnenolone
↓
17 OH Δ^5 pregnenolone
↓
DHA
↓ (to placenta)

PLACENTAL

Mid-trimester

DHA ---->16α-OH-DHA
↓ (DHA) → E_1 & E_2 -----> E_3 ← ↓ (16α-OH-DHA)

Late Pregnancy

DHA -/-> 16α-OH-DHA
↓ (DHA) → E_1 & E_2 --/--> E_3 ← ↓ (16α-OH-DHA)

FETAL

Late Pregnancy

Adrenal -----> DHA-S
↓
DHA
↓
(liver)
↓
16α-OH-DHA (to placenta -->
-/->
E_3

Mid-trimester

DHA-S <--- Adrenal
16α-OH-DHA-S
Adrenal
↓
DHA
↓
(liver)
↓
E_3

Figure 2

B. Mechanism of Aromatization

Biosynthesis of estriol from C_{19} neutral steroids necessarily involves several enzymatic reactions. Thus the conversion of DHA to estriol would involve the following reactions, but not necessarily in the order given: (1) 16α-hydroxylation (2) Dehydrogenation at C-3 (3)Δ^{5-4} isomerization (4) Aromatization of ring A (5) 17β-reduction. The proposed sequence in ring A aromatization of C_{19}, 3β-hydroxy-Δ^5-neutral steroids is: The oxidation of the 3β-hydroxyl function to a 3-ketone by 3β-hydroxy steroid dehydrogenase — a reaction which is NAD dependant; shift of the Δ^{5-6} double bond to the Δ^{4-5} position: 19-hydroxylation followed by elimination of the C-19 angular methyl group as formaldehyde or formic acid and finally elimination of a C-1 hydrogen.

The exact fate of the C_{19} carbon has not been clearly established. Breuer (8) provided some evidence for the removal of C_{19} methyl group as formaldehyde; however, Axelrod et.al (1) used testosterone-19-^{14}C with a placental microsomal preparation and found the radioactivity in formic acid but failed to demonstrate radioactive CO_2.

Structural requirements for ring A aromatization have been studied by Gual et. al. (33). They found that (1) rings C & D are necessary and that (2) unsaturation of ring A is also necessary; (3) presence of axial substituents at C-11 interferes with aromatization; (4) 11α-OH and 9α substituents do not interfere with ring A aromatization; however, a 2β-hydroxyl decreases aromatization. Townsley (69) further clarified the aromatization process when he incubated placental homogenate (10,000 g supernatant) with A^4-^{14}C selectively tritiated at C-1β or

C-1α. The 1β hydrogen was lost in conversion to estrone. Incubation with estra-4-ene-3, 17-dione also caused loss of the C-1β hydrogen showing that this loss is not dependant on the C-10 methyl group. He speculated that 1β hydrogen may be eliminated by hydroxylation. Breuer has demonstrated the conversion of Δ^7-DHA and 7α-OH-DHA to non-phenolic ring B aromatic C-18 compounds by perfusion studies of full term placentas. He suggested that 19-hydroxylation may occur without Δ^5-Δ^4 isomerization or oxidation of the 3β-hydroxyl groups (8).

C. Steroid 19-hydroxylation in vivo and vitro

The proposed role of 19-hydroxylation in the transformation of neutral to phenolic steroids has brought about a search for 19-hydroxylated intermediates and stimulated investigators to synthesize these compounds. Meyer (1955) first demonstrated the in vitro 19 hydroxylation of A^4 with cell free placental homogenates and the conversion of 19-OH-A^4 to estrogens was carried out by Longchampt (46). Recently, O'Kelly and Grant have reported the isolation of 19-OH-DHA and 19-OH-DHA-S from a placental microsomal system, incubated with DHA and DHA-S (62).

Further evidence for the role of 19-hydroxylation in placental aromatization of neutral steroids was brought about by the use of metapyrone (known as SU-4885). This compound has been shown to decrease 19-hydroxylase activity as well as that of 11β-hydroxylase (40) Giles and Griffiths showed inhibition of estrone formation from testosterone using placental homogenates (29), NADPH and O_2.

The 19-hydroxy derivatives of DHA, A^4, and T have been studied in vitro with a placental aromatizing system.

The aromatization of DHA to estrogens has been explained by the reaction sequence DHA --> A^4 or T--> 19-OH-A^4 or 19-OH-T --> estrogens. Attempts to characterize the 19-hydroxylated intermediates have been carried out by several investigators (51, 54). Morato (51) studied the quantitative conversions of 19-OH-A^4, and 19-oxo-A^4 to estrogens _in vitro._ He found, using placental homogenates, the following percent conversions to estrogens: 19-oxo-A^4 (100%); 19-OH-A^4 (50-60%): A^4 (30-40%); and 10β-carboxy estr-4-ene-3, 17-dione (<5%): this lends support to the above reaction sequence. This reaction sequence, however, does not account for the direct 19-hydroxylation of DHA. Wilcox and Engel (73) demonstrated that the _in vitro_ placental conversion of 19-OH-DHA and 19-OH-A^4 to estrone was greater than the conversion of C-10-CH_3 steroids. Starka (67), using a placental microsomal preparation showed that 19-OH-DHA was converted to estrogens in higher yields than were A^4 or T.

A study of the early reactions kinetics by Wilcox et al., (72), again using placental microsomes, NADPH and O_2, showed that when using androstenedione 4 C^{14} radioactivity appeared in 19-OH-A^4 but not in estrone or 17β-estradiol in the first minute of the reaction.

D. Steroid Sulfates in Estrogen Biosynthesis

The role steroid sulfates play in estrogen biosynthesis, particularly in the fetal-maternal-placental unit, has only recently received attention. Baulieu (4) et al., studied pregnant individuals and demonstrated that DHA-S was converted in vivo to estrone and estradiol more efficiently than were testosterone or androstenedione. They postulated

the possibility that the sulfate may more easily cross the cell membrane. Barlow (2) was unable to demonstrate significant incorporation of DHA-S into estrogens in the non-pregnant individual. The problem seemed to be partially clarified when Diczfalusy (8) demonstrated hydrolysis of DHA-S by the intact maternal or fetal placenta. It is known that fetal tissues have extensive sulfokinase activity, and that the placenta contains sulfatases: therefore, the sulfated steroids were suggested as conjugates for fetal placental transfer. That in vivo conversion of DHA-S to estrogens was greater than that of the free steroid was partially explained by Siteri (65) when he reported that the half-life of plasma DHA-S in normal subjects was approximately nine hours and that of the free steroid only 15 minutes; therefore, DHA-S was present in the circulation longer and more of it could be converted to estrogens. It should be noted, however, that he gave no data for this statement.

Kirchner et al., (43) administered 7α-^{3}H-DHA-S and estrone ^{14}C sulfate to the intact midterm fetal-placental and maternal circulations. They found conversion to estriol was greater from DHA-S than from estrone sulfate in the fetal placental circulation while the opposite was true in the maternal circulation, thus indicating different pathways of synthesis. Charreau (11) perfused human term placentas through both fetal and maternal circulations with DHA-4-^{14}C-S and DHA-7α-^{3}H-sulfate. They found a difference in H^{3}/C^{14} ratios between 19-OH-A^{4} and andostenedione, thus indicating that DHA or its sulfate could be hydroxylated before conversion to the Δ^{4}-3-ketone; however, no labeled 19-OH-DHA was found in the placentas or the perfusates.

Morato (55) further studied the 19-hydroxylation of the steroid sulfates with placental microsomal preparations, using DHA-4-^{14}C, DHA-7α-^{3}H, and A^4-4-^{14}C and studying the $^{3}H/C^{14}$ ratios in estrone, androstenedione suggested that 19-hydroxylation could take place not only in compounds with Δ^4-3-keto structures but also in DHA and its sulfate. His studies suggested three routes of synthesis as follows:

1. --DHA-S --> DHA --> A^4 --> 19-OH-A^4 --> E_1
2. --DHA-S --> 19-OH-DHA-S --> 19-OH-DHA--> 19-OH-A--> E_1
3. --DHA-S --> 19-OH-DHA--> 19-OH-A^4 --> E_1

O'Kelly and Grant have isolated both 19-OH-DHA and 19-OH-DHA-S using DHA as substrate in a placental microsomal system (62).

A new concept for the role of sulfurylated neutral steroids in estrogen biosynthesis arose with the work of Oertel (60). He injected [7α-^{3}H]-DHA-[^{35}S]-sulphate in female subjects and found that the isolated urinary estrone had the original $^{3}H/^{35}S$ ratio. He further synthesized the labeled 3,5,-dienol sulphate of androstenedione (7α-^{3}H-A^4-^{35}S-Na) and 7α-^{3}H DHA -^{35}S-Na) then using placental microsomes DPNH and O_2 was able to show that the $^{3}H/S^{35}$ ratio was nearly the same in estrone as in the labelled 3, 5, dienol sulphate and DHA sulphate (32). The yields of estrogens from the 3-5 dienol-A^4-sulphate were higher than from DHA-sulphate thus suggesting that the biosynthesis of estrogens from DHA-S may proceed via androstenedione sulphate. Figure III gives the general scheme of the aromatization process within the placenta. For purposes of clarity, all pathways are included for DHA. The synthesis of estriol is assumed to involve the same mechanisms, the only addition being that of the 16α-OH-group on all intermediates.

Figure No. 3

Biosynthetic pathway of estrone and 17β estradiol from DHA. The structural formula for estradiol - 17β is omitted because it is assumed that estradiol -17β is synthesized directly from estrone by 17β reduction rather than from a 17β-OH derivative.

OHA-S

A^4-S

19-OH-DHA-S

19-OH-A^4-S

19-OH-OHA

(?)

19OA A^4

A^4

T

19-OH-T

Estrone and/or Estradiol

OHA

A^4

19-OH-A^4

19-OXO-A^4

19-OH-DHA

(?)

E. 16α-Hydroxylation

1. Placental 16α-hydroxylation

The interaction of 16α-hydroxylation in the aromatization process within the placenta has only been studied very recently (Diczfalusy 18, 1967). In vivo, perfusion of midterm placentas with 16α-OH-A^4-4-^{14}C, 16α-OH-T-4-^{14}C and 16α-OH-DHA-7α-^3H, gave estriol in high yields; however, there was no interconversion between 16α-OH-A^4 or 16α-OH-T. The extent of aromatization (based on the amount of estrogen isolated from the placenta and perfusate) was much less with 16α-OH-T than with either 16α-OH-DHA or 16α-OH-A^4. The Δ^5-triol also gave a less yield of estrogens as compared to DHA and T. Diczfalusy explains these findings by postulating that the vicinal 16α, 17β-hydroxyl groups of 16α-OH-T and Δ^5-triol interfere with the removal of the angular methyl group at C-10, leading to an impeded aromatization.

2. Rat liver 16α-hydroxylation in vitro

Rat liver 16α-hydroxylation of DHA was demonstration in 1962 (13). The 16α-hydroxylase activity in male rat liver slices was increased with addition of NADPH, was found to require oxygen, and to be virtually absent in female rats. This subject was extensively reviewed by Heinrichs (35).

3. Bacterial 16α-hydroxylation

Microbiological hydroxylation of steroids has been well documented, practically every position on the steroid nucleus can be hydroxylated by exposure to a suitable microorganism. Streptomyces roseochromogenus has been shown to introduce 16α-hydroxyl

groups on a variety of C_{18}, C_{19}, and $C_{21,}$ steroids (19). Recently Solomon and Younglai (67) have used this organism to hydroxylate a conjugated steroid, 16α-OH-DHA-S. Further, Kogan and Elin have demonstrated 16α-hydroxylase activity with a cell-free preparation (22, 27), of S. roseochromogenus.

III. MATERIALS AND METHODS

A. Animals

Sprague Dawley rats were purchased from Berkeley Pacific Laboratories, Berkeley, California. Male rats, 11 - 14 weeks of age, were used. The animals were fed Purina rat chow and maintained by the Animal Care Department in protective isolation for five days after purchase.

1. Preparation of rat liver microsomes

Rats were killed by a blow on the head and the livers removed, weighed, washed, and perfused with 0.25 M sucrose solution. The liver was then cut into approximately one centimeter slices and homogenized in 0.88 M sucrose solution (1/3 w/v) with a Teflon pestle inside a smooth glass homogenizer (1.25×10^{-2} mm of clearance).

Nuclei and mitochondrial fractions were removed simultaneously by centrifugation at 10,000 x g for 35 minutes at $4^{\circ}C$ in a Lourdes refrigerated centrifuge. The supernatant was made isotonic by the addition of 5 volumes of 0.04 M KCL and re-centrifuged at 10,000 x g for 25 minutes in the Lourdes centrifuge. The 10,000 x g supernatant was collected and re-centrifuged at 78,000 x g (Spinco #30 rotor at 30,000 rpm) for 60 minutes. The resultant pellet was resuspended by homogenization in 0.1 M phosphate buffer, pH 7.4 for incubation experiments with a volume equal to the original suspension volume.

B. Bacteria

Streptomyces roseochromogenus (AT CC 3347) was obtained from American Type Culture Collection. The media for growth of S. roseochromogenus was Starch Salt Media (22) as follows:

1. Starch 10 g
2. $(NH)_2 SO_4$ 2 g
3. $MgSO_4$ 1 g
4. Na Cl 1 g
5. K_2HPO_4 1 g
6. Distilled H_2O q. s. 1, 000 ml.

The above media was inoculated with 5 ml. of S roseochromogenus culture in nutrient broth and incubated @ 28° C for 24 hours with air as the gas phase. After the initial 24 hour growth phase, the media was diluted 8-fold with starch salt media; divided into 8 equal portions and incubated an additional 6 hours in air at 28^oC. All incubations were carried out in a Gyrotory Shaker (model G76) with air as the gas phase.

Induction of 16α-hydroxylase activity was carried out by adding 5 mg. 11α-hydroxyprogesterone (in 0. 5 ml. methanol) to each of the 8 flasks and incubating for 18 hrs. at 28^oC. At this peak of the growth phase, the mycelia were filtered through cheese cloth, washed successively with 50 ml. 0. 5% Na Cl, 50 ml. distilled H_2O and 50 ml of 0.001 M EDTA. The washed mycelia was resuspended in 200 ml of 0.05 M phosphate buffer, pH 7.4, containing 0.001 M EDTA and 0.001 M GSSH.

C. Placenta

All placentas were obtained immediately following full term deliveries, placed in 0.9% NaCL at $0^{o}C$, and perfused with 0.25 M sucrose solution to remove the remaining fetal blood. After perfusion, the placentas were taken to the cold room and all remaining work carried out at $4^{o}C$. Placentas were selected from patients without evidence of disease or without known complications of pregnancy.

1. Crude Microsomes

The human placentas were dissected free of fetal membranes, sliced, into 1 cm chunks, weighed and added to 1.5 volumes of the following buffer solution:

0.05 M phosphate buffer
0.25 M sucrose
0.01 M nicotinamide
pH 7.0

Homogenization was then carried out with a Virtis-homogenizer at setting #6 for 2 minutes in a 300 ml flask. Following homogenization, differential centrifugation was carried out as follows:

1. Centrifuge for 20 minutes at 700 x g in 9 RA head of Lourdes centrifuge 2000 rpm.

2. Supernatant from one recentrifuged for 15 min. at 10,000 x g Lourdes centrifuge (9 RA head, 8875 rpm).

3. The supernatant from the 10,000 x g centrifugation was decanted and centrifuged 60 minutes at 30,000 rpm (78,000 x g) in the #3 head of the Beckman model L centrifuge.

4. The pellet from the 78,000 x g spin was resuspended in the 0.05 M phosphate buffer solution so that 4.0 cc was equivalent to 12 gms of wet placental tissue.

The microsomes resuspended as outlined in 4 above were used for placental incubation experiments.

D. Reagents

All reagents were reagent-grade unless otherwise specified. Special reagents are listed as follows:

1. NADPH was obtained from Sigma Chemical Company.

2. Androst-4-ene-3, 17-dione-4-C^{14} in benzene 0.05 millicuries in 0.5 ml benzene (0.283 mg) was purchased from New England Nuclear Corp., Boston, Mass., Lot #302-87.

3. Dehydroepiandrosterone-7 H^3 1.0 millicurie in 1.0 ml. benzene (0.2 mg) was obtained from New England Nuclear Corp., Boston, Mass., Lot #184-63-8.

4. DHA-3-Acetate was obtained from Mann Research Lab. New York, N.Y. Lot T 3917.

5. Estrone, Estradiol, and Estriol were obtained from Sigma Laboratories.

6. Androstenedione was purchased from Calbiochem, Lot #71864.

7. Reference 16α-OH-DHA, supplied by Dr. Colás, M.P. 183 to 188° C.

8. Purified chloroform: chloroform was washed with water, then 0.1 N NaOH and distilled.

E. Incubation Techniques

All incubations were carried out in duplicate, and a blank was prepared for each sample. The blank was the same as the sample except that the substrate was added after the incubation.

1. Rat Liver Microsomal Incubations

To a 25 ml Erlenmeyer flask was added 0.2 ml. of suspended microsomes, Krebs Ringer Phosphate q.s. to 6.0 ml., NADPH (12 μmoles and 1 μmoles substrate. The resultant suspension was incubated with shaking (120 rpm) at 37-38°C in a Model 2156, Research Specialty Co., water bath. The duration of incubation

varied from 30 to 60 minutes, depending on the experimental conditions required. The reaction was stopped by placing the flask in a methanol, dry-ice bath at -60°C.

2. S. roseochromogenus Mycelial Incubations

Thirty mg. of 19-OH-DHA or DHA in 3 ml methanol was added to 200 ml of the suspended mycelia. After addition of 120 μ moles of NADPH, the incubation was carried out for 24 hours @ 28°C with O_2 as the gas phase. The reaction was stopped by placing the culture in a deep freeze at -18°C.

F. Extraction procedures

All extraction procedures were carried out after the proper substrate was added to the sample blank. The quantity of substrate added to the blank was the same as for the incubation. For recovery experiments, a specified amount was added depending on the amount of steroid expected in the incubation sample.

1. Rat Liver Microsomes

a. Extract sample 2 times with 40 ml purified chloroform.

b. Wash pooled extract with 15 ml. saturated $NaHCO_3$ and two times with distilled water.

c. Chloroform extract dried with 9 g Na_2SO_4 for 30 min. and evaporated with a Rotovac evaporator.

d. The residues were transferred to conical test tubes with five separate washings of methanol and evaporated under N_2. The residues were then ready for spotting on thin-layer plates of silicagel.

2. S. roseochromogenus

Extraction procedure was similar as that outlined for rat-liver microsomes except all quantities were increased ten-fold.

3. Placental Microsomal Extractions

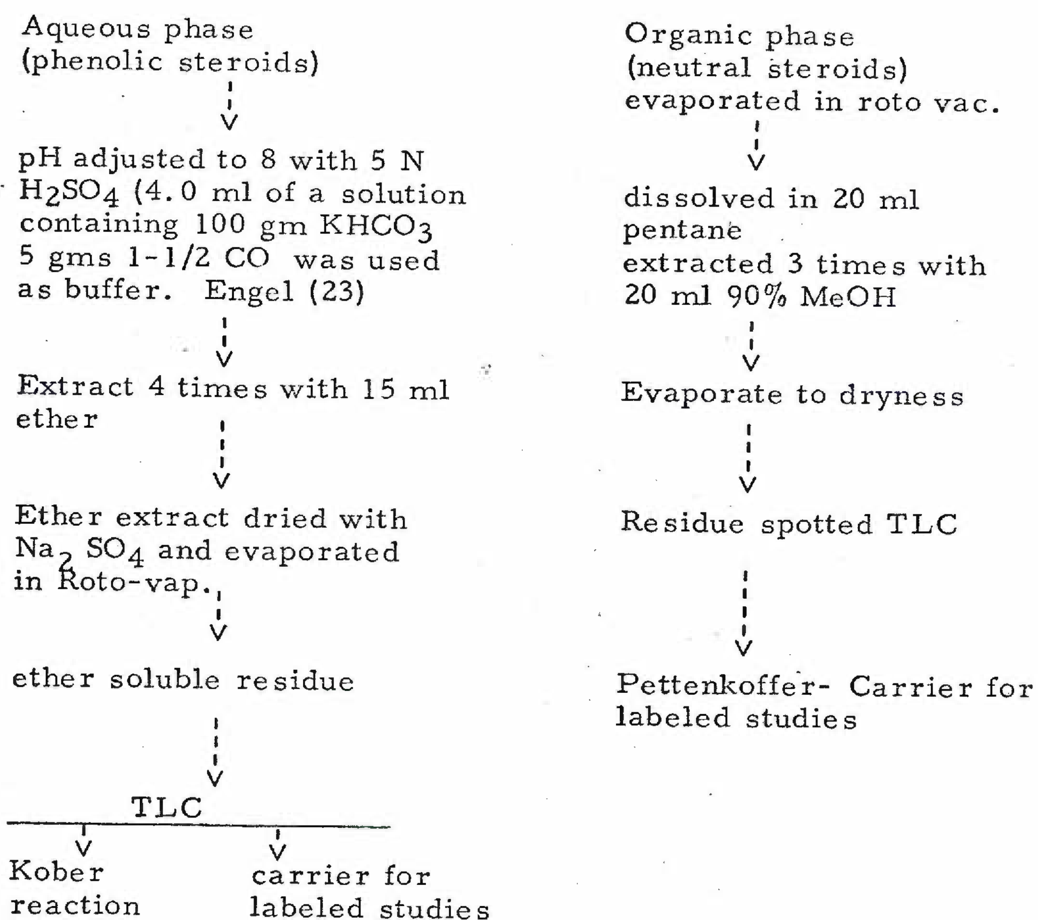


FIGURE NO. 4

G. Miscellaneous Procedures

1. Melting points (uncorrected) were obtained with a Koffer hot-stage microscope. The rate of temperature change through each melting point was $2^{o}C$/minute. A minimum of three determinations was carried out on each synthetic steroid.

2. Infrared Spectra of steroids in KBr pellets were determined using a Perkin Elmer Model 221 infrared spectrophotometer. For IR-spectra with less than 100 μg of steroid, a special micro adaptor for making pellets was utilized.

3. Protein content of the microsomal preparations was determinded by the biuret method (31). Sodium deoxycholate 2% (0.2 ml) was incubated with 0.2 ml of the crude microsomal suspensions for 2 hours at 37° C; 0.9% NaCl was added to a sample, bringing the volume to 1 ml. Four ml of biuret reagent was added to each sample, and the solution allowed to stand at room temperature for 30 minutes. A sample blank was prepared as outlined above except that 0.45 N NaOH was added instead of the biuret reagent. The sample was read against the blank at 540 μmoles in a Zeiss PMQ II spectrophotometer. Duplicate samples containing 4.0 mg. of serum albumin were used as reference standards for calculation of the amount of protein.

4. Carbon, Hydrogen, Oxygen and Bromine quantitiative analyses were done by Micro-Analysis, Inc., Wilmington, Delaware.

5. Mass spectroscopy was done by West Coast Technical Service, San Gabriel, California.

6. Modified Pettenkofer Reactions (37) was done as follows: To the dry residues after extraction 1.0 ml of the following freshly mixed reagents was added:

1 part glacial acetic acid

4 parts 1% furfural in 50% acetic acid (by weight)

15 parts 17 N H_2SO_4

The tubes were shaken, incubated 12 minutes at 67°C, immersed in an ice bath for 1 minute and read in a Zeiss PMQ II spectrophotometer at 635, 660 and 685 μmoles. The samples were read against a reagent blank. Duplicate standard samples were run with each experiment in an amount corresponding to the quantity of steroid expected in the unknown sample.

H. Thin-layer Chromatography

All plates were 0.25 mm thick and were run in glass tanks 8 inch x 8 inches. The inside of the tanks was lined with Whatman #1 chromatography paper to increase the rate of saturation of the gas phase. Three hundred ml. of the appropriate solvent system was placed in the tank and allowed to equilibrate for 24 hours before use. Aliquots of the residue dissolved in methanol or ethanol were applied with the use of Hamilton micro syringes. The solvent front traveled 15 cm from the starting spot before the plates were removed from the tanks and allowed to dry in air.

1. Silica Gel G., Lot F 913, Brinkmann Instruments, Inc., Westbury, N.Y. 11590 (40 gms and 60 ml H_2O was used to separate most neutral steroids. Identification of neutral steroids was carried out with one or more of the following color reagents:

a. Sulfuric acid spray. Plates were sprayed with 2% (w/v) solution of concentrated sulfuric acid in aqueous ethanol (50: 50 w/v), and heated 15 min. at 100° C. All steroids were identified by comparison with standard.

b. Picric acid reaction. Plates were sprayed with a solution of 100 mg picric acid in 36 ml glacial acetic acid to which was added 6 ml of 70% perchloric acid. Plates were gently heated and air dried (3β-OH-Δ^5 steroids).

c. Blue tetrazolium. A mixture (1:9) of 1% (w/v) solution of blue tetrazolium in methanol was freshly prepared and sprayed on the plates which were then heated to 85° C for 5 minutes.

2. Silica Gel HF 254, Lot F 3676, Brinkman Instruments Inc., Westbury, N.Y., 40 gms, 60 ml H_2O was used for identifying Δ^4-3 ketosteroids. These steroids were spotted on the plates, run in the appropriate solvent system, allowed to dry in air and qualitative identity established by viewing the plates in a Chromato-Vue model C-3 UV light.

3. Dichlorofluorescein plates (70). These plates were prepared by spreading a slurry of 40 gm of Brinkman silica gel H containing 15 mg of 2:7 dichlorofluorescein dissolved in 20 ml of 95% ethanol and 80 ml H_2O. Thorough mixing was necessary. The plates were activated by heating in an oven for 1 hour @ 100° C. After chromotography, the fluorescent plates were air dried and estrone, estradiol, and estriol located with the aid of ultraviolet light. The sensitivity of this method of detection was less than 5 μg of estrogen

spot when the plate was examined with ultraviolet light.

4. Solvent systems for T.L.C.

a. System C

Ethyl acetate	50%
Cyclohexane	50%

b. System M

Ethyl acetate	75%
N-Hexane	20%
Glacial acetic acid	5%

c. Ethanol-Benzene Systems. Variable composition (% by volume) depending on polarity required for separation.

Et OH	10	15	25
Benzene	90	85	75

d. System B

Ethyl acetate	45%
n-Hexane	45%
Et OH	10%

5. <u>Elution of steroids from thin-layer plates</u>

a. Neutral steroids: After identification, the area containing the steroid was removed and placed in a conical test tube. The steroids were extracted twice with 2 ml aliquots of 1:9 (v/v) dichloromethane, methanol, centrifuged, decanted and evaporated to dryness in vaccuum.

b. Phenolic steroids were extracted from the dichloro-flourescein plates with diethyl ether at 37°C for 10 minutes. The ether extracts were pooled and evaporated to dryness. Recovery experiments will be given in the results section.

IV. RESULTS

A. Synthesis of 5α-bromo-3β-acetoxy-6β-hydroxyandrostan-17-one

The synthesis was carried out according to the method of Grenville et al., (32). 6.6 g of 3β-acetoxyandrost-5-ene-17-one MRL R 1847 was dissolved in 50 ml p-dioxane (MCB-CB 368) and to this solution was added 2.8 g N-bromoacetamide (K & K - 70515) dissolved in 15 ml H_2O and 1.0 ml. 72% perchloric acid (Merk). After allowing the reaction to proceed at room temperature for 18 hours, 435 ml of water was added, the precipitate was collected by filtration and dissolved in 360 ml chloroform. The chloroform extract was washed as follows:

1. 35 ml 0.1 N NaI (aqueous phase yellow-chloroform phase purple)
2. 35 ml 0.1 N $Na_2S_2O_3$, with resultant clearing of both chloroform and aqueous phases.
3. 35 ml. 0.1 N Na_2CO_3
4. 35 ml H_2O, three times. The pH of the third washing was 7.

The chloroform extract was then dried with 9 g anhydrous Na_2SO_4, filtered, and the solvent evaporated in a roto-vac at room temperature. The resultant oily residue was dissolved in 30 ml of warm acetone and approximately 100 ml boiling hexane was added until precipitation started and just cleared with further heating. The crystals (needles) formed after 24 hours were collected by filtration and dried in vacuum for 12 hours at room temperature. Yield 2.2 g (33%) 3β-acetoxy-5α-bromo-6β-hydroxyandrostan-17-one, m.p. 173-175°C (reported 173-175°C), R_F's 0.68 and 0..44 in systems M and C respectively.

Infrared Spectra is shown as figure 5 A.

λmax 3500, 2940, 1740, 1380, 1250, 1030.

The above reaction was carried out 6 separate times. The total starting material was 39.6 gms and the over-all yield 13.2 gms or 33% of the theoretical yield.

B. Synthesis of 3β-acetoxy-5α-bromo-6β-19 oxideandrostan-17-one

Synthesis was done according to the method of Kalvoda et al., (41). To 33 grams of lead tetracetate (MCH LX200) was added 1.5 ml of glacial acetic acid, 15.0 grams of anhydrous calcium carbonate and 1350 ml of cychlohexane. The reaction mixture was stirred with a magnetic stirrer and boiled under reflux for 30 minutes. During this period a black precipitate formed. After 30 minutes, 7.6 gms of 3β-acetoxy-5α-bromo-6β-hydroxyandrostan-17-one, m.p. 173-175°C, and 9.0 grams of iodine (Merk 7300) wereadded to the reaction mixture. The reaction was then carried out under constant illumination with a Sylvania 650 watt movie light providing sufficient heat to maintain constant boiling. The reaction was allowed to proceed until the purple, iodine color disappeared, or for 90 minutes, whichever was first. The mixture was then cooled to room temperature, filtered, and the filtrate washed with 90 ml of 0.1 N sodium thiosulfate, three times with 100 ml of water, and dried with 25 grams anhydrous sodium sulfate. The cyclohexane extract was evaporated in a rotavac and the resultant oily residue was crystallized from ether. Four grams of 3β-acetoxy-5α-bromo-6β, 19-oxidoandrostan-17-one were obtained. A double M.P. was found: 177-179 / 186-189° C (reported 174-178 / 184-187° C) and the yield was 51%. R_F: System C, 0.45; system M, 0.69.

Infrared Spectra is Figure 5 B.

λmax 2930, 2890, 1740, 1440, 1370, 1250, 1030, 920.

C. Synthesis of 3β, 19-diacetoxyandrost-5-en-17-one

Synthesis was according to the method of Kalvoda et al., (41). Two grams of 3β-acetoxy-5α-bromo-6β, 19-oxidoandrostan-17-one, M.P. 177-179 / 186-189° C, was heated under reflux at 100° C with 6.0 grams of zinc dust (Mallinkrodt 8681) and 60 ml of glacial acetic acid. The reaction was stopped after 12 hours by cooling in an ice bath, the mixture was filtered and the zinc dust and white precipitate in the filter was washed with 240 ml of dichloromethane-glacial acetic acid solution; the aqueous phase with the acetic acid was discarded and the organic phase was neutralized with 45 ml of 1% sodium bicarbonate, and washed twice with 45 ml water. The dichloromethane was then dried with 10 g of anhydrous sodium sulfate, filtered and evaporated in a rotovac. The oily residue left after evaporation of the dichloromethane was crystallized from 30% aqueous methanol. 900 mg (yield 45%) of 3β, 19-diacetoxy-androst-5-ene-17-one was obtained, m.p. 103-105°C, (reported 103-105°C) R_F's system M, 0.7 ; system C, 0.49.

Infrared Spectra is Figure 5 C.

λmax 2950, 1740, 1435, 1390, 1370, 1250, 1040.

D. Synthesis of 3β, 19-dihydroxyandrost-5-en-17-one

Hydrolysis of 3β, 19-dihydroxyandrost-5-en-17-one to 19-hydroxy-dehydroepandrosterone was carried out as follows: 2.8 grams of 3β, 19-diacetoxyandrost-5-en-17-one was dissolved in 75 ml of 5% methanolic sodium hydroxide and left at room temperature for one hour. Neutralization was carried out with 6 N sulfuric acid. The sodium sulfate

Figure No. 5

A. Infrared spectra of

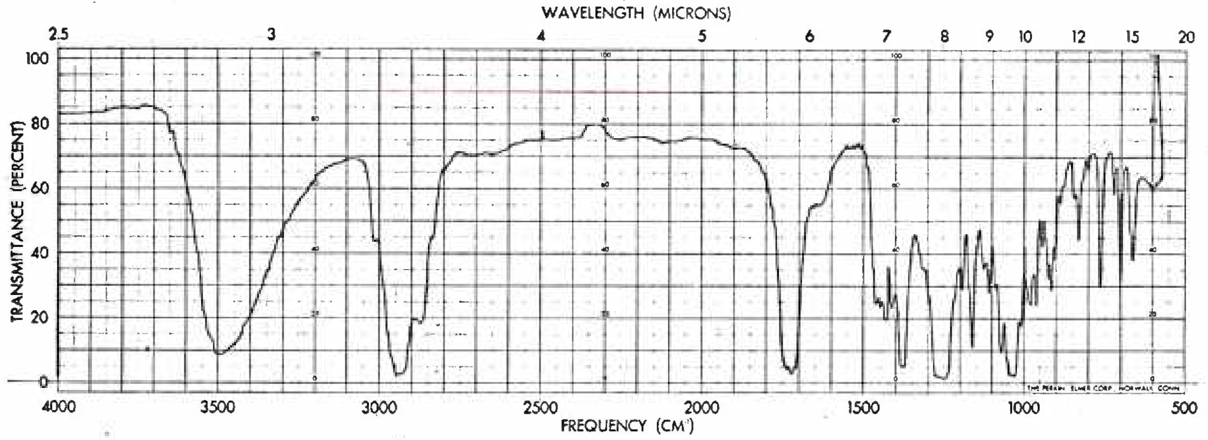
5α-bromo-3β-acetoxy-6β-hydroxyandrostan-17-one.

B. Infrared spectra of

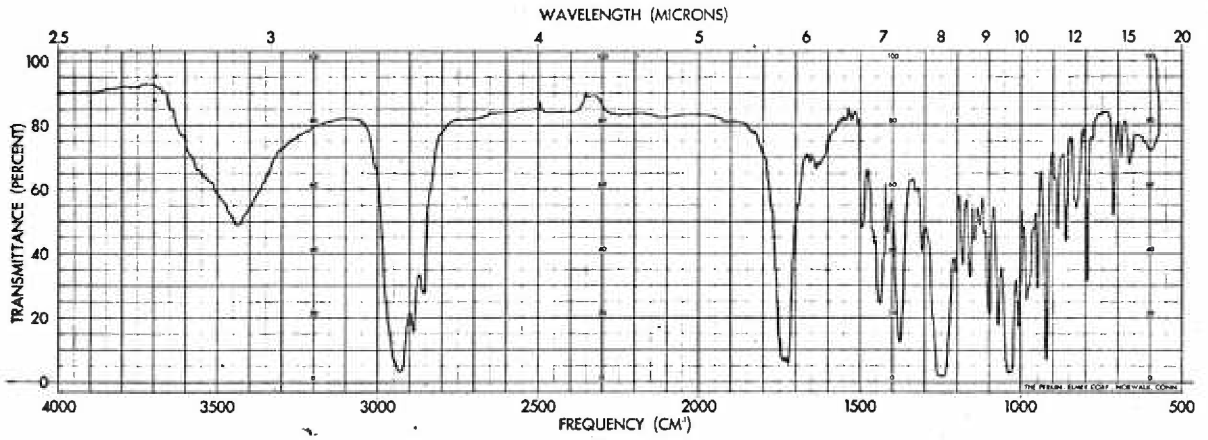
3β-acetoxy 5 bromo-6β-19 oxidoandrostan-17-one.

C. Infrared spectra of

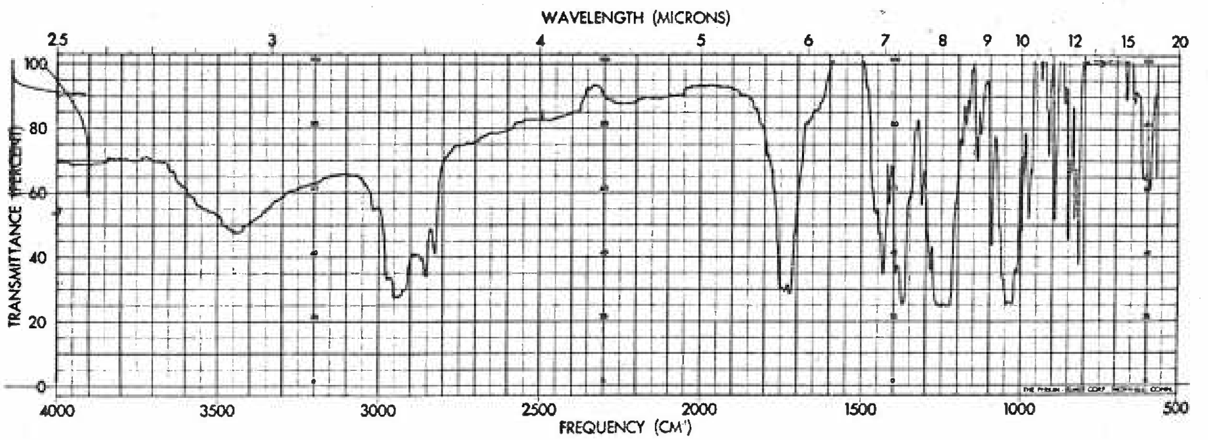
3β-19-diacetoxyandrost-5-in-17-one.



A



B



C

crystals were washed with anhydrous methanol and the pooled methanol filtrate evaporated to 30 ml in the rotovac at room temperature. The remaining methanol was extracted twice with 300 ml of purified chloroform. The chloroform extract was then washed with 30 ml of 1% sodium bicarbonate, twice with 30 ml water, and dried with 15 g anhydrous sodium sulfate. Evaporation of the chloroform left an oily residue which was crystallized from ethyl acetate to give 1.5 g product m.p. 214-220° C, (personal communication from Dr. O. Helpern, Syntex Research Laboratories, 219-221° C). Analysis: C74.84, H9.09, 015.83 (calculated: C74.96, H 9.27, O15.76). In thin-layer chromatography (silica gel G) with system M, the R_F was 0.3 migrating as a single spot (with 50 μg and sulfuric acid spray). Infrared spectra is Figure No. 6 A, Sulfuric acid spectra is Figure No. 7. λ max 3400, 2950, 2935, 2900, 2890, 1740, 1065, 1035, 1025.

E. Synthesis of 3β, 17-diacetoxyandrost-5, 16-diene

The synthesis was carried out according to the method of Kelly and Sykes (42). 3β-acetoxyandrost 5-ene-17-one (7.5 g) and 600 mg p-toluene-sulphonic acid (MCB 2736) were dissolved in 90 ml of isopropenyl acetate (K & K). The solution was heated (91°C) under reflux with a 10 cm fractionating column so that any acetone formed would be removed. The reaction was heated for 8 hours and 10 ml of fresh isopropenyl acetate was added each hour. After 8 hours the solution was evaporated to 45 ml, poured into 100 ml cold 0.1 N sodium carbonate and extracted three times with 150 ml ether. The ether extract was washed with 30 ml 0.1 N sodium carbonate,

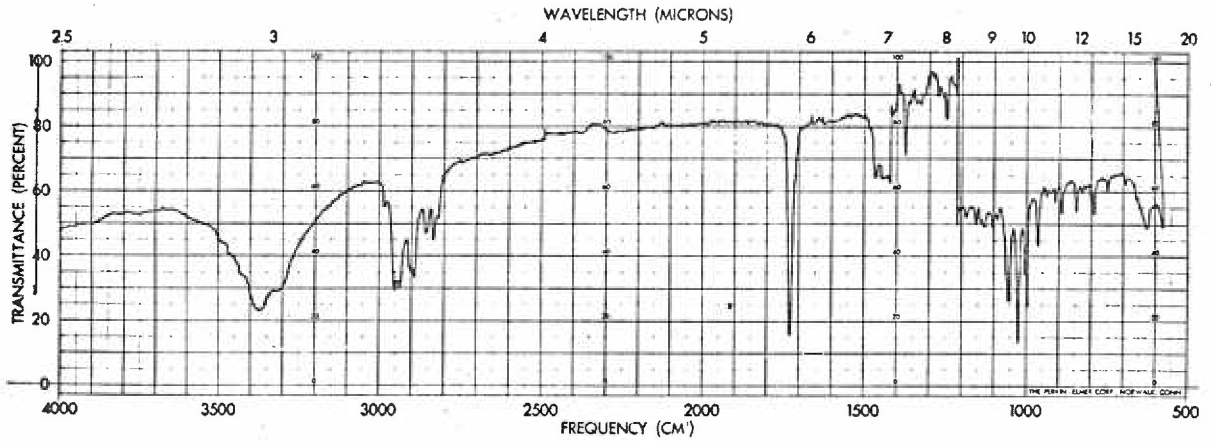
Figure No. 6

A. Infrared spectra of 19 hydroxy DHA

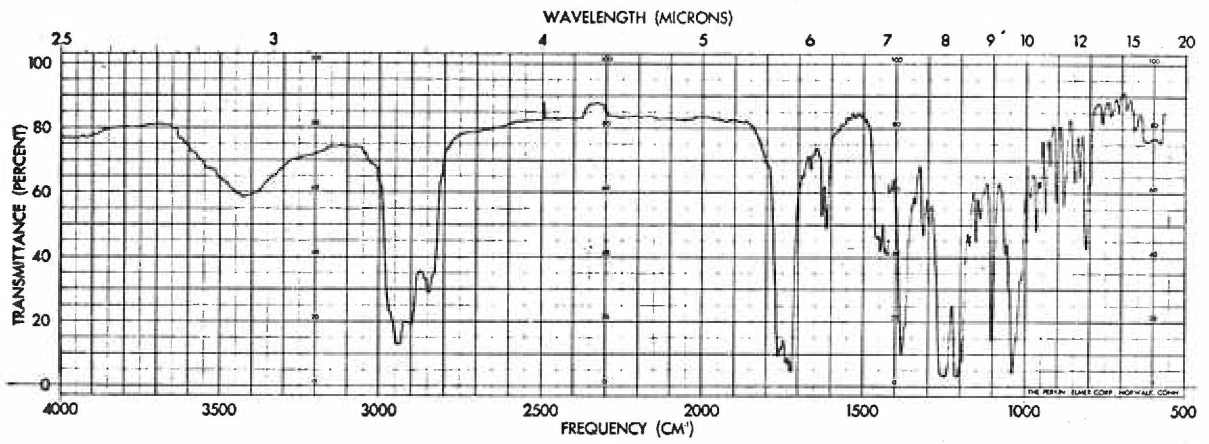
B. Infrared spectra of 3β, 17 diacetoxyandrost-5-16 diene.

C. Infrared spectra of

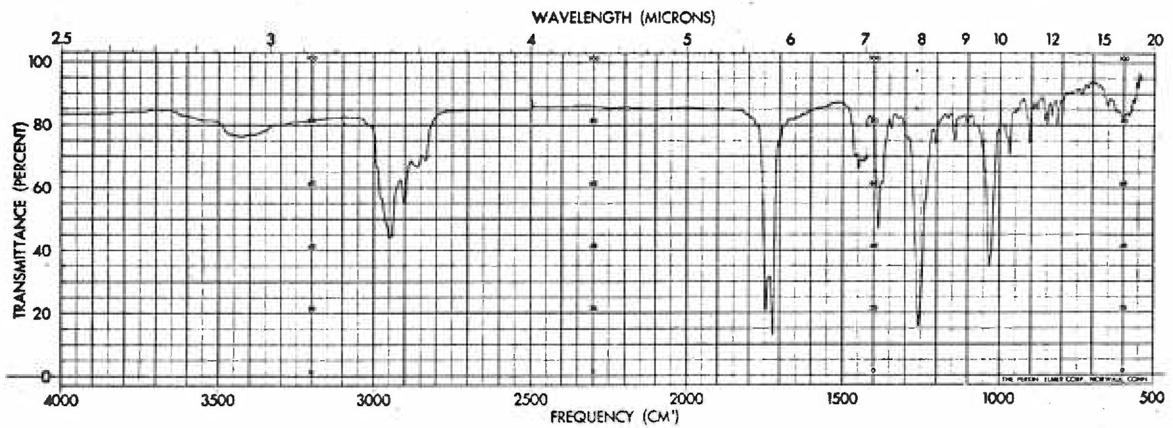
3β-acetoxy 16α-bromoandrost-5-ene-17-one.



A



B



C

30 ml of saturated sodium chloride solution, dried with 15 g anhydrous sodium sulfate, and evaporated to an oily residue in the rotovac. The oily residue was crystallized from 60% petroleum ether. Yield 5.1 g (68%) m.p. 146-150°C (reported, 147-148°C). R_F is: System C, 0.68; system M, 0.83.

Infrared spectra is Figure No. 6B.

λ max 2940, 2900, 1760, 1735, 1720, 1470, 1250, 1210, 1190, 1100, 1040.

F. Synthesis of 3β-acetoxy-16α-bromoandrost-5-ene-17-one

The method of Kelly and Sykes (42) was followed. 50 grams of 3β, 17-diacetoxyandrost-5, 16-diene (m.p. 146-150°C) was dissolved in 200 ml carbon tetrachloride and cooled to 14°C. The solution was stirred and 2.12 g bromine was added in 25 ml carbon tetrachloride over 2 minutes. The solution was stirred for an additional 2 minutes and 100 ml of 5% aqueous sodium hydrogen sulphite was added. The steroid was extracted twice with 250 ml chloroform. The chloroform extract was washed with 50 ml 0.1 N sodium carbonate, and with 50 ml saturated sodium chloride, dried with 10 grams anhydrous sodium sulfate, and evaporated to dryness in the rotovac. The residue was crystallized from ethanol (yield 3.8 gms, 70%) : m.p. 173-175°C; after recrystallization from ethanol 176-178°C (reported 181-183°C). R_F is: System, M 0.82; system C 0.67.

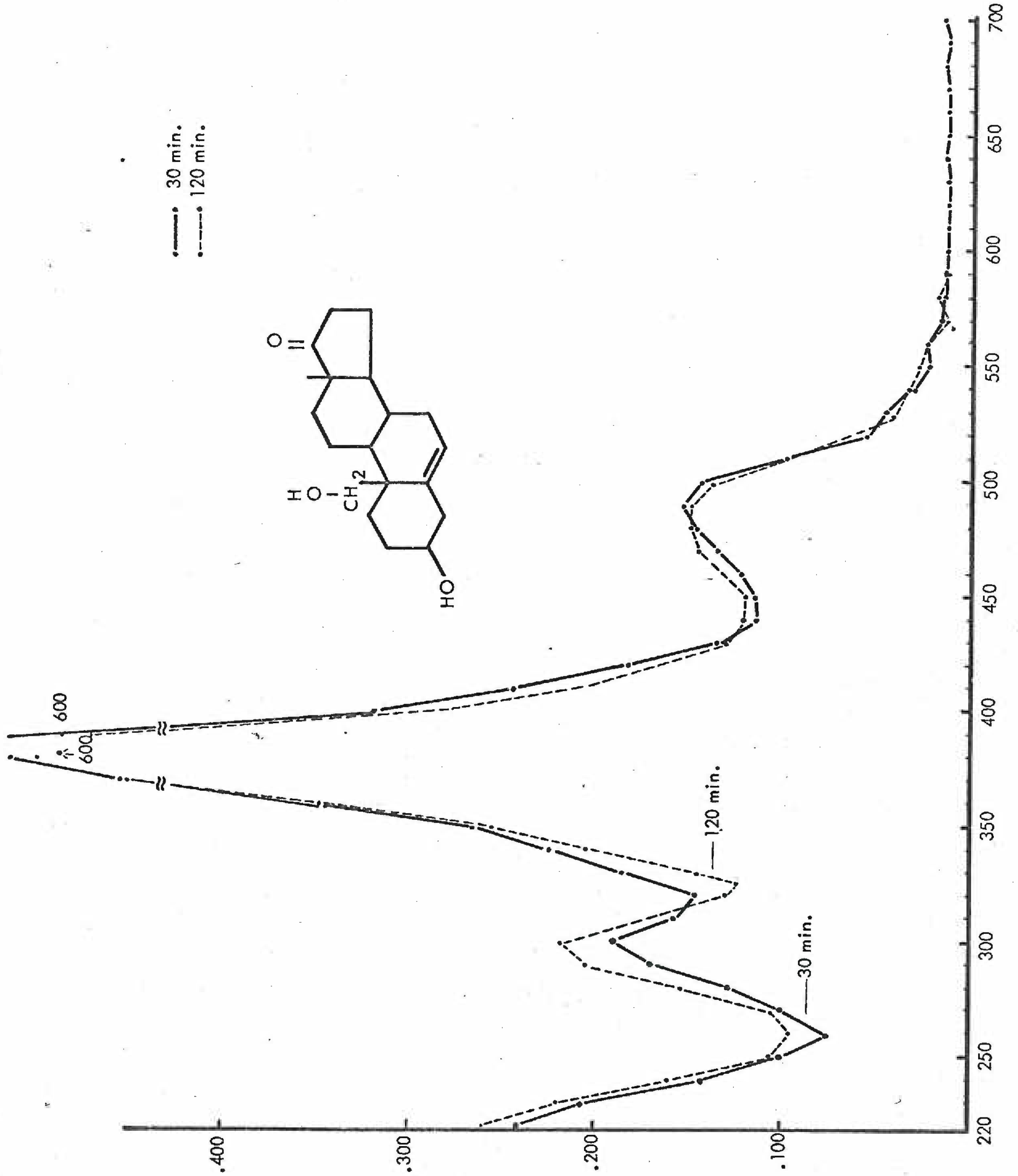
Infrared Spectra is Figure 6 C.

λ max is 2950, 1740, 1720, 1260, 1030.

Figure No. 7

Sulfuric acid spectra

25°C 30 min. and 120 min. of 19 hydroxy DHA



30 min.
120 min.
600
600
120 min.
30 min.
.400
.300
.200
.100
220
250
300
350
400
450
500
550
600
650
700
HO
H
O
CH2
O

G. Synthesis of 17, 17-dimethoxyandrost-5-ene-3 , 16 -diol (42)

3β-acetoxy-16α-bromoandrost-5-en-17-one (3.8 g) was dissolved in 80 ml. boiling methanol and added to 100 ml. hot sodium methoxide (4.0 g sodium / 100 ml. methanol). After one hour of boiling under reflux,the solution was poured into 600 ml distilled water at 0° C. The precipitated steroid was extracted three times with 250 ml ether. The combined ether extract was washed successively with 100 ml. each, 0.1 N hydrochloric acid, 0.1 N sodium carbonate and saturated sodium chloride. The ether extract was then dried with anhydrous sodium sulfate, evaporated to dryness and the residue crystallized from ether (double m.p. 155-160/176-182° C.) Upon recrystallization from ether, the m.p. was 156-160/179-181° C (reported, 177-179° C (32). R_F is: System C, 0.31; system M, 0.60.

Infrared spectra is Figure No. 8 A.

λ max 3400, 2930, 2900, 2870, 1180, 1110, 1060.

H. Synthesis of 16α-hydroxy-dehydroepiandrosterone (42)

3β, 16α-dihydroxy-17, 17-dimethoxyandrost-5-ene (1.5 g) was dissolved in 250 ml of acetone and added to 25 ml of water containing 1.0 g p-toluene-sulphonic acid. The solution was kept at 40° C for 12 hours, 50 ml of water was then added, and evaporation was carried out in the rotovac to one-half its original volume. Extraction was then carried out three times with 250 ml chloroform. The chloroform extract was washed with 100 ml 0.1 N sodium carbonate and 100 ml of a saturated solution of sodium chloride, dried with 20 g anhydrous sodium sulfate, and evaporated to dryness. The oily residue was crystallized

from 30% acetone-hexane, yielding 1.1 gm (73%). The m.p. was 180-183°C and the product did not depress the m.p. of authentic 16α-OH-DHA. R_F values were identical with those of the reference in system C, system M, and ethanol 25%,benzene 75% .

I. Synthesis of 3β, 19, 17-triacetoxyandrost-5, 16-diene

3β, 19-diacetoxyandrost-5-en-17-one (m.p. 103-105°C), 7.35 gms. were dissolved in 95 ml acetone-enolacetate (K & K Lab. Lot 78572). 645 mg p-Toluene sulfonic acid monohydrate was added to the solution and the reaction boiled in a fractionation column with a side condenser. Sixty ml of fresh acetone-enol acetate was added to maintain constant volume. After 6 hours the solution was evaporated to 45 ml and poured into 200 ml. 0.1 N sodium carbonate. The steroid was extracted three times with 250 ml ether. The combined ether extracts were washed with 100 ml 0.1 N sodium carbonate and saturated sodium chloride solution and dried with anhydrous sodium sulfate (9 g). The ether was evaporated to dryness and the dryness and the oily residue crystallized from 60% petroleum ether. The yield was 2.45 g (33%) and the m.p. 134-137° C (Found: C, 69.47; H, 7.86, requires C, 69.74; H, 7.95). R_F is: System C, 0.63; M, 0.81; EOH-Benzene (25:75) 0.75.

Infrared Spectra is Figure No. 8 B.

λmax 2960, 2930, 1730, 1240, 1210.

J. Synthesis of 3β, 19-diacetoxy-16α-bromoandrost-5-en-17-one

3.45 g, 3β, 19, 17-triacetoxyandrost-5, 16-diene was dissolved in 150 ml dry carbon tetrachloride and cooled to 15°C. A solution

Figure No. 8

A. Infrared spectra of

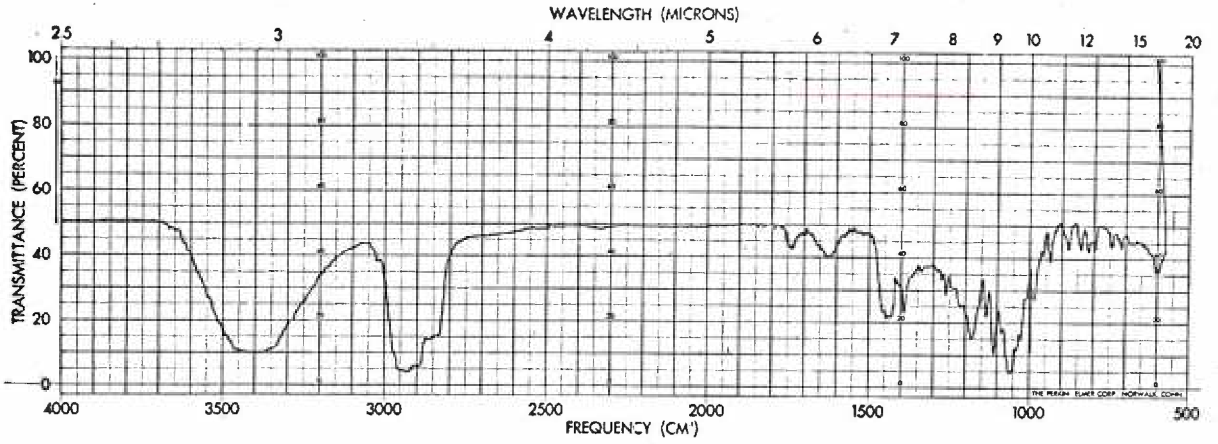
3β, 16α-diOH 17, 17 dimethoxyandrost-5-ene.

B. Infrared spectra of

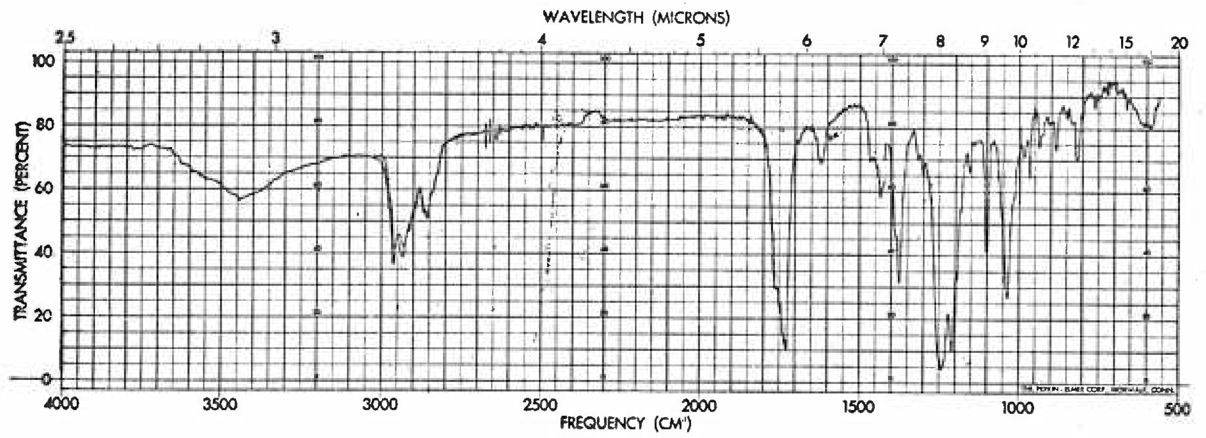
3β, 19, 17, triacetoxyandrost 5-16 diene

C. Infrared spectra of

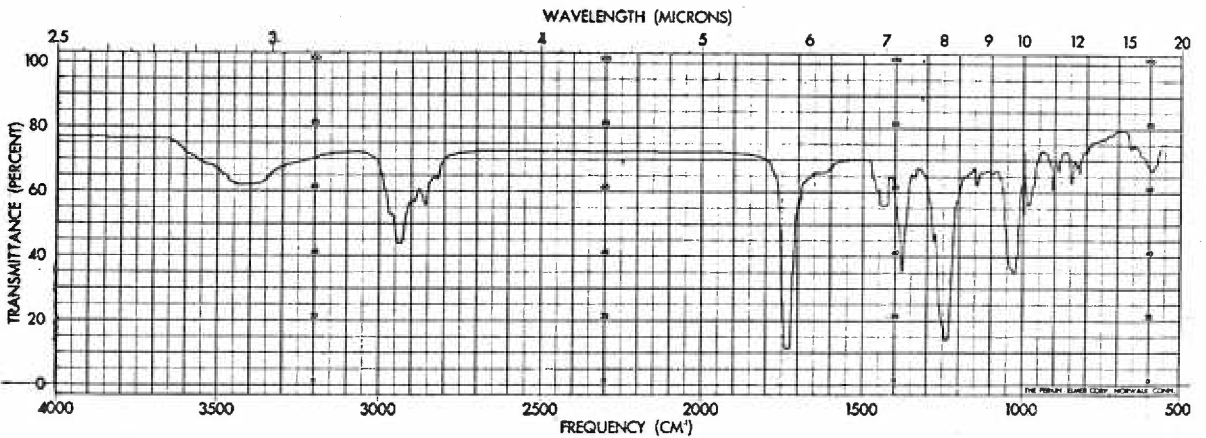
3β, 19 diacetoxy 16α-bromoandrost-5-en-17-one



A



B



C

containing 2.47 grams bromine in 20 ml. carbon tetrachloride was added with constant stirring over 2 min. After allowing the reaction to proceed for an additional 2 min., 0.1 N sodium hydrogen sulphite, 50 ml., was added and the steroid extracted three times with 250 ml. chloroform. The combined chloroform extracts were washed with 50 ml. 0.1 N sodium carbonate and 50 ml. saturated sodium chloride solution, dried with 10 g anhydrous sodium sulfate, and evaporated to dryness in the rotovac. The residue was crystallized from ethanol to give 2.6 g of product, (yield 75%) m.p. 139-141°C. Found: C, 59.62; H, 6.60; Br, 16.01; required: C, 59.21: H, 6.70; Br, 16.95 R_F is: System C, 0.57; M, 0.78.

Infrared spectra is Figure No. 8 C.

λmax 1740, 1380, 1240, 1030.

K. Synthesis of 17, 17-dimethoxyandrost-5-ene-3β, 16α, 19-triol

3β, 19-diacetoxy-16α-bromoandrost-5-en-17-one (2.6 g) was dissolved in 70 ml of hot methanol and added to a solution of sodium methoxide (2.8 grams sodium in 112 ml anhydrous methanol). The solution was boiled under reflux for 1 hr. and then poured into 450 ml. water at 0°C. The precipitated steroid was extracted three times with 300 ml. ether and the pooled ether extract washed with 0.1 N hydrochloric acid (90 ml), 0.1 N sodium carbonate (50 ml), saturated sodium chloride solution (50 ml.), dried with 10 grams anhydrous sodium sulfate, and evaporated to dryness. From the residue, crystallized from acetone, 600 mg. of product (yield 23%), m.p. 190-193° C, was obtained. Found C, 69.30, 69.07: H, 940, 9.45: requires C, 68.82; H, 9.35. R_F's system M, 0.3; system C, 0.08.

Infrared spectra is Figure No. 9 A.

λmax is 3400, 2940, 1185, 1110, 1050.

L. Synthesis of 16α, 19-dihydroxydehydroepiandrosterone

Two hundred fifty (250) mg. of 17, 17-dimethoxyandrost-5-ene-3β, 16α, 19-triol was dissolved in acetone (50 ml) and added to 5 ml water containing p-toluene sulfonic acid monohydrate (200 mg). The solution was kept at 40° C and 20 ml. aliquots taken and run on thin-layer chromotography at 30 min. intervals to determine the extent of hydrolysis. After 270 minutes, the hydrolysis was nearly complete and the reaction was stopped by adding 10 ml. of water and evaporating to one-half volume in vaccuum. The steroid was extracted three times with 100 ml chloroform. The chloroform extract was washed with 25 ml 0.1 N sodium carbonate, saturated solution sodium chloride and then dried with 9 g sodium sulfate. The chloroform extract was evaporated to dryness and the residue crystallized from acetone-hexane (yield 150 mg. m.p. 210-216). An analysis of its mass spectrum (Figure 8) performed by West Coast Technical Service, San Gabriel, Calif., showed the parent ion at 320 m/c as a minor peak, due to two major cleavage reactions; the dehydration at the 16α-position, as shown by a peak at 302 m/c, and the removal of CH_2O from the 19-hydroxymethyl group. There was no spectral evidence for the presence of the starting ketal in the sample. The modified Pettenkofer as compared to 16α-OH-DHA is given in Figure No. 11. The spectrum in concentrated sulfuric acid (25°, 30 min.) had λmax of 279, 412, and 480 nm. and min 260, 320, and 420 nm. and is given on Figure No. 10. Infrared spectra is given in Figure 9 B. Analysis calculated for $C_{19} H_{28} O_4$; C, 71.20; H, 8.81; O, 19.98. Found: C, 71.50; H, 8.87; O, 19.94.

Figure No. 9

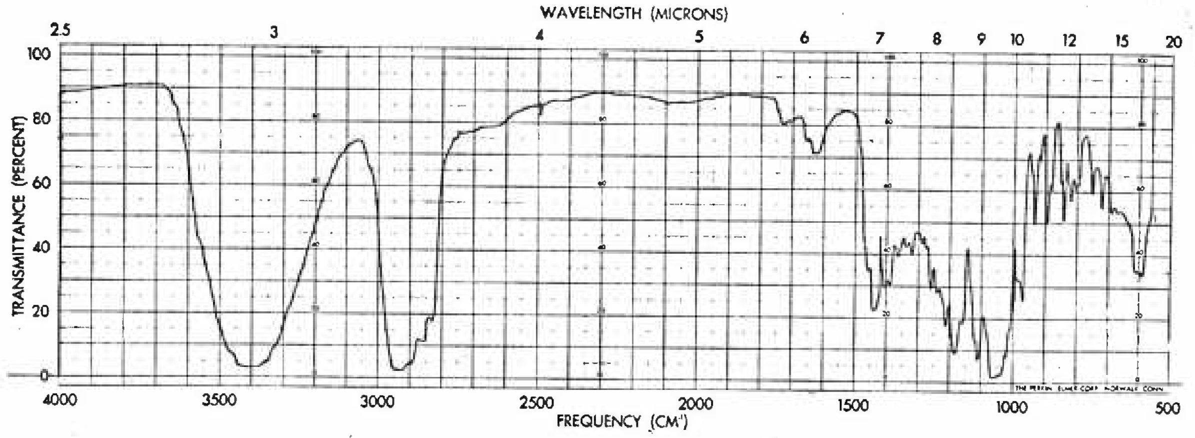
A. Infrared spectra of

17, 17-dimethoxyandrost-5-ene 3β, 16α, 19 triol

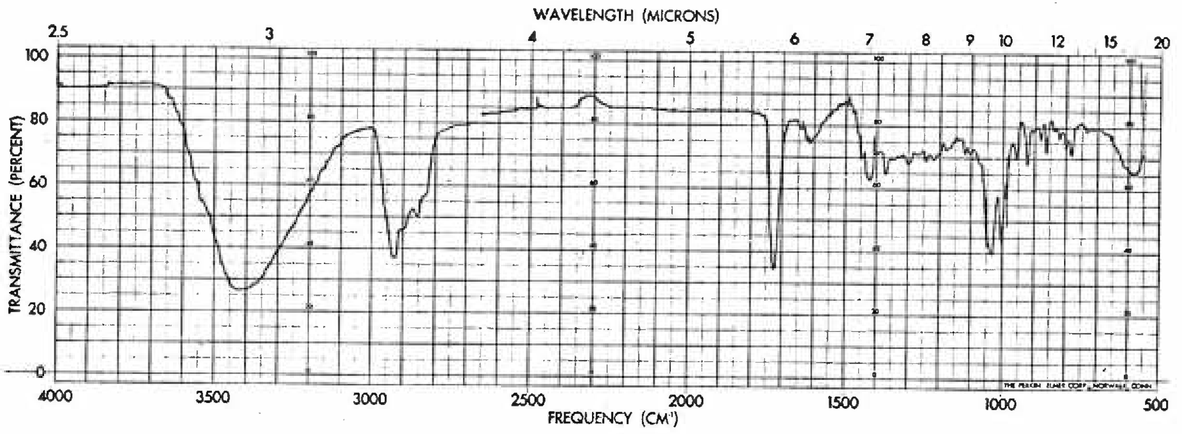
B. Infrared spectra of 16α, 19 dihydroxy DHA

C. Infrared spectra of

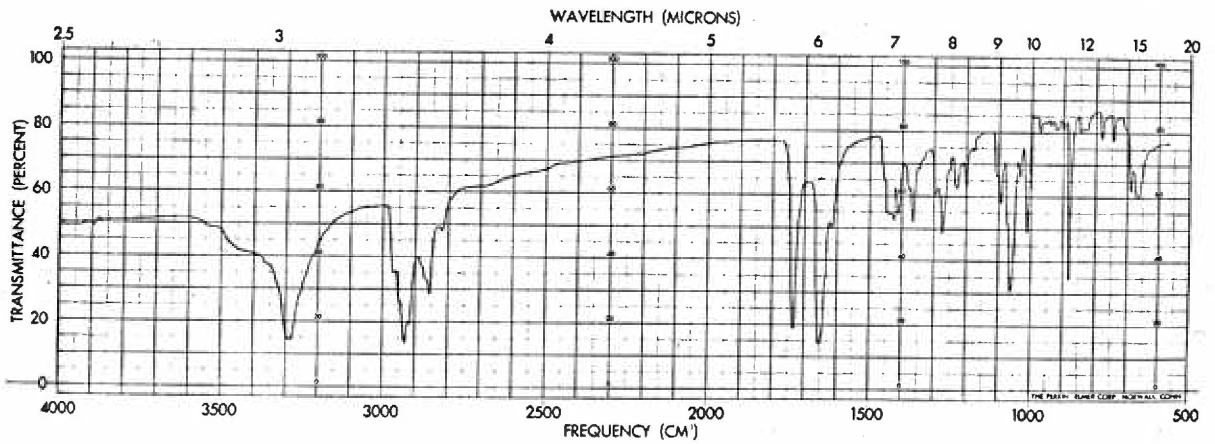
19 hydroxyandrostenedione



A



B



C

Figure No. 10

Sulfuric acid spectra. 25°C, 30 minutes and 120 minutes of 16α, 19-dihydroxy DHA.

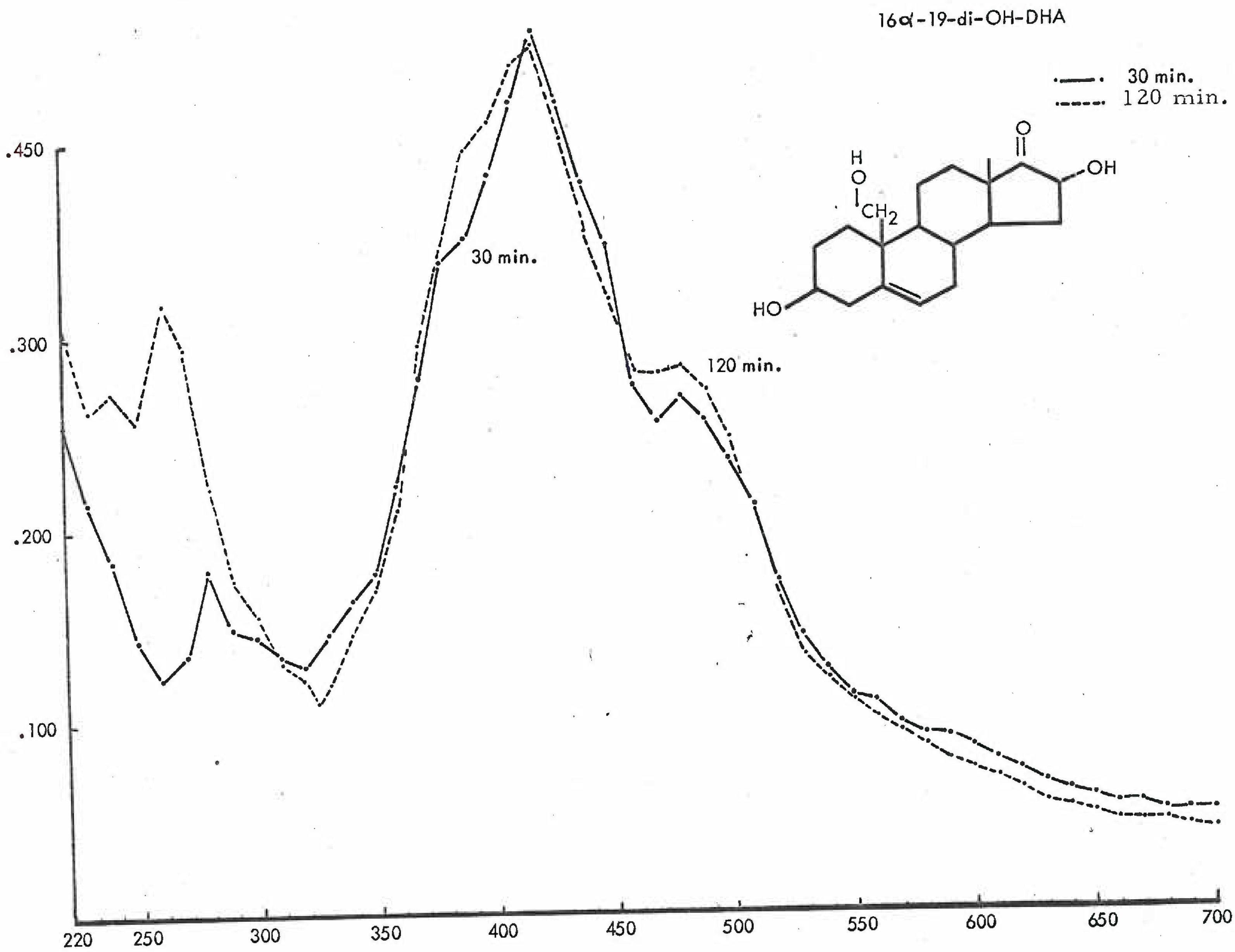
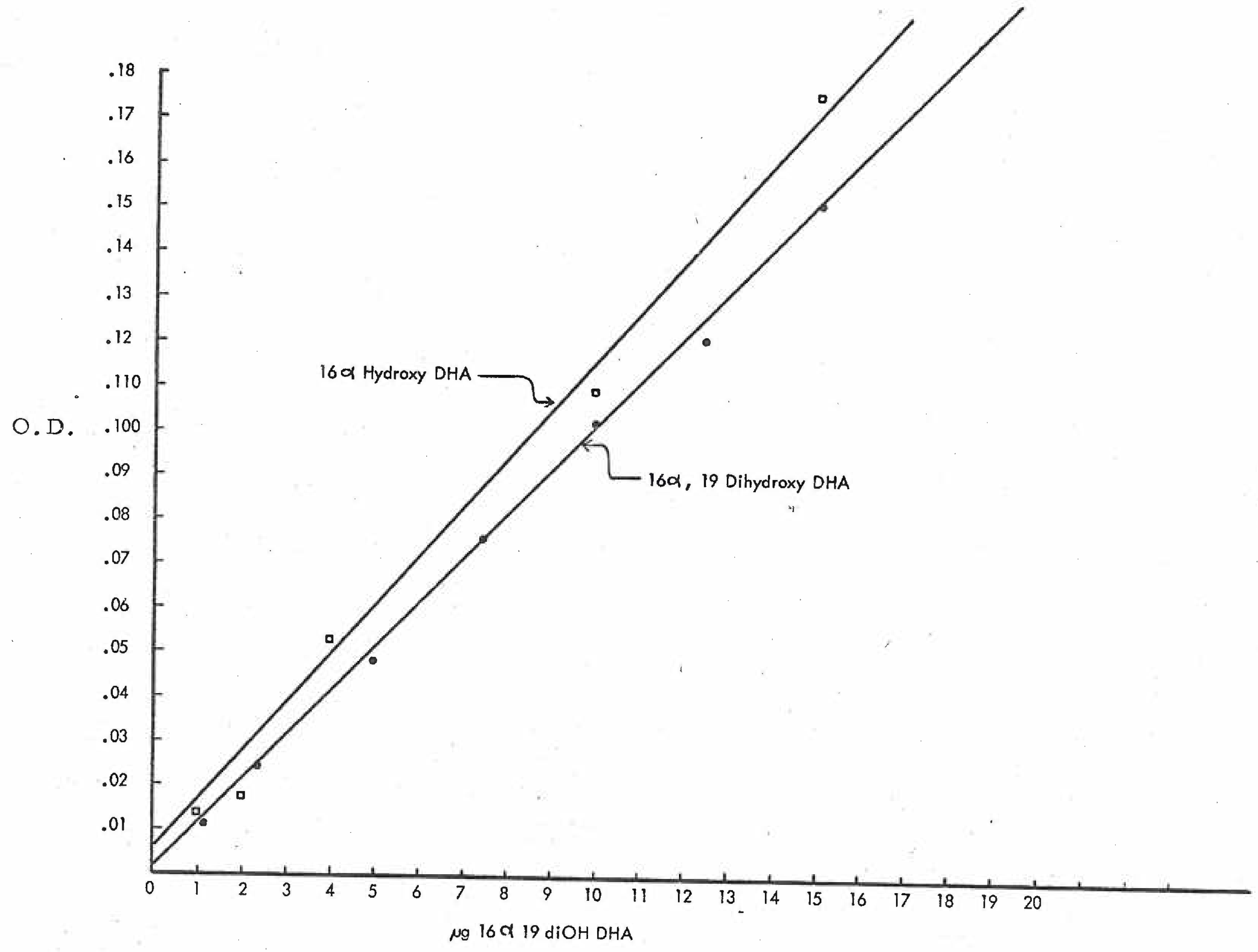

16α-19-di-OH-DHA
30 min.
120 min.
.450
.300
.200
.100
220
250
300
350
400
450
500
550
600
650
700
30 min.
120 min.
HO
OH
O
H
O
CH2

Figure No. 11.

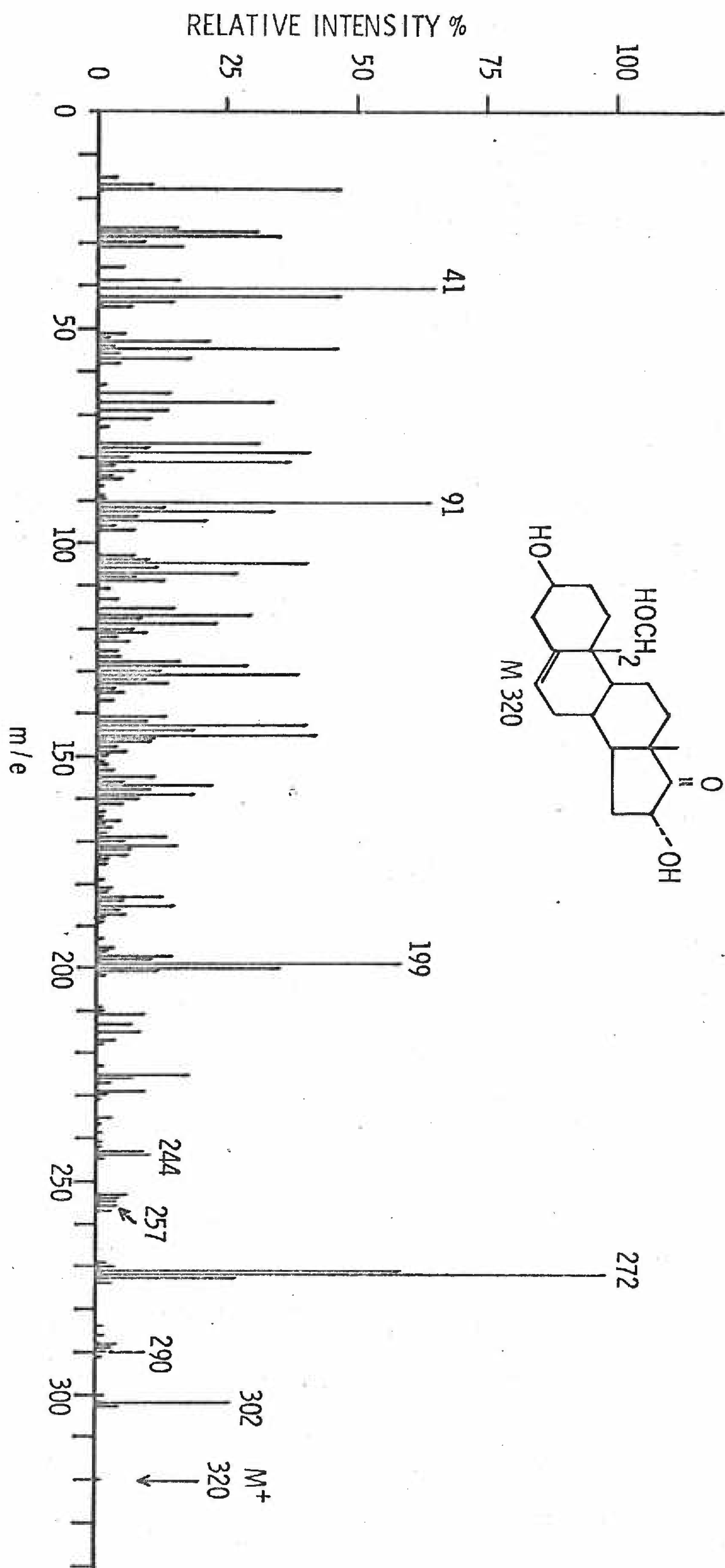
Modified Pettenkofer comparing 16α-hydroxy DHA and 16α, 19 dihydroxy DHA. Each point is the average of 3 separate determinations.



O.D.
.18
.17
.16
.15
.14
.13
.12
.110
.100
.09
.08
.07
.06
.05
.04
.03
.02
.01
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
16α Hydroxy DHA
16α, 19 Dihydroxy DHA
μg 16α 19 diOH DHA

Figure No. 12

Mass spectroscopy of 16α, 19-dihydroxy DHA.


RELATIVE INTENSITY %
0
25
50
75
100
0
50
100
150
200
250
300
m/e
41
91
199
244
257
272
290
302
M+
320
HOCH2
HO
O
OH
M 320

M. Synthesis of 19-Hydroxyandrost-4-ene-3-17-dione

Oppenhuer oxidation of 3β, 19-dihydroxyandrost-5-en-17-one. 19-OH-DHA (0.775 grams), was heated in 20 ml. dry benzene and 10 ml. cyclohexanone and 4 ml. of the boiling mixture was distilled off. 0.500 g $Al(P_rO)_3$ was added and refluxed for one hour and 10 minutes. The reaction was stopped by addition of 100 ml 2 N sodium hydroxide. The steroid was extracted twice with 300 ml. ethylacetate. The pooled extract was washed three times with distilled water and evaporated. The residue was crystallized from acetone-hexane. The yield was 0.418 g (54%).

Infrared Spectra is given in Figure No. 9 C.

N. Incubation of 19-Hydroxy DHA with Streptomyces Roseochromogenes

1. Starch salt medium (100 ml.) was inoculated with 5 ml of *Streptomyces roseochromogenes* culture and incubated at 28°C for 24 hours with air as the gas phase. *S. roseochromogenes* was obtained from the American-type Culture Collection. After the prescribed incubation, the culture media was diluted 8-fold with starch salt media, divided into 8 equal portions, and incubated for an additional 6 hours at 28°C in air. Induction of 16α-hydroxylase activity was carried out by adding 5 mg. 11α-hydroxyprogesterone (in 0.5 ml. methanol) to each incubation flask. The incubation was allowed to continue for 18 hours at which time the pooled mycelia were filtered through cheese cloth, washed with 0.5 % sodium chloride (50 ml), distilled water (50 ml) and EDTA (0.001 M) (50 ml). The collected mycelia (wet weight 2.34 grams) was resuspended in 0.05 M phosphate buffer (pH 7.4) containing 0.001 M EDTA

Table No. 1

R_F values for 13 synthetic steroids in three different solvent systems.

6. -R_F values for steroids in different solvent systems

		C System	M	ETOH 25 Benzene 75
1.	5α Bromo, 3 ACO, 6β-OH-androstane -17-one	.45	10.3	10.7
2.	5 Bromo, 3 Acetoxy, 6-19 epoxy, androstane-17-one	.45	10.4	10.7
3.	3β 19-diacetoxy androst 5-en-17-one	.49	10.8	10.8
4.	19 OH-DHA	.06	4.5	7.2
5.	3β, 17-diacetoxyandrost 5, 16-dien	.68	12.5	11.3
6.	3β Acetoxy, 16α-bromo-androst 5-ene-17-one	.66	12.4	11.4
7.	3β, 16α-di-OH, 17-17 dimethoxy-androst-5-ene	.31	9.0	9.0
8.	16α-OH-DHA	.20	8.1	8.3
9.	3β, 19-17 tri-ACO androst 5, 16, dien	.62	12.2	11.3
10.	3β, 19 diACO, 16α bromo androst 5-ene-17-one	.56	11.8	11.5
11.	3β-19, 16α-tri-hydroxy, 17-17 dimethoxy-androst-5-ene	.08	4.5	7.3
12.	16α-19, di-OH, DHA	.03	2.7	5.6
13.	DHA, 15 CM solvent traveled	.34	9.5	9.5

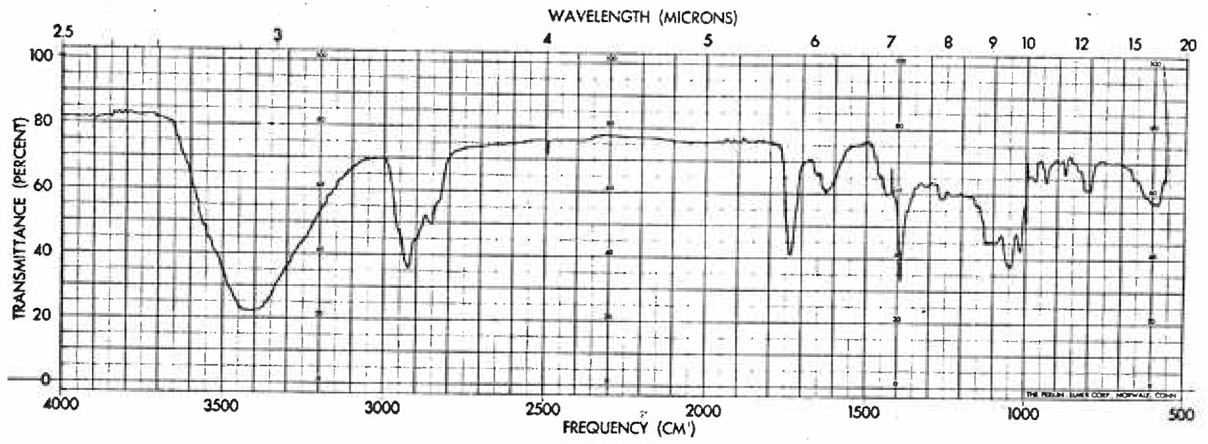
and 0.001 M GSSH (total volume 200 ml). 19-hydroxy DHA, 30 mg. (in 3 ml. ethanol, and TPNH (120 mM) were added to the incubation flask; the preparation was then incubated aerobically for 24 hours at 28°C. The incubation mixture was extracted twice with 400 cc chloroform; the combined chloroform extract was washed with a saturated solution of sodium bicarbonate (75cc) and then water, and finally dried with anhydrous sodium sulfate, and evaporated to dryness. The residue from the chloroform extract was dissolved in methanol. Thin-layer chromatography (silica gel G, systems M and ethanol-benzene, 1:4) gave a product with the same R_F as a standard sample of 16α, 19-dihydroxy-DHA. Purification and separation of 16α, 19-dihydroxy-DHA from the starting material (19-hydroxy DHA) was carried out by thin-layer chromatography in system M and system ETOH-benzene. The infrared spectra of the purified product was identical to that of standard 16α, 19-dihydroxy DHA. See Figure No. 9. Quantitation by the B.T. reaction gave 1.470 mg. of 16α, 19-dihydroxy DHA. (Yield 4.9%, 1.9 mμmoles/ mg. wet mycelium/ 24 hours incubation).

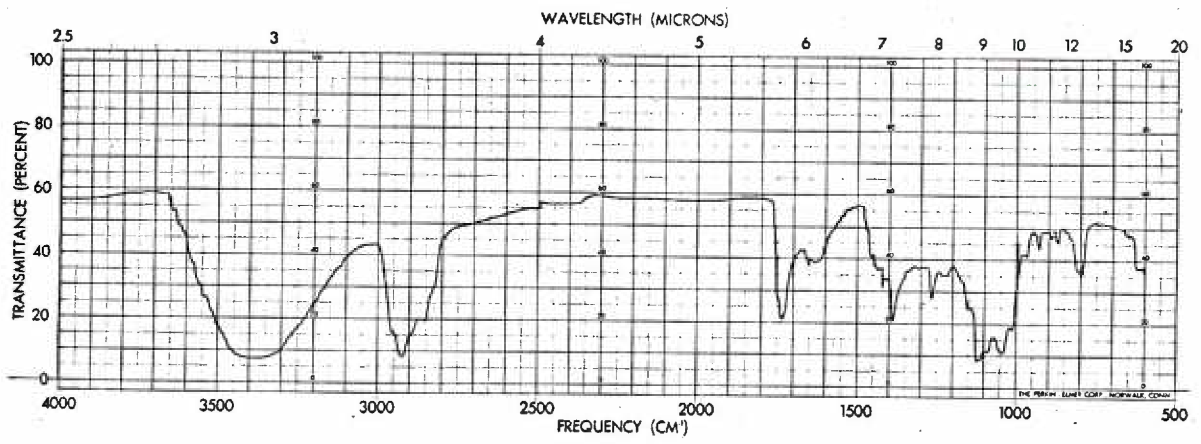
Another incubation was carried out under the same conditions with DHA. A product with the same R_F as 16α-hydroxy-DHA in two TLC systems was obtained that, on quantitation by the B.T. reaction, represented an overall yield of 1.56%.

O. Incubation of 19-hydroxy-DHA with male rat liver microsomes was carried out as previously described. Partial characterization of the product (16α, 19-dihydroxy-DHA) produced from the incubation

Figure No. 13.

Infrared spectra of 16α, 19 dihydroxy DHA chemical synthesis both from silical gel G.


WAVELENGTH (MICRONS)
2.5
3
4
5
6
7
8
9
10
12
15
20
TRANSMITTANCE (PERCENT)
100
80
60
40
20
0
4000
3500
3000
2500
2000
1500
1000
500
FREQUENCY (CM⁻¹)


WAVELENGTH (MICRONS)
2.5
3
4
5
6
7
8
9
10
12
15
20
TRANSMITTANCE (PERCENT)
100
80
60
40
20
0
4000
3500
3000
2500
2000
1500
1000
500
FREQUENCY (CM⁻¹)

by means of TLC (the same R_F as that of the standard in three different systems), reaction with picric acid and quantitative color reaction with blue tetrazolium. The net conversion of 19-hydroxy-DHA to 16α, 19-dihydroxy-DHA was 4% for a 30 min. incubation of 0.6 mμmoles/min./mg. microsomal protein. To check for the hydroxylation ability of the microsomal preparation, another incubation with DHA was also carried out. Under the same conditions and with the same microsomal preparation, DHA was converted to 16α-hydroxy-DHA at a rate of 2.7 mμmoles/mg. microsomal protein/minute --a net yield of 17.5% .

P. Incubation studies with placental microsomes.

The experimental design of placental microsomal incubations with DHA-7-α-^{3}H and androstenedione 4-^{14}C is given in Figure No. 14.

The incubations were carried out in duplicate at 37° in air for 8, 15, 30 and 60 minutes in the first experiment. An incubation lasting 4 min. was added to the second experiment. Blanks to which the radioactive steroids and carriers were added after the incubation and immediately before extraction were also included to determine the extent of recovery of each compound. The samples contained microsomes to the equivalent of approximately 13 g. of placental tissue in 7 ml. of phosphate buffer, pH 7.0; 5 ml. (12 μmoles) of NADPH solution; 100 μg. (4μc) of 4-^{14}C-androst-4-ene-3, 17-dione; and 100 μg. (10μc.) of 7-^{3}H-3β-hydroxyandrost-5-en-17-one.

The simultaneous determination of tritium and carbon -14 radioactivities was accomplished with the Packard spectrometer model 3315 using the following settings:

Figure No. 14

Experimental design: 7α ^{3}H-DHA (1) and 4-^{14}C-A^4 (2) were simultaneously incubated with placental microsomes. "Cold" DHA, A^4, 19-OH-DHA (3), 19-OH-A^4 (4), 17β estradiol (5) and estrone (6) were added as carriers. Solid arrows depict reactions known to take place in placental tissue.

(1) (2) (3) (4) (5) (6)

Forty percent gain and 50-200 window in the tritium channel (channel 1) and 7% gain and 250-1000 window in the carbon-14 channel (channel 2). With the above settings and with the scintillation fluid used, (*) the following counting efficiencies were obtained: 15.6% for tritium and 2.7% for carbon in channel 1 and 37% for carbon and $< .001$ for tritium. The background was 7 CPM in channel 1 and 9 CPM in channel 2.

Recovery of the six steroids (DHA, 19-OH-DHA, 19-OH A^4, E_1, and E_2) was not expected to be similar or complete because of their difference in polarity, elution from silica gel, solubility in the different extraction agents, etc. Therefore, in each experiment separate incubation flasks were used to estimate the recovery of each steroid. These incubation flasks were treated exactly as the experimental flasks except no radioactive substrate was added and a known quantity of the steroid under question was added just prior to the extraction procedure. The quantity of steroid added to these recovery flasks was approximated to be what was expected in the experimental flask. In general 10 μg and 50 μg. quantities were used for the recovery experiments.

The recovery of DHA was estimated by adding a known quantity of tritium labeled DHA to the recovery flask and determining the quantity recovered after the complete extraction procedure. A similar approach was used for determining the recovery of A^4.

(*) Toluene solution of 5 grams of P.P.O. (2,5-diphenyloxazole) and 0.3 grams of dimethyl P.O.P.O.P. (1, 4-bis-2-4-methyl-5-phenyl-oxazolyl-benzene) per liter.

Since no labeled compounds were available for E_1, E_2, 19-OH-DHA and 19-OH-A^4, known quantities of these steroids were added to the recovery flasks and the percent of recovery determined as follows:

The quantitative recovery of E_1, and E_2, was determined by the Kober reaction, 19-OH-DHA by the modified Pettenkofer and 19-OH-A^4 by its ultraviolet absorption properties. A scan of 19-OH-A^4 in the ultraviolet wave length in a Zeiss PMQ II spectrophotometer gave a maximum absorption at 242, and its recovery was calculated on the basis of the direct relationship between quantity of 19-OH-A^4 and its absorbency.

The recovery of each steroid was as follows: At least triplicate determinations were done, and except for the phenolic fraction, the more polar the neutral steroid the lower the % recovery.

DHA: - -	46.4 %
A: - -	35.6 %
19-OH-A^4 -	26.8 %
E_1 - -	79 %
E_2 - -	79 %

The identity of each steroid was established by running in a separate lane a known standard of the steroid on thin-layer chromatography with the unknown, using (Silical Gel HF 254 for A^4 . 2', 7' dichlorofluorescein for E_1 and E_2) and appropriate spray (picric acid for DHA and 19-OH-DHA).

The purity of each steroid was established by adding a known quantity of non-radioactive carrier and crystallizing to constant

specific activity, further the melting point of each steroid (radioactive with carrier) was then determined and compared with a pure sample of the steroid under consideration.

1. Estrogen Formation. Data on crystallization, specific activity, recovery, H^3/C^{14} ratios, and quantitative conversion of A^4 and DHA to estrone and estradiol is given in tables 2 and 3. This data is graphically depicted in Figure No. 15. The $^3H/^{14}C$ ratio was similar in the isolated estrone and 17β-estradiol. This result indicates a rapid equilibrium between the two estrogens. The ratio increased during the first 30 min. of incubation and then leveled off or even declined (experiment 1) . An interesting but unexplained observation is that, while estrone accumulated during the incubation, the amount of 17β, estradiol decreased.

2. Neutral Steroids. The significant experimental data, crystallization, specific activity and tritium carbon ratios for DHA, A^4, 19 OH-A^4, and 19 OH-DHA are given in Tables 4, 5, 6, and 7. Graphic illustrations of the time course of the reactions and the changing $^3H/^{14}C$ ratios is given in Figure 16. Only a small amount, of 0.5-1.7μ g of 19-OH DHA was estimated to be present at any given time during the incubation. The carrier-added fraction contained a small but significant amount of ^{14}C-labeled material which could not be eliminated after repeated recrystallizations. The purity and even the identity of the ^3H-labeled material accompanying the 19-OH-DHA carrier must therefore be regarded as unproven.

The $^3H/^{14}C$ ratios of A^4 and 19-OH-A^4 are shown in Table 6 and 7

and also in Figure 16. These ratios are larger than those for the estrogens at all time intervals. The $^{3}H/^{14}C$ ratios of A^4 are also higher than those of 19-OH-A^4, except for the incubations lasting 8 min. in both experiments, where the opposite is true.

Since the specific activity with regard to both labels and the amount of each compound present at the end of each time interval are known, it is possible to calculate the contribution made by DHA and A^4 to these intermediates and products. This is graphically shown in Figure 17. The black portion of each bar represents the contribution of DHA, the rest being contributed by A^4. Figure 17 also shows the rapid decline in the amount of DHA recovered unchanged at the various time intervals (dotted line).

Table No. 2

Crystallization and specific activity of estrone and estradiol.

ESTROGENS

A. Estrone E_1 (79% Recovery)

Min	Mg. of carrier	Mg. of carrier counted crystallization 1	2	3	CPM C^{14}/mg carrier crystallization 1	2	3	CPM C^{14}/mg carrier	CPM H^3/mg carrier	Specific Activity DPM C^{14}/mg carrier	Specific Activity DPM H^3/mg carrier
8	10.0	.50	.30		48,500	47,150		47,150	5,281	127,432	33,853
	9.86	.27	.69		24,160	22,848		22,848	4,076	61,752	26,130
15	10.0	.70	.60		67,145	66,023		66,023	13,922	178,440	89,243
30	10.04	1.07	.84		68,111	68,101		68,101	23,213	184,058	148,806
60	10.0	.60	.81		175,104	173,175		173,175	34,173	468,040	219,057
	10.26	.91	1.3		86,012	85,926		85,926	30,658	232,234	196,529

B. Estradiol E_2 (79% Recovery)

Min	Mg. of carrier	Mg. of carrier counted crystallization 1	2	3	CPM C^{14}/mg carrier crystallization 1	2	3	CPM C^{14}/mg carrier	CPM H^3/mg carrier	Specific Activity DPM C^{14}/mg carrier	Specific Activity DPM H^3/mg carrier
8	10.0	1.08	.46		31,400	32,396		32,396	3,706	87,556	23,756
	10.26	.63			28,032	27,605		27,605	4,618	74,609	29,606
15	10.0	.63	.46		15,064	14,943		14,943	3,175	40,386	20,352
	10.21	.64	1.23		7,180	7,063		7,063	2,559	19,090	16,406
30	10.0	.61	.50		1,720	1,648		1,648	460	4,454	2,949
	10.02	.94	.76		5,004	4,873		4,873	1,819	13,172	11,665
60	10.0	.92	.32		2,862	2,762		2,762	520	7,464	3,333
	9.97	1.15	.43		3,412	3,332		3,332	1,155	9,006	7,406

Table No. 3

Data: $^3H/^{14}C$ ratios and quantitative conversion of DHA-7α-^3H and androstenedione-4-^{14}C to estrone and estradiol.

ESTROGENS

A. Estrone E_1 (79% Recovery)

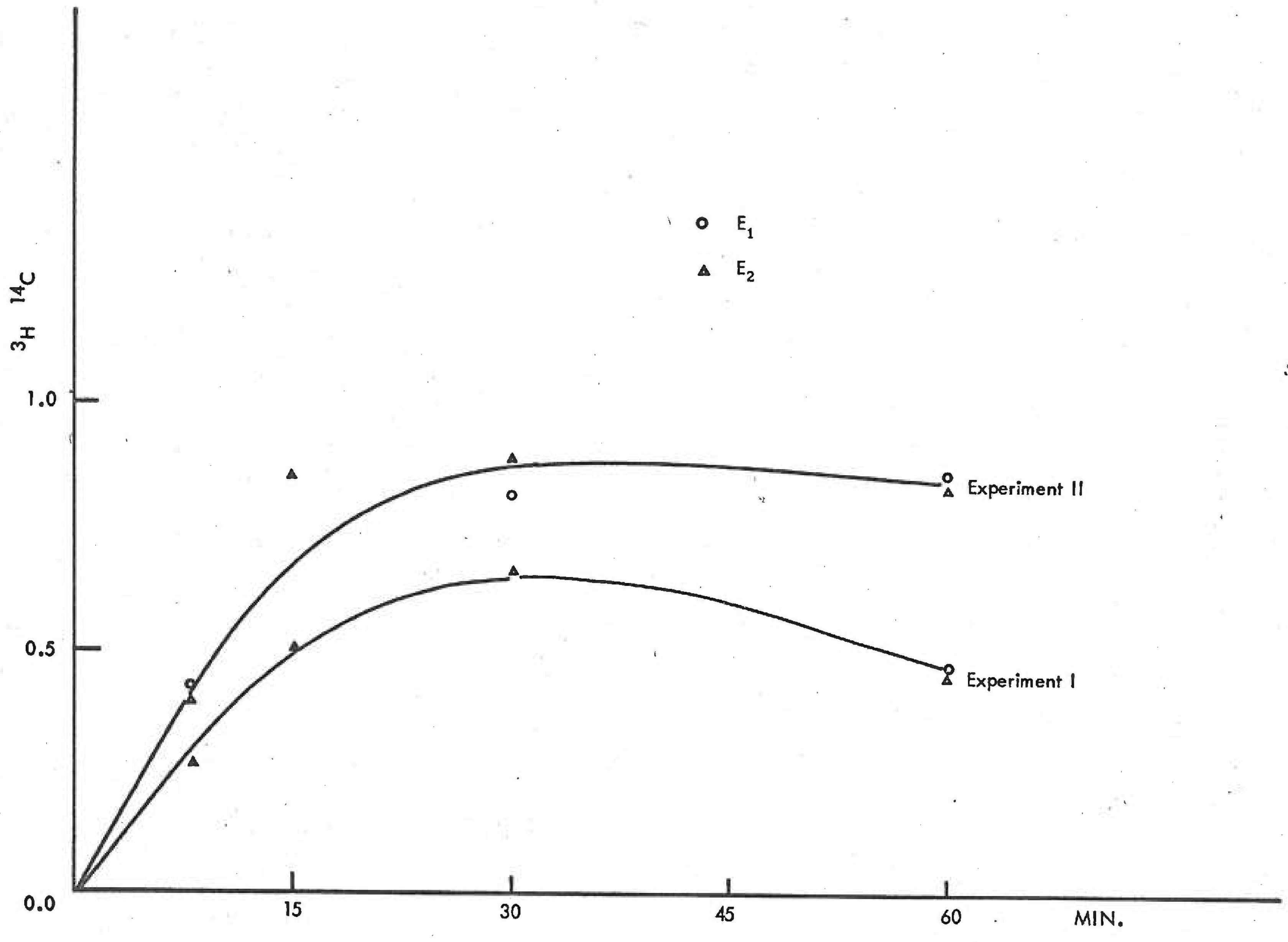
	Total $DPMC^{14}$ Recovered	Total DPM H^3 Recovered	H^3/C^{14}	Total µg. Recovered	Corrected Total µg. Recovered	µg. from A^4	µg. from A^4 Corrected	µg. from DHA	µg. from DHA Corrected
8 min	1,274,320	338,530	.266	15.20	19.24	13.66	17.29	1.54	1.95
	608,881	257,650	.423	8.0	10.13	6.9	8.73	1.1	1.39
15 min	1,784,400	892,430	.500	23.22	29.39	19.14	24.23	4.08	5.16
30 min	1,840,580	1,480,806	.808	26.9	34.05	20.9	26.45	6.07	7.68
60 min	4,680,400	2,190,570	.468	60.24	76.25	50.21	63.56	10.03	12.70
	2,322,234	1,965,290	.846	35.20	44.55	26.30	33.29	8.90	16.07

B. Estradiol E_2 (79% Recovery)

	Total $DPMC^{14}$ Recovered	Total DPM H^3 Recovered	H^3/C^{14}	Total µg. Recovered	Corrected Total µg. Recovered	µg. from A^4	µg. from A^4 Corrected	µg. from DHA	µg. from DHA Corrected
8 min	875,560	237,560	.271	10.54	13.34	9.45	11.96	1.09	1.38
	746,090	296,060	.396	9.70	12.28	8.40	10.63	1.30	1.64
15 min	403,860	203,520	.504	5.29	6.70	4.36	5.52	.93	1.18
	190,090	164,060	.859	2.81	3.56	2.10	2.69	.71	.89
30 min	44,540	29,490	.662	.57	.72	.46	.58	.11	.12
	131,172	116,650	.885	1.93	2.44	1.42	1.79	.51	.64
60 min	74,640	33,330	.447	.93	1.18	.79	1.00	.14	.18
	90,060	74,060	.822	1.35	1.71	1.01	1.28	.34	.43

Figure No. 15

Time course of ratios of $^{3}H/^{14}C$ in estrone and estradiol.


^3H / ^{14}C
1.0
0.5
0.0
o E_1
▲ E_2
Experiment II
Experiment I
15
30
45
60
MIN.

NEUTRAL STEROIDS

A^4 (35 6% Recovery)

Min.	Mg. of carrier	Mg. of carrier counted crystallization 1	2	3	CPM C^{14}/mg carrier crystallization 1	2	3	CPM C^{14}/mg carrier	CPM H^3/mg carrier	Specific Activity DPM C^{14}/mg carrier	Specific Activity DPM H^3/mg carrier
4	10.0	.77	1.85		71,004	69,939		69,939	1,728	150,731	10,225
8	10.73	1.09	.66	.99	28,000	32,160	31,837	31,837	7,417	86,045	47,544
	13.48	.85	.50		42,176	41,550		41,550	722	89,547	4,273
15	10.96	.74	1.08		17,030	16,696		17,696	11,799	47,827	75,634
	9.56	.83	.84		30,104	29,539		29,539	14,370	79,834	92,117
30	10.68	.71	.53		16,954	17,015		17,015	28,788	45,986	184,538
60	10.79	.78	.51	.45	32,400	17,604	17,493	17,493	34,913	47,278	233,801
	9.00	.82	.75		25,003	24,721		24,721	49,411	66,814	316,738
					DHA (46.4% Recovery)						
4	10.0	.74	.81		2,123	2,037		2,037	60,704	5,506	389,129
8	10.71	.48	.62	.85	8,065	6,310	6,101	6,101	52,235	16,489	334,839
15	10.82	.69	.77	.60	4,006	3,300	3,075	3,075	37,734	8,310	241,884
30	10.42	.68	.54	.61	700	1,100	1,000	1,000	3,072	2,702	19,692
60	10.0	.79	.93	.48	300	920	850	850	4,463	2,297	28,608

Table No. 5

Ratios of tritium and carbon in A^4 and DHA

NEUTRAL STEROIDS

A^4 (35.6% Recovery)

	Total $DPMC^{14}$ Recovered	Total DPM H^3 Recovered	H^3/C^{14}	Total µg. Recovered	Corrected µg. Recovered	µg. from A^4	µg. from A^4 Corrected	µg. from DHA	µg. from DHA Corrected
4 min	1,507,310	102,250	.068	17.5	49.1	17.1	48.0	.4	1.0
8 min	923,263	510,147	.553	12.8	35.9	10.4	29.2	2.49	6.99
	1,207,380	576,100	.477	16.30	45.78	13.7	38.4	2.60	7.30
15 min	524,184	828,949	1.581	10.01	28.11	5.96	16.74	4.05	11.37
	763,214	880,640	1.153	12.70	35.67	8.60	24.15	4.10	11.51
30 min	491,130	1,970,865	4.013	15.21	42.72	5.58	15.67	9.63	27.05
60 min	510,130	2,522,713	4.945	18.33	51.48	5.80	16.29	12.33	34.63
	601,330	2,850,642	4.740	19.70	55.33	6.80	19.10	12.90	36.23
DHA (46.4% Recovery)									
4 min	55,060	3,891,290	70.67	18.24	39.31	.62	1.33	17.62	37.97
8 min	176,597	3,586,126	20.30	19.53	42.09	2.01	4.33	17.52	37.75
15 min	89,914	2,616,752	29.10	13.81	29.76	1.02	2.19	12.79	27.56
30 min	28,155	205,191	7.28	1.32	2.84	.32	.68	1.00	2.15
60 min	22,970	286,080	12.45	1.66	3.57	.26	.56	1.40	3.01

Table No. 6

Crystallization and specific activity of 19-OH-A^4 and 19-OH-DHA.

Min.	Mg. of carrier	Mg. of carrier counted crystallization 1	2	3	CPM C^{14}/mg carrier crystallization 1	2	3	CPM C^{14}/mg carrier	CPM H^3/mg carrier	Specific Activity DPM C^{14}/mg carrier	DPM H^3/mg carrier
19 OH A^4 (26.8% Recovery)											
8	10.03	.61	.82	.76	8,045	5,640	5,827	5,827	1,729	1,575	11,088
	10.00	.96	.54	.68	3,052	2,300	2,391	2,391	780	6,463	5,000
15	10.77	1.2	.63	.52	2,154	1,780	1,878	1,878	774	5,076	4,964
	10.02	.74	.88	.75	780	590	615	615	2,358	1,662	15,113
30	10.55	.52	1.10		3,624	3,513		3,513	2,662	9,494	17,064
60	11.15	.88	.67	.55	4,014	3,406	3,327	3,327	3,084	8,991	19,769
	9.40	1.11	.82		3,041	2,977		2,977	3,496	8,046	22,409
19 OH DHA (42.2% Recovery)											
4	10.03	1.42	.65	.61	405	230	204	204	894	551	5,729
8	10.20	1.36	.43	.67	780	690	672	672	1,541	1,817	9,878
15	9.72	.88	.72		420	415		415	617	1,121	3,955
	9.90	.65	.81		624	604		604	1,736	1,632	11,129
30	10.82	.76	1.30		380	279	268	268	267	724	1,711
60	10.21	.81	.76	.84	430	312	323	323	303	740	1,942

Table No. 7

Tritium carbon ratios in 19 OH-A^4 and 19 OH-DHA.

19 OH A^4 (26.8% Recovery)

	Total DPMC^{14} Recovered	Total DPM H^3 Recovered	H^3/C^{14}	Total µg. Recovered	Corrected µg. Recovered	µg. from A^4	µg. from A^4 Corrected	µg. from DHA	µg. from DHA Corrected
8 min	157,540	110,880	.70	2.29	8.54	1.79	6.67	.50	1.86
	64,630	50,000	.77	.96	3.58	.73	2.72	.23	.85
15 min	54,670	53,470	.97	.86	3.20	.62	2.31	.24	.89
	166,200	151,135	.91	2.56	9.55	1.88	7.01	.68	2.53
30 min	100,162	180,025	1.79	2.08	7.76	1.17	4.36	.91	3.39
60 min	100,250	220,424	2.19	2.29	8.54	1.17	4.36	1.12	4.17
	75,640	210,650	2.78	1.85	6.90	.85	3.17	1.00	3.73

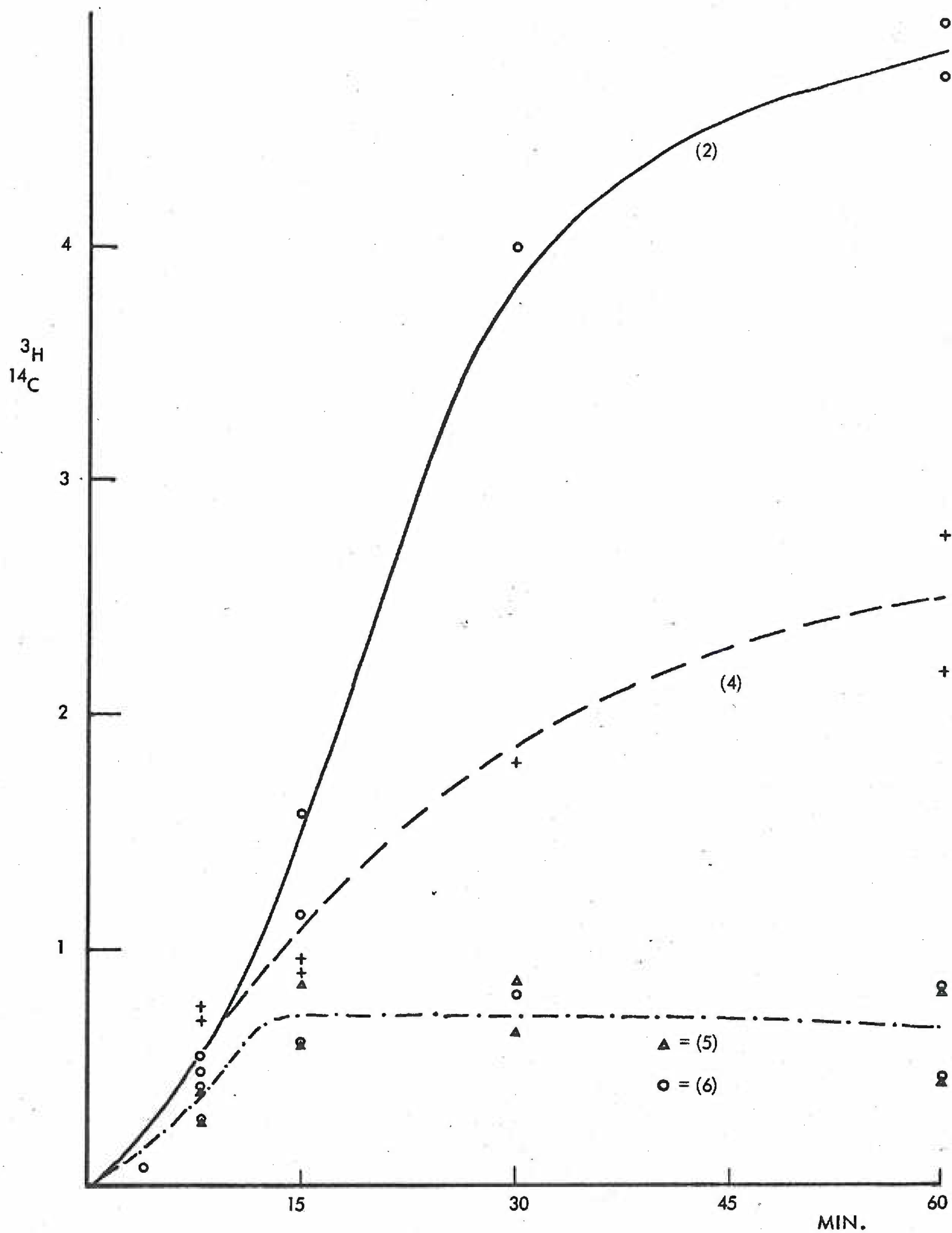
19 OH DHA (40.2% Recovery)

	Total DPMC^{14} Recovered	Total DPM H^3 Recovered	H^3/C^{14}	Total µg. Recovered
4 min	5,530	57,470		.26
8 min	18,530	100,760		.60
15 min	10,896	38,442		.30
	16,160	110,180		.68
30 min	7,834	18,513		.18
60 min	7,555	19,828		.18

Figure No. 16

Graphic illustration of tritium carbon ratios changing with time: Composite experiements 1 and 11.

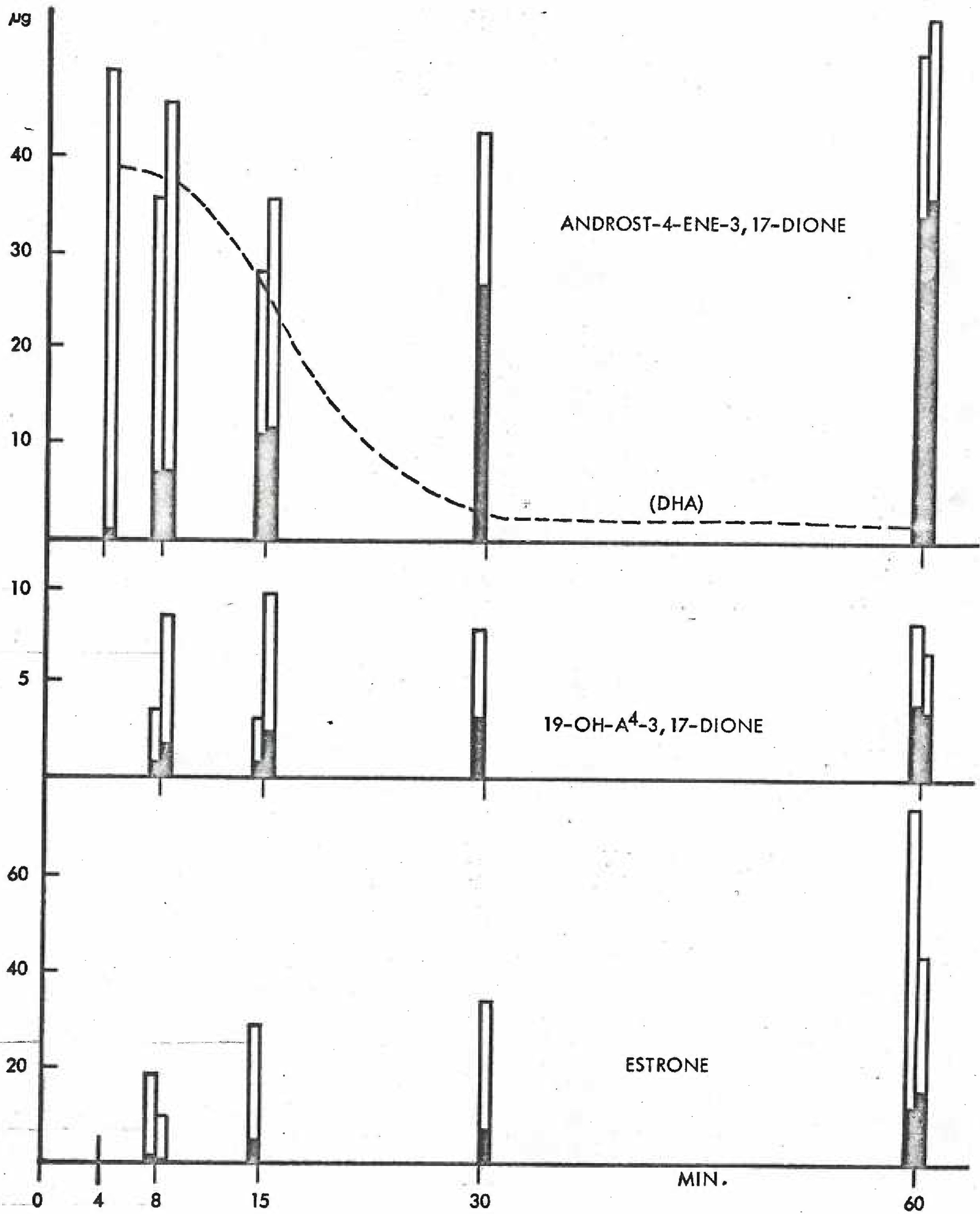
(2) androstenedione
(4) 19 hydroxyandrostenedione
(5) estrone
(6) estradiol



3H
14C
4
3
2
1
15
30
45
60
MIN.
(2)
(4)
▲ = (5)
○ = (6)

Figure No. 17

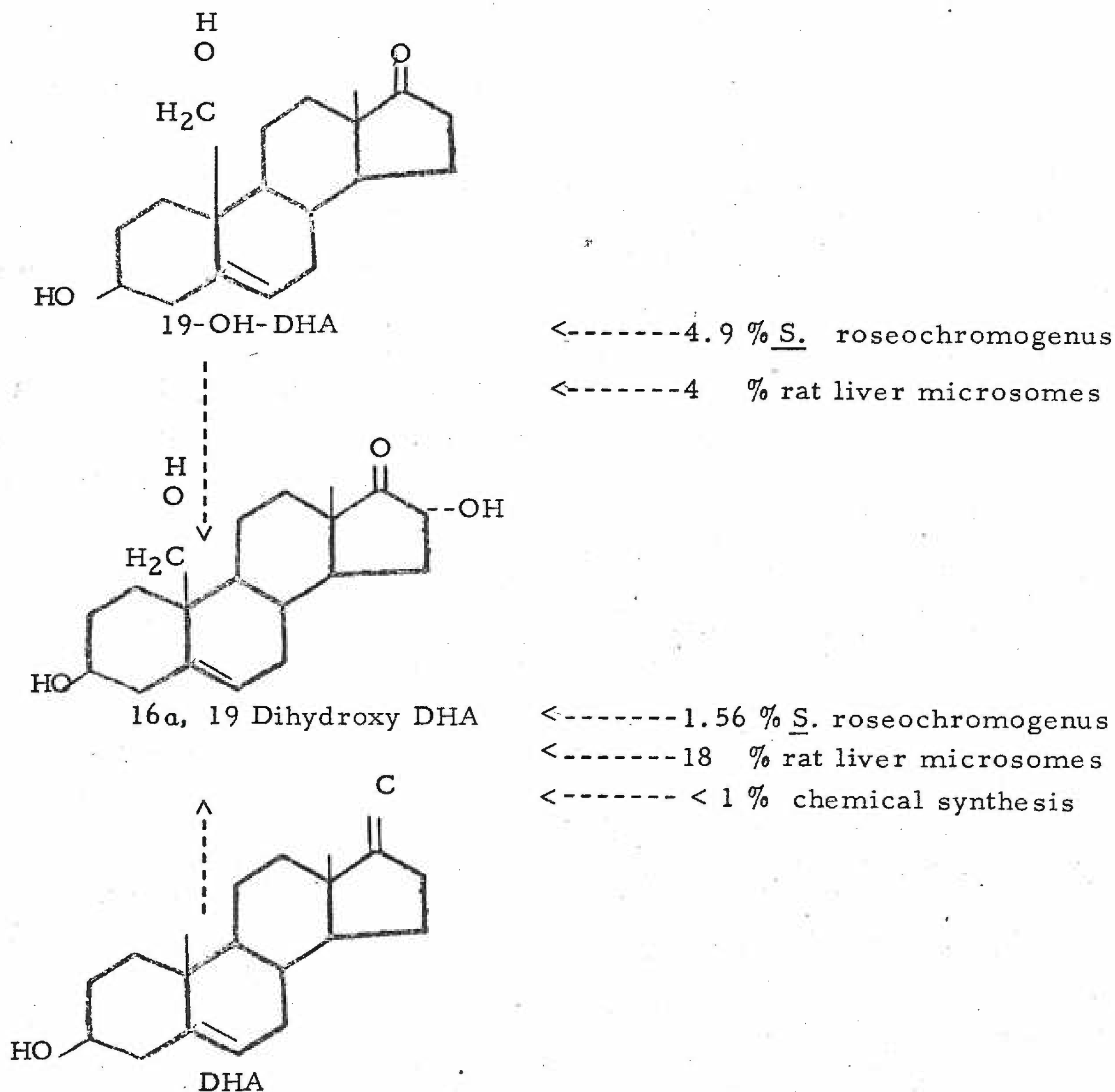
Contribution of DHA to A^4, 19-OH-A^4 and estrone. Dotted line on androst-4-ene-3, 17 dione figure represents disappearance of DHA.


µg
40
30
20
10
ANDROST-4-ENE-3, 17-DIONE
(DHA)
10
5
19-OH-A4-3, 17-DIONE
60
40
20
ESTRONE
MIN.
0
4
8
15
30
60

V. DISCUSSION

A. Chemical and Biological Synthesis of 16α, 19-Dihydroxy-DHA

The chemical synthesis of 3β, 16α, 19-trihydroxyandrost-5-en-17-one was accomplished by starting with DHA and following the reaction sequence (1-7) outlined on page 3. A brief summary of the chemical and biological synthesis is as follows:



Both the chemical and physical properties studied indicated that the synthesized compound was 3β, 16α, 19 trihydroxyandrost-5-ene-17 one. Chemical synthesis of the other neutral steroid (19-OH-A^4) used in the placental microsomal incubation studies was accomplished with a modified Oppenhuer oxidation of 19-OH-DHA. Thus one previously undescribed steroid (16α, 19 dihydroxy-DHA) and three steroids not yet commercially available (19-OH-DHA, 16α-OH-DHA, and 19-OH-A^4) were successfully snythesized from the starting compound DHA. The availability of these steroids made possible the placental microsomal incubation studies. More important however, Oppenhuer oxidation of 16α, 19 dihydroxy DHA could lead to 16α, 19 dihydroxy ^{4}A. With this later compound and tritium labeled 16α-OH-DHA, and ^{14}C labeled 16α-OH androstenedione studies relevant to estriol biosynthesis could be carried out and must be done to establish the pathway of estriol biosynthesis in the placenta.

The aromatization of Δ^5 3 -16α-dihydroxysteroids may proceed in part by bypassing androst-4-ene-3, 17, dione through an alternative route involving the free or sulfated Δ^{5-3} β, 19-dihydroxysteroid. The availability of 3β, 16α, 19 di OH-DHA now makes it possible to explore these possible routes to estriol biosynthesis.

The significance of the pathway in the conversion of neutral steroids to estriol may lie in sexual differentiation of the fetus. For example, if the Δ^{4-3} keto configuration is the preferred pathway then the major intermediates would be weak androgens (androstenedione and 16 hydroxyandrostendione). 17β reduction would then give testosterone or 16α-hydroxy testosterone. It is becoming clear that without androgens

the fetal sexual differentiation is primarily female. For example the work of Neumann, Elger and Kramer (58) indicates that during the second half of gestation and of the newborn during the first 3 post-natal weeks, male rats would develop a vagina when treated with an antiandrogen cyproterone acetate (1, 2-methylene-6-chloro-$\Delta^{4,6}$-pregnadiene-17α ol-3, 20-dione -17α-acetate). They were also successful in preventing the differentiation of the male genitalia in male fetuses by administering cyproterone acetate during pregnancy (59). Further they were able to induce the differentiation of mammary glands such as usually develops only in female rats (57) therefore, it is also conceivable that the pathway of estriol biosynthesis may play some role in providing the androgens necessary for male sexual differentiation.

In the introduction we discussed the estriol levels in pregnancy complicated by an anencephalic fetus. It is extremely interesting that 70% of such fetuses are female (74). It is possible that these "female" fetuses are actually male fetuses which without the adrenal androgens, fail to achieve male genitalia. I am currently exploring this possibility by examing the anencephalic fetus with respect to histology of the gonads and genitalia and comparing to the genetic sex.

B. <u>Biological synthesis of 16α, 19 Dihydroxy DHA.</u>

1. <u>Bacterial</u> experiments incubating 3β, 19-dihydroxyandrost-5-en-17-one with <u>S</u>. roseochromogenes (AT CC 3347) gave a product which had essentially the same infrared spectra (Figure No. 13) as the chemically synthesized 16α, 19 Dihydroxy DHA. The chemical

properties and the chromatographic behavior were also the same as those from the chemically synthesized compound.

The increased yield of 4.9 % for the 19 OH-DHA as compared to only 1.6 % for 16_α-OH DHA from DHA in a similar experiment is not easily explained. However, this maybe in the quality of the mycelia used for the different experiments.

2. Liver microsomes. The hepatic incubations failed to yield a pure compound. The main products of the reaction, however, had the same R_F values in 3 systems of thin-layer chromatography and the same color reactions as the chemically synthesized 16α, 19-dihydroxy DHA. The estimated yield was less than 4 % while the yield of 16_α-OH-DHA from DHA was 18 % with a similar microsomal preparations. The low yield of the 16α-, 19-OH-dihydroxy DHA was expected because in general each additional hydroxyl group on the steroid nucleus decreases the likelihood of further hydroxylation. Further work must be carried out to study the reactions of 19 OH-DHA with hepatic microsomes. Our experiments indicated that incubation of 19 OH-DHA with hepatic microsomes gave at least three products. One had the same R_F as did 16α, 19 dihydroxy DHA, the second was less polar, while the third was more polar, and turned blue with picric acid spray and was blue tetrazolium positive. This latter compound could have been 7β, 16α, 19, trihydroxy DHA ; however further characterization was not attempted. No estrogens could be identified.

It is known that midterm human fetal liver can aromatize neutral steroids with the Δ^{4-3} keto configuration but not the Δ^{5-3} hydroxy derivatives. The assumption has been made that the fetal liver does

not possess 3β-hydroxydehydrogenase. We carried out preliminary experiments with 19-OH-DHA and mid trimester human fetal liver microsomes to determine if aromatization took place with the 19 hydroxy derivative. Estrogens could be identified in only small amounts for both androstenedione and 19 hydroxy DHA. Therefore, the results were inconclusive and further work must be carried out here as well.

C. Placental microsomal incubations with DHA-7α ^{3}H and androstenedione-4-^{14}C.

The formation of estrogens from androst-4-ene-3, 17-dione (A^4) by placental mocrosomes has been reported to proceed through 19-hydroxyandrost-4-ene-3-17-dione (19-OH-A^4) as an obligatory intermediate (46). However, it seems clear that the physiological precursors for the three 'classical' estrogens that pregnant women at term excrete in large quantities are free or sulfated Δ^5-3 -hydroxysteroids, notably 3β-hydroxyandrost-5-en-17-one (DHA) and 3β, 16α-dihydroxyandrost-5-en-17-one, reaching the placenta from the fetal side, rather than Δ^4-3 ketosteroids. Since the yields of estrogens obtained in vitro with DHA-sulfate are greater than with DHA or A^4 (3), it seemed worthwhile to consider the possibility of pathways which would bypass either A^4 or 16α-OH-A^4, and which would have Δ^5-3 , 19-dihydroxysteroids as intermediates (Fig. 14). The conversion of 3β, 19-dihydroxyandrost-5-en-17-one (19-OH-DHA) to 17β-estradiol was shown in experiments (4) from which it was also concluded that C-10- hydroxymethyl compounds were aromatized to a greater extent than C-10-methyl compounds.

Later, the direct aromatization of C_{19}-steroid sulfates was reported (61) and it was proposed that androst-3, 5-dien-17-one sulfate (not shown in Fig. 18) was the intermediate.

The availability of 19-OH-DHA allowed us to carry out the experiments reported here in an attempt to measure the production of 19-OH-DHA in incubation mixtures of DHA with placental microsomes, and to explore the possibility that a small amount of 19-OH-DHA might be aromatized excluding 19-OH-A^4 as an intermediate. The relevant reactions are shown in Figure 18.

While this work was in progress, the identification of 19-OH-DHA and of its sulfate in incubation experiments with human placenta (62) and corpus luteum (24) was achieved by other investigators.

These experiments were conceived as preliminaries to a series where the role of various 3β, 19-dihydroxy-Δ^5-steroids and their sulfates in the placental biosynthesis of estrogens would be explored. It was assumed that any 17β-estradiol resulting from these incubations would originate from estrone, and not from the reduction at C-17 of the neutral steroids precursors with subsequent aromatization. While the correctness of this assumption cannot be demonstrated in this experiment, the fact that estrone and 17β-estradiol had the same $^3H/^{14}C$ ratios is certainly consistent with it. In view of the large variability in placental aromatizing ability (73), the yields of estrogen obtained in these experiments (up to approximately 39% conversion in one hour) can be considered acceptable. On the other hand, it does not seem possible to explain the decreasing amounts of 17β-estradiol recovered by any known property of the estradiol-specific 17β-

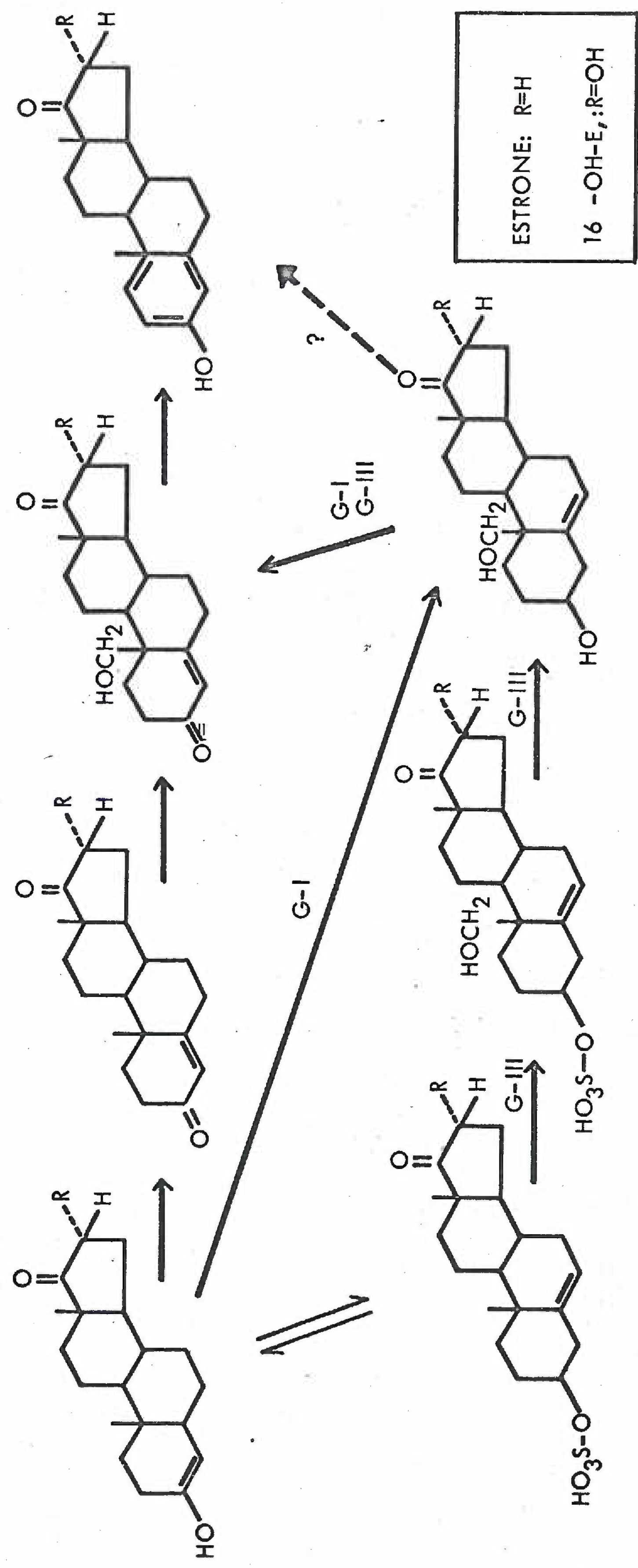
hydroxysteroid dehydrogenase of placental tissue. Perhaps an increasing degree of transformation of 17β-estradiol to other unmeasured compounds is responsible for the observation. It should be noted that no effort was made to measure the incorporation of the labels into other products than those already mentioned.

In view of the higher $^3H/^{14}C$ ratios of Δ^4-3-ketosteroid intermediates in comparison with those of the two estrogens, it seems correct to conclude that the contribution of the pathway from 19-OH-DHA to estrone bypassing 19-OH-A^4 was insignificant in these experiments. This supports the conclusion that 19-OH-A^4 is an obligatory intermediate in thebiosynthesis of estrone (72). It is still possible that a pathway not involving A^4 exists for the conversion of DHA-sulfate to estrone (61, 62). The fact that in the first 8 minutes of our incubations the $^3H/^{14}C$ ratio of 19-OH-A^4 was greater than that of A^4 is compatible with the view that, at least in the initial stages of these experiments, 19-OH-A^4 was enriched with ^3H not coming from A^4, with 19-OH-DHA being the most likely source. It is also reasonable to assume that the fast disappearance of DHA may be responsible for the decreasing significance of this route through 19-OH-DHA after the first 8 min. These considerations again emphasize the importance of studying the kinetics during the first few minutes of incubation (73), which we plan to do in similar and related experiments.

Lastly, the fact that so little '19-OH-DHA' could be detected during the incubations might be due to fast disappearance and conversion to other compounds, for which there is evidence in the literature (73), or to a greater affinity of the 19-hydroxylase system for A^4 than for DHA. These possibilities will be examined in future experiments.

Figure No. 18

Possible pathways from Δ^5-3 -hydroxysteroid sulfates to estrogens. G-1 and G-111 are pathways 1 an 111 depicted by O1Kelly and Grant, J.K. (6).



ESTRONE: R=H
16 -OH-E1 ;R=OH
G-I
G-III
G-I
G-III
G-III
G-III
?
HOCH2
HO
HO3S–O

VI SUMMARY AND CONCLUSIONS

1. Chemical synthesis of the possible intermediate (16α, 19 dihydroxy DHA) in estriol biosynthesis was successful by starting with DHA-3 acetate. The physical and chemical properties of this hitherto undescribed steroid is given in the text.

2. Twelve additional steroids were chemically synthesized. The most significant of these are:

   1. 16α-OH-DHA
   2. 19-OH-DHA
   3. 19-OH-A^4

These three steroids were utilized in biological experiments involving incubations with liver microsomes (16α-OH-DHA, and 19-OH-DHA), placental microsomes (19-OH-DHA, 19-OH-^{4}A), and bacteriological mycelia (19-OH-DHA).

Three other intermediate compounds in the synthesis of 16α, 19 dihydroxy DHA have not been previously described. Their chemical and physical characterization is also given in the text. They are-

   1. 3β, 19, 17-triacetoxyandrost 5, 16-diene
   2. 3β, 19-diacetoxy-16α-bromoandrost-5-en-17-one.
   3. 17, 17-dimethoxyandrost-5-ene-3β, 16α, 19 triol.

3. The bacterial synthesis of 16α, 19 dihydroxy dehydroepiandrosterone was accomplished by incubating 19-OH-DHA with _S._ roseochromogenus.

4. Incubation experiments were carried out with 19-OH-DHA and rat male-liver microsomes.

   a. At least three products resulted from these experiments: One which chemically and chromatographically resembled 16α, 19 dihydroxy DHA, the second compound was more polar

and may have been a 7β-OH derivative, and a third less polar derivative but its idenity could not be established.

5. The placental microsomal incubation studies of DHA-7α ^{3}H and androstenedione-4-^{14}C indicate that little 19-OH-DHA accumulates during the conversion of DHA to estrone.

6. No evidence could be obtained for a pathway from 19-OH-DHA to estrone not involving 19-OH-A^4. Thus the role of the latter as an obligatory intermediate in the biosynthesis of estrone is confirmed to either the precursor of A^4 or DHA.

7. There were indications that part of the 19-OH-A^4 was derived from DHA in a pathway not involving A^4 during the first 8 min. of incubation, when there was still a considerable amount of DHA available.

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