The in vitro Biosynthesis of Steroids by Ovarian and Adrenal Tissue of C57BL/6J Normal (+/+) and C57BL/6J Tortoise (To+/+) Mice

Alice Ming-Hsien Tang
Northern Michigan University
TITLE OF THESIS

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AND ADRENAL TISSUE OF C57BL/6J NORMAL (+/+ ) AND
C57BL/6J TORTOISE (To/+) MICE

by

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This thesis is recommended for approval by the student's thesis committee.

Chairman

Approved by__________________________, Dean of Graduate Studies.

(date)

Submitted in Partial Fulfillment of the Requirements for the Degree of
Master of Arts.

Northern Michigan University
Marquette, Michigan

(date)
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A Thesis
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School of Graduate Studies
Northern Michigan University
Marquette
June, 1971
ABSTRACT

This study was conducted to determine the in vitro biosynthesis of progesterone, androstenedione, and testosterone by ovarian tissue of C57BL/6J normal (+/+) mice and the production of androstenedione, testosterone, estrone, and estradiol by adrenal glands of normal and C57BL/6J tortoise (To/+) mice.

Ovarian and adrenal tissue from mice were incubated with cholesterol-4-C$^{14}$ and progesterone-4-C$^{14}$ respectively. Using the double label method, individual steroids were isolated by extraction and thin layer chromatography. Recrystallization to constant specific activity was used for positive identification.

The double label method demonstrated that the biosynthesis of progesterone, androstenedione, and testosterone by the ovarian tissue from normal mice were detectable. The adrenal glands of tortoise mice were possibly more efficient in the synthesis of androgens than the adrenal glands of normal mice.
ACKNOWLEDGEMENTS

I sincerely wish to thank the members of my graduate advisory committee, Dr. Frank A. Verley (Chairman), Dr. Lewis Peters of the Department of Biology and Dr. Roger D. Barry of the Department of Chemistry. Their assistance and advice facilitated the successful completion of this research work and preparation of this thesis.

I would like to give special recognition to Dr. F. A. Verley, my major advisor, and Dr. R. D. Barry under whose direction and guidance this investigation was conducted. I wish to thank the Department of Biology for the use of the animal room and supplies and the Department of Chemistry for the use of their laboratory materials and equipment.
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INTRODUCTION

In a previous study it was shown that ovaries of normal mice produce more estrone and \(17\beta\)-estradiol but less estriol than ovaries of tortoise mice (1). In order to determine the point in the biosynthesis when the differences occur, an experiment was designed permitting the isolation of some of the intermediates. The investigation also provided information on the qualitative and quantitative differences in \textit{in vitro} biosynthesis of androgens and estrogens by adrenal glands of normal and tortoise shell mice. Since the tortoise gene is prenatally lethal for hemizygous males, it was felt that differences in steroid hormone production may contribute to the observed lethality.

The tortoise gene, first reported by Dickie in 1954 (2), has recessive, sex-linked characteristics. The tortoise females produce both normal and tortoise female offspring because the females are heterozygous (To/\(+\)). The absence of tortoise males (To/\(Y\)) prevents the production homozygous tortoise females (To/To). The biological differences between the normal and tortoise mice are apparent in the coloring. The tortoise is variegated, black, brown, yellowish, gray, or almost white. The hair is silkier and vibrissae are slightly curled. There are skeletal abnormalities of the thoracic
cavity, vertebrae, and fore and hind limbs. All these effects result from a mutation at one locus.

Mice are polyestrous mammals and their estrous cycle is completed every four to six days (3). Each cycle is a complex of related cycles (behavioral, secretory, and anatomical) in which the pituitary and ovarian hormones interact and together serve the function of insuring fertilization (3,4). The factors which govern the cyclic reproductive activities are thought to be tied to the hypothalamic portion of the brain (5) which communicates with the adenohypophysis via the pituitary portal veins (6).

Steroid hormones are secreted mainly by the ovaries, testes, placenta, and adrenal cortex and are believed to have the same general route of biosynthesis (7,8). In the presence of enzymes from these various tissues, a common precursor such as cholesterol or pregnenolone can be converted into any one of the known steroid hormones. While there is some relationship between biological activity and chemical configuration of the molecule, it is known that a particular steroid hormone may exert multiple actions. The major source of estrogen production is the ovarian follicle in the non-pregnant female (9). In the pregnant female the placenta is the main site of estrogen production (10). A minor source of estrogen in both sexes is the adrenal. The chief site of androgen production is the testis (9).
Androgens and estrogens are members of the large group of chemical compounds called steroids, substances which contain the 1,2-cyclopentanoperhydrophenanthrene nucleus. The general steroid skeleton is given below.

![1,2-Cyclopentanoperhydrophenanthrene](image)

Typical configuration of a steroid

In the natural steroids, rings B and C are often trans fused, in that the hydrogen atom at C-8 is β-oriented and at C-9 α-oriented. Ring C and D are trans fused with the methyl group at C-13 β-oriented, and H at C-14 α-oriented. Most of the hormonally active steroids have a double bond between carbons 4 and 5, and therefore have no hydrogen at C-5.
However, the methyl group at C-10 is β-oriented. When the 4-5 double bond is reduced, the H at C-5 may be α(trans A/B ring) or (cis A/B ring) (11-14).

Steroids such as androgens, estrogens, progesterone, and the corticoid hormones are derived from cholesterol. Cholesterol, a common steroid in animals, is unsaturated and has the following structure.

![Chemical Structure of Cholesterol](image)

**Progesterone:**

The principal endocrine tissue that secretes progesterone in the normal female is the corpus luteum of the ovary. The placenta produces very large quantities of progesterone, especially during late pregnancy. In addition, this steroid hormone has been isolated from adrenal tissue and testes. Progesterone is required for the maintenance of the implanted blastocyst and is necessary for the preservation of a viable
fetus during the latter stages of pregnancy.

The de novo (from acetate or cholesterol) synthesis of progesterone from cholesterol is presented in Fig. 1. The reactions involve two important steps. One is the scission of the side chain of 20,22-dihydroxycholesterol, which yields 5-pregnen-3β-ol-20-one and isocaproic aldehyde. The reaction, which requires NADPH and oxygen, is in some way mediated by the adrenocorticotropic hormone (ACTH) and inhibited by 20-hydroxycholesterol, pregnenolone, and progesterone. In the second step a Δ⁵-3β-hydroxysteroid dehydrogenase (and Δ⁵, Δ⁴-isomerase), which requires NAD, promotes oxidation of the Δ⁵-3β-hydroxysteroid to the Δ⁴-3-ketosteroid, progesterone (15).

Progesterone is a key intermediate in the biosynthesis of steroids with 21 or fewer carbon atoms, e.g. androgens and estrogens.

**Androgens:**

Androgens are produced chiefly by the interstitial cells of the testis under normal conditions and in small amounts from the adrenal cortex and ovaries. Androgens are primarily responsible for stimulating the development of the male reproductive organs and secondary sex characteristics. In addition, they are anabolic, increasing tissue growth and muscle mass, with an increased retention of nitrogen, potassium,
Fig. 1. Biosynthesis of Progesterone
phosphorus and calcium.

A significant portion of the androgen present in the mammalian organism arises from the adrenal cortex (16). A number of organs possess the necessary enzymatic equipment to produce androgenic steroids (17-19). It is reasonably well established that all of the steroid hormones, whether adrenocortical, testicular, or ovarian, are produced by biosynthetic pathways common to all secreting cells of endocrine organs (20). Thus, the endocrine differences between testis, ovary and adrenal cortex are quantitative rather than qualitative. This accounts for the fact that the adrenal cortex, for example, may function abnormally and liberate an excess of gonadal steroids as in the case of adrenocortical virilism in females.

Like other steroid hormones, the androgens are cyclopentanoperhydrophenanthrene derivatives. All contain 19 carbon atoms and possess methyl groups at C-10 and C-13. Biological activity is altered by making relatively small additions or substitutions at the various carbon positions. Androstene represents the fully saturated hydrocarbon, and the introduction of double bonds may have a great effect on biological activity. Among these steroids, stereoisomerism is possible at carbon atoms 3, 5, 11, and 17. In the capon comb test the order of biological activity is testosterone > androsterone > dehydroepiandrosterone > epiandrosterone (15).

The biosynthesis of androgens is shown in Fig. 2. The key intermediate is androstenedione.
Fig. 2. Biosynthesis of Androgens
Fig. 3. Metabolism of Androgens
The C₁₉ steroids are chiefly metabolized by the liver and excreted in the urine. Testosterone in the circulation is bound to the blood proteins and hence does not normally filter through the kidney glomeruli. It is not stored in the body but is quickly utilized or degraded into relatively inactive androgens that are excreted in the urine or through the bile and feces. The principal catabolic pathway of androgens is shown in Fig. 3.

**Estrogens:**

Estrogens are a group of sex hormones characterized by their ability to produce estrus in females of various mammalian species. In the non-pregnant female, the major source of estrogen production is the ovarian follicle and a minor source of estrogen production for both sexes is the adrenals. (21). In the pregnant female, the placenta and fetus are the main sites of estrogen production. (22-24).

Estrogens are important in life processes, specifically in reproduction. They appear to have a greater affinity for the uterus than for other tissue (27,28). Some of their physiological effects are (1) to stimulate ovocytogenesis and follicular growth, (2) to initiate typical cyclic changes of the vaginal epithelium, (3) to produce growth of the uterine tissue, (4) to prime the uterus and fallopian tubes for ovulation, (5) to induce proliferation of the mammary gland ducts;
and (6) to maintain the secondary sexual characteristics of the female (21, 25, 26). The metabolism of protein, nucleic acids, lipids and terpenes (29-31), and inorganic metabolism of calcium and phosphorus are all affected by estrogens (32).

Steroids with estrogenic activity belong to the C18 series, contain an aromatic A ring, a hydroxyl group at C-3 which imparts phenolic characteristics, and an oxygen atom at C-17. These functional groups and the unique steric configuration of the rings determine the degree of physiological activity of the estrogens. The relative activity of the estrogens is believed to be 17β-estradiol > estriol > 17α-estradiol > estrone, as shown by the vaginal smear and uterine weight methods. (13)

Isotope studies have demonstrated that both acetate and cholesterol may serve as precursors in the biosynthesis of estrogens (33-35). The pathway is outlined in Fig. 4. The estrogens are synthesized by the pathway 5-pregnenolone to 17α-hydroxyprogrenolone to dehydroepiandrosterone to androstenedione or by the pathway progesterone to 17β-hydroxyprogesterone to androstenedione. The latter androgen is converted by aromatization to estrone. Estrone and 17β-estradiol constitute a reversible oxidation-reduction system in which estradiol is rapidly oxidized to estrone and then slowly converted back to estradiol (36); and both are irreversibly converted to estriol.
Fig. 4. Biosynthesis of Estrogens
Estrogens are believed to be produced in the free state, but approximately 50-66 percent of the estrogens circulating in the vascular system are bound to proteins and usually conjugated with glucosiduronic acid or sulfuric acid (7, 37). The site of these bindings is in the liver. Elimination of the estrogen is either through the bile or by the kidneys (15). The metabolic relationship between the estrogens is shown in Fig. 5.
Fig. 5. Some Metabolic Relationships for Estrogens

(1) 17β-Dehydrogenase  (2) 17α-Dehydrogenase
(3) 2β-Hydroxylase      (4) 16α-Hydroxylase
MATERIALS AND METHODS

1. Chemicals

All solvents were redistilled analytical grade. \textsuperscript{14}C \textsuperscript{14} C, progesterone-4-\textsuperscript{3}H, androstenedione-3 \textsuperscript{1,2-\textsuperscript{H}}, progesterone-1,2-\textsuperscript{H}, testosterone-1,2-\textsuperscript{H}, estrone-3 \textsuperscript{2,4,6,7-\textsuperscript{H}}, and estradiol-6,7-\textsuperscript{H} were purchased from the Nuclear Chicago and New England Nuclear Corporations. All radioactive steroids were kept in benzene and checked for specific activity immediately before use. Nonlabeled steroids, cofactors, and glucose-6-phosphate dehydrogenase were purchased from commercial sources.

2. Animals

The female mice used in these experiments were 60-120 day old C57BL/6J normal (+/+) mice and C57BL/6J tortoise shell (To/) mutants, obtained from the Biology Department of Northern Michigan University. They were fed ad libitum on a complete diet (Rockland Mouse/Rat Diet, Winfield, Iowa).

3. Tissue Preparation

Mice were killed by cervical dislocation. Ovaries and adrenal glands were removed immediately and placed in a Petri dish with chilled (\textsuperscript{-1\degree}C) physiological saline (0.9\% NaCl in distilled water). After removing the fatty tissue, the
ovaries and adrenal glands were blotted with filter paper and weighed to determine the wet weight. Using a razor blade, ovaries or adrenal glands were then cut into small sections in Krebs-Ringer phosphate buffer or in Krebs bicarbonate buffer, respectively.

4. Incubation

Ovarian tissue was put in 2 ml of Krebs-Ringer phosphate buffer with cofactors (Table I), pH 7.4; this was followed by the addition of 0.1 ml of cholesterol-4-\textsuperscript{14}C (about $1.0 \times 10^5$ cpm). The incubation was carried out at 37° for 3 hours in an atmosphere of 95% O\textsubscript{2} - 5% CO\textsubscript{2} in a Dubnoff incubator.

Adrenal glands were put in 2 ml of Krebs bicarbonate buffer solution with cofactors (Table I), pH 7.4; this was followed by the addition of 0.5 ml of progesterone-4-\textsuperscript{14}C (about $1.0 \times 10^5$ cpm). The incubation was carried out according to the method described for the ovaries.

5. Extraction

After the incubations, trace amounts of progesterone-1,2-\textsuperscript{3}H\textsubscript{3}, testosterone-1,2-\textsuperscript{3}H\textsubscript{3}, and androstenedione-1,2-\textsuperscript{3}H\textsubscript{3} were added to the unextracted incubated ovarian mixture. Trace amounts of estrone-2,4,6,7-\textsuperscript{3}H\textsubscript{3}, estradiol-6,7-\textsuperscript{3}H\textsubscript{3}, testosterone-1,2-\textsuperscript{3}H\textsubscript{3}, and androstenedione-1,2-\textsuperscript{3}H\textsubscript{3} were added to the
unextracted incubated adrenal gland mixture to enable correction to be made for losses of the steroid during extraction and purification.

Each incubate was processed in an identical manner as follows. The incubate was diluted with 10 volumes of physiological saline and extracted once with one volume and twice with one-half volume ethyl acetate. The remaining tissue was further extracted by grinding to a slurry and extracting with 2 ml aliquots of ethyl acetate. The combined ethyl acetate extracts were dried over sodium sulfate and evaporated to dryness.

6. Phenolic Partition

The residue was dissolved in 50 ml dichloromethane and extracted once with 20 ml and twice with 15 ml 2N sodium hydroxide. The estrogens were extracted into the alkaline solution. The pH of the combined extract was adjusted to 7-8 by adding dilute hydrochloric acid. The aqueous mixture was subsequently extracted once with 20 ml and twice with 15 ml of dichloromethane. The extracts were combined, dried and evaporated as previously described. The dichloromethane remaining after extraction of estrogens was dried and evaporated.
II. Incubation Media for Ovaries

A. Solution: Krebs-Ringer Phosphate Buffer Solution
(use within 2.5 hours)

(1). To prepare solution #7, place 17.8 g of Na₂HPO₄·2H₂O into a 1 liter volumetric flask. Add 20 ml of
1.0 N HCl Dilute to 1.0 liter with distilled H₂O.

(2). 10 ml of solution #7 (NaH₂PO₄) and 488 mg of
Nicotinamide are added to 50 ml of dilute isotonic
mixture in a 100 ml volumetric flask.

(3). When this is in solution, fill up to the 100 ml
mark with dilute isotonic mixture, check pH and
adjust, if necessary, to 7.4. Gas this final
solution 10 minutes with 95% O₂ - 5% CO₂ just
prior to use.

B. Cofactors

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Mg. (per 50 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN (NAD)</td>
<td>13.28</td>
</tr>
<tr>
<td>TPN (NADP)</td>
<td>15.0</td>
</tr>
<tr>
<td>ATP</td>
<td>101.4</td>
</tr>
<tr>
<td>Fumaric acid (Na salt)</td>
<td>4.06</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>4.5</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>10 cori units</td>
</tr>
</tbody>
</table>

Note: Tissue weight incubation media ratio should be
1 g tissue to 10 ml of media.
III. Incubation Media for Adrenal Glands

A. Solution: Krebs Bicarbonate Buffer Solution (use within 2.5 hours).

1. 16 ml of solution #6 (NaHCO₃) and 488 mg of Nicotinamide are added to 50 ml of dilute isotonic mixture in a 100 ml volumetric flask.

2. When this is in solution, fill up to the 100 ml mark with dilute isotonic mixture. Gas the final solution 10 minutes with 95% O₂ - 5% CO₂ just prior to use.

B. Cofactors

<table>
<thead>
<tr>
<th>Cofactor</th>
<th>Mg. (per 50 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPN (NAD)</td>
<td>13.28</td>
</tr>
<tr>
<td>DPNH (NADH)</td>
<td>14.18</td>
</tr>
<tr>
<td>TPN (NADP)</td>
<td>3.75</td>
</tr>
<tr>
<td>TPNH (NADPH)</td>
<td>4.17</td>
</tr>
<tr>
<td>ATP</td>
<td>10.14</td>
</tr>
<tr>
<td>Glucose-6-phosphate</td>
<td>6.50</td>
</tr>
<tr>
<td>Fumaric acid (Na salt)</td>
<td>5.80</td>
</tr>
<tr>
<td>Glucose</td>
<td>100.00</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>10 cori units</td>
</tr>
</tbody>
</table>
TABLE I. INCUBATION MIXTURES FOR OVARIES AND ADRENAL GLANDS

I. Standard Mixtures

A. Stock Solutions - (stable one month in refrigerator)

<table>
<thead>
<tr>
<th>Stock solutions no.</th>
<th>Final per cent concentration</th>
<th>Gms/total c.c. H₂O</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9% NaCl</td>
<td>22.5/500c.c.</td>
</tr>
<tr>
<td>2</td>
<td>1.15% KCl</td>
<td>5.75/100</td>
</tr>
<tr>
<td>3</td>
<td>1.62% CaCl₂·2H₂O</td>
<td>8.1/100</td>
</tr>
<tr>
<td>4</td>
<td>2.11% KH₂PO₄</td>
<td>10.55/100</td>
</tr>
<tr>
<td>5</td>
<td>3.8% MgSO₄·7H₂O</td>
<td>19.1/100</td>
</tr>
<tr>
<td>6</td>
<td>1.3% NaHCO₃</td>
<td>1.3/100 (Gas with CO₂, 1 hr just prior to use)</td>
</tr>
<tr>
<td>7</td>
<td>1.76% NaH₂PO₄·2H₂O</td>
<td>see below to prepare</td>
</tr>
</tbody>
</table>

B. Concentrated Isotonic Mixture - (mix just prior to use)

<table>
<thead>
<tr>
<th>Solution no.</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

C. Dilute Isotonic Mixture

Dilute 100 ml of concentrated isotonic mixture to 500 ml with distilled H₂O.
Incubation materials (containing \(^{14}C\)-steroids) + \(^3H\)-steroids

Extract with Ethyl Acetate (2,1,1)

Ethyl Acetate extract
(Neutral + phenolic)

- Evaporate to dryness

Aqueous (discarded)

Residue
dissolve in 50 ml Dichloromethane

Extract with 2N Sodium Hydroxide (20,15,15)

Dichloromethane extract
(Neutral)

Sodium Hydroxide (Phenolic)

- Adjust to pH 7-8 (by Hydrochloric Acid)

Extract with Dichloromethane

Dichloromethane extract
(phenolic steroids)

Aqueous (discarded)

**Fig. 6.** Scheme for the Extraction of Steroids from Ovaries and Adrenal Glands
7. Preparation of Thin Layer Chromatoplates

The 20 x 20 cm thin layer plates were prepared by using 30 g of Silica Gel H in 65-70 ml of distilled water. The spreader (Model S-11 Applicator, Desage-Brinkman) was set for a thickness of 250. The plates were air-dried for one-half hour, dried in an oven at 120-125°C for two hours then stored in a dessicator.

8. Chromatography

The residues from the phenolic partition were dissolved in a minimal amount of dichloromethane (1-2 ml) and transferred quantitatively to small test tubes (5 ml capacity). Excess dichloromethane was evaporated under a gentle stream of nitrogen at 37-38°C. At this point, 0.5 mg each of progesterone, androstenedione, testosterone, estrone, and estradiol were added as carrier. Using a micropipet, the concentrated solution was spotted on a starting line approximately 2.5-3.0 cm from the bottom edge of the plate. (a). Separation of Progesterone, Androstenedione, and Testosterone

For positive reference, standard progesterone, androstenedione, and testosterone were spotted in a column on either side of the biological extract. The first plate was run in a solvent system of
dichloromethane/ethyl acetate/absolute methanol (85:11:4) to separate testosterone from progesterone and androstenedione. Developing was done at room temperature. When the solvent reached the top of the plate (approximately 40 minutes), the plate was removed from the chamber and solvents were evaporated in the hood. The respective areas of testosterone, progesterone and androstenedione were identified under ultraviolet light, scraped from the plate, eluted with dichloromethane/methanol (1:1), and filtered through Whatman No. 42 filter paper. The filtrates were evaporated, spotted on separate plates, and run in the system cyclohexane/ethyl acetate (1:1), which purified the testosterone and separated progesterone from androstenedione. Remaining traces of cholesterol also were removed. Progesterone and androstenedione were purified in a chamber of chloroform/ether (9:1). In each run the sample was spotted on two plates in order to prevent overloading.

(b). Separation of Estrone and Estradiol

For positive reference, standard estrone and estradiol were spotted, one on each side of the biological extract. The estrogens, extracted by sodium hydroxide solution and dissolved in dichloromethane as previously described, were chromatographed on thin
layer plates in the solvent system of dichloromethane/ethyl acetate/absolute methanol (85:11:4). Estrogens were detected on a thin layer chromatography (TLC) plate with Pauly's reagent (38) prepared just prior to use by mixing equal volumes of sulfanilic acid solution (0.9 g sulfanilic acid, 9 ml conc. hydrochloric acid and 90 ml H₂O) with 5% sodium nitrite solution. The mixture was allowed to react a few minutes, then an equal volume of 10% sodium carbonate solution was added. Only the side lanes containing the reference estrogens were sprayed with the mixture. Estrogens give permanent yellow to red spots with Pauly's reagent. The corresponding area for each estrogen in the sample lane was marked 2 cm above and below the center of each respective standard spot. The areas were scraped from the plate, eluted with dichloromethane/methanol (1:1), filtered and evaporated as previously described. Estrone and estradiol were purified respectively in the solvent systems dichloromethane/methanol (99:1) and dichloromethane/methanol (95:5).
Fig. 7. Scheme for Separation of Progesterone, Testosterone, and Androstenedione
Fig. 8. Scheme Separation of Estrone and Estradiol
9. Preparation of Sample for Liquid Scintillation Counting

Each eluted residue was dissolved in 10 ml of methanol, and 0.1 ml of each solution was pipetted to a tared counting vial. Methanol was evaporated by a gentle stream of nitrogen. The residue contained in the counting vial was dissolved in 15 ml scintillation solution consisting 0.3% (W/V) of 2,5-diphenyloxazoyl (PPO) and 0.01% of 1,4-bis-2-(5-phenyloxazoyl)benzene (POPOP) in toluene. Radioactivity was measured using a Packard Tri-Carb Liquid Scintillation Spectrometer (Model 3310). The instrument was adjusted for single isotope determinations to efficiencies of approximately 34% and 84% for H³ and C¹⁴ respectively, as shown in Table II. For counting the isotopes, settings were adjusted to efficiencies of about 36 per cent for H³ and 67 per cent for C¹⁴.

TABLE II. Setting for Tri-Carb 3310

<table>
<thead>
<tr>
<th></th>
<th>Red Channel</th>
<th>Green Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>C¹⁴</td>
<td>% Gain Window</td>
<td>9.95%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50-1000</td>
</tr>
<tr>
<td>H³</td>
<td>% Gain Window</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50-1000</td>
</tr>
<tr>
<td>H³+C¹⁴</td>
<td>% Gain Window</td>
<td>9.95%</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200-1000</td>
</tr>
</tbody>
</table>
10. Recrystallization to Constant Specific Radioactivity

The radioactive material in each sample was mixed with 20 mg of pure steroid by dissolving it in hot acetone or hot methanol (estrogens). Hexane (water if methanol was used) was added as the acetone and the mixture were heated permitting the lower boiling acetone to evaporate until crystals began to form slowly at room temperature. The mixture was chilled; then the mother liquor was removed and the crystals rinsed with hexane and dried in a vacuum dessicator. One milligram of each dried crystal sample was weighed and counted in the scintillation solvent. The remaining crystals of each steroid were recrystallized a second and third time using the same solvent.
RESULTS

1. Conversion of Cholesterol-4-C\textsuperscript{14} to Progesterone, Androstenedione and Testosterone by Ovarian Tissue.

The data in this study came from three trials as shown below:

TABLE III. Animals and Amounts of Cholesterol-4-C\textsuperscript{14} Used in Ovarian Tissue Experiment

<table>
<thead>
<tr>
<th>Trials</th>
<th>Animals</th>
<th>Amount of cholesterol-4-C\textsuperscript{14} in dpm</th>
<th>Weight of Ovaries (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype</td>
<td>Number</td>
<td>Age in days</td>
</tr>
<tr>
<td>1</td>
<td>++</td>
<td>8</td>
<td>120±10</td>
</tr>
<tr>
<td>2</td>
<td>+/-</td>
<td>10</td>
<td>90±10</td>
</tr>
<tr>
<td>3</td>
<td>+/-</td>
<td>10</td>
<td>90±10</td>
</tr>
</tbody>
</table>

An average of 89 per cent (range 84-93 per cent) of the radioactivity of the added substrate was recovered from the incubation mixture. The steroid extracts were chromatographed on thin layer plates. The radioactivity was more efficiently recovered in the chromatographic fraction containing testosterone than in fractions with progesterone and androstenedione. The results are summarized in Tables V, VI, and VII. Mouse ovarian tissue produced testosterone in larger quantity than either progesterone or androstenedione.

29
2. Identification of Progesterone, Testosterone and Androstenedione.

Progesterone, androstenedione and testosterone were identified by recrystallization to constant specific activity. Recrystallization data for each identified product are shown in Tables VIII, IX, and X. Because the amount of ovarian tissue was small and the loss of radioactive material during the processes was unavoidable, it was necessary to use the double label method in order to determine the percentage loss. Therefore, in the third trial this method was used and the results are believed to be more accurate.

Further scrutiny of these tables reveals that a large amount of radioactivity remained in the mother liquid, compared to the crystals in the initial crystallization. This was expected and probably was due to sample contamination by the starting materials.

These recrystallization data represent conclusive identification of isolated progesterone, androstenedione and testosterone, and show that testosterone occurred in the largest amount in two of three trials.
3. Conversion of Progesterone-$4\text{-C}^{14}$ to Androstenedione, Testosterone, Estrone, and Estradiol by Adrenal Glands of Normal and Mutant Mice.

The data in this study came from both normal and mutant mice as shown below:

**TABLE IV. Animals and Amounts of Progesterone-$4\text{-C}^{14}$ Used in Adrenal Glands Experiment**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number</th>
<th>Age in days</th>
<th>Amount of progesterone-$4\text{-C}^{14}$ in dpm</th>
<th>Weight of Adrenal glands (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>10</td>
<td>90+10</td>
<td>$1.282 \times 10^5$</td>
<td>41.35</td>
</tr>
<tr>
<td>To/+</td>
<td>10</td>
<td>90+10</td>
<td>$1.282 \times 10^5$</td>
<td>44.43</td>
</tr>
</tbody>
</table>

The products were extracted and purified by chromatography on thin layer plates. The results are summarized in Tables XI and XII. Adrenal glands of normal and tortoise mice are capable of *in vitro* conversion of progesterone-$4\text{-C}^{14}$ to androgens and androstenedione is synthesized to a greater extent than testosterone.

The adrenal glands of the mutant mouse produced more total androgens than the normal mouse.
The areas on thin layer plates corresponding to estrone and estradiol were eluted and the residues contained negligible amounts of radioactive material. Both these steroids were not further purified because of the minute amounts available as shown by the low radioactivity.

4. Identification of Androstenedione and Testosterone.

Androstenedione and testosterone were recrystallized to constant specific activity after the addition of authentic non-labelled material to confirm their identity. Recrystallization data for each identified product are shown in Table XIII.

DISCUSSION

The results in the Tables V through X show that mouse ovaries synthesize progesterone, androstenedione and testosterone. Therefore, the conversion of cholesterol into estrogens in mice probably occurs by way of progesterone, androstenedione and testosterone. Further studies are required to explain differences in estrogen biosynthesis between normal and tortoise mice.

In the experiment dealing with the synthesis of androgens and estrogens by adrenal glands, the tortoise mouse adrenal glands produced more androgens than the normal mice. The observed difference in androgen biosynthesis may have signifi-
cant biological effects. It is probable that in the tortoise mouse some of the enzyme systems are changed and therefore cause a difference in androgen production by adrenal cells.

The fact that androstenedione is synthesized to a greater extent than testosterone was not surprising because the adrenal glands are not mainly responsible for testosterone production. Testosterone is made mainly in the testis. The ovary in the tortoise produces less estrone and estradiol than in normal mice, but the adrenal gland produces more androgens in the tortoise than in normal mice. This suggests an interesting relationship for steroid hormone production between organs. Probably, the synthesis of androgens influence the synthesis of estrogens via an hypothalamic-hypophyseal feedback mechanism.

The minute quantity of estrogens isolated from adrenal incubations was expected because the adrenal glands are not directly responsible for estrogen synthesis and the estrogens are produced farther along the biosynthetic path than androgens.
TABLE V. Conversion of Cholesterol-4-C\(14\) (1.047 \times 10^5 dpm) to Progesterone, Androstenedione, and Testosterone by 8 Normal Mice Ovaries after TLC (Trial I)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total activity in dpm</th>
<th>% Conversion</th>
<th>% Conversion per g Wet Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>906</td>
<td>0.866</td>
<td>1.837 \times 10^{-2}</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>505</td>
<td>0.482</td>
<td>1.023 \times 10^{-2}</td>
</tr>
<tr>
<td>Testosterone</td>
<td>1200</td>
<td>1.146</td>
<td>2.432 \times 10^{-2}</td>
</tr>
<tr>
<td>Total</td>
<td>2611</td>
<td>2.494</td>
<td>5.293 \times 10^{-2}</td>
</tr>
</tbody>
</table>
TABLE VI. Conversion of Cholesterol-4-C<sup>11</sup> (1.083 × 10<sup>5</sup> dpm) to Progesterone, Androstenedione, and Testosterone by 10 Normal Mice Ovaries after TLC (Trial II).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Activity in dpm</th>
<th>% Conversion</th>
<th>% Conversion per g Wet Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>375</td>
<td>0.346</td>
<td>5.662 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>381</td>
<td>0.352</td>
<td>5.761 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Testosterone</td>
<td>402</td>
<td>0.371</td>
<td>6.072 × 10&lt;sup&gt;-2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>1158</td>
<td>1.069</td>
<td>1.750 × 10&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
TABLE VII. Conversion of Cholesterol-$\Delta^{4}$-C$^{14}$ (1.09$\times$10$^5$ dpm) to Progesterone, Androstenedione, and Testosterone by 10 Normal Mice Ovaries after TLC (Trial III).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Activity in dpm</th>
<th>% Conversion</th>
<th>% Conversion per g Wet Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>561</td>
<td>0.513</td>
<td>1.804$\times$10$^2$</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>790</td>
<td>0.722</td>
<td>1.098$\times$10$^2$</td>
</tr>
<tr>
<td>Testosterone</td>
<td>986</td>
<td>0.902</td>
<td>1.372$\times$10$^2$</td>
</tr>
<tr>
<td>Total</td>
<td>2336</td>
<td>2.155</td>
<td>3.279$\times$10$^2$</td>
</tr>
</tbody>
</table>
TABLE VIII. The Specific Activity of Crystals in dpm/mg by 8 Normal Mice Ovaries (Trial I).

<table>
<thead>
<tr>
<th>Recrystallization Number</th>
<th>Sample</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frogesterone</td>
<td>Androstenedione</td>
<td>Testosterone</td>
</tr>
<tr>
<td>1</td>
<td>16.5</td>
<td>25.3</td>
<td>42.7</td>
</tr>
<tr>
<td>2</td>
<td>6.45</td>
<td>10.7</td>
<td>16.2</td>
</tr>
<tr>
<td>3</td>
<td>4.26</td>
<td>8.53</td>
<td>14.3</td>
</tr>
</tbody>
</table>
TABLE IX. The Specific Activity of Crystals in dpm/mg by 10 Normal Mice Ovaries (Trial II).

<table>
<thead>
<tr>
<th>Recrystallization Number</th>
<th>Sample</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progesterone</td>
<td>Androstenedione</td>
<td>Testosterone</td>
</tr>
<tr>
<td>1</td>
<td>20.6</td>
<td>18.2</td>
<td>25.5</td>
</tr>
<tr>
<td>2</td>
<td>7.52</td>
<td>8.31</td>
<td>14.1</td>
</tr>
<tr>
<td>3</td>
<td>6.94</td>
<td>8.56</td>
<td>13.5</td>
</tr>
</tbody>
</table>
TABLE X. The Specific Activity of Crystals in dpm/mg by 10 Normal Mice Ovaries (Trial III).

<table>
<thead>
<tr>
<th>Recrystallization Number</th>
<th>Sample</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progesterone</td>
<td>Androstenedione</td>
<td>Testosterone</td>
</tr>
<tr>
<td>1</td>
<td>28.5</td>
<td>27.7</td>
<td>31.7</td>
</tr>
<tr>
<td>2</td>
<td>7.46</td>
<td>10.9</td>
<td>18.1</td>
</tr>
<tr>
<td>3</td>
<td>8.52</td>
<td>10.3</td>
<td>16.4</td>
</tr>
<tr>
<td>4</td>
<td>7.68</td>
<td>9.16</td>
<td>15.9</td>
</tr>
<tr>
<td>5</td>
<td>8.01</td>
<td>9.32</td>
<td>15.0</td>
</tr>
</tbody>
</table>
TABLE XI. Conversion of Progesterone-4-\textsuperscript{14}C (1.282 × 10\textsuperscript{5} dpm) to Androstenedione, Testosterone, Estrone, and Estradiol by 10 Normal Mice Adrenal Glands after TLC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Activity in dpm</th>
<th>% Conversion</th>
<th>% Conversion per g Wet Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>876</td>
<td>0.6829</td>
<td>1.652 × 10\textsuperscript{2}</td>
</tr>
<tr>
<td>Testosterone</td>
<td>331</td>
<td>0.2580</td>
<td>0.624 × 10\textsuperscript{2}</td>
</tr>
<tr>
<td>Estrone</td>
<td>-4.6</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Estradiol</td>
<td>-7.5</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Total</td>
<td>1207</td>
<td>0.9409</td>
<td>2.276 × 10\textsuperscript{2}</td>
</tr>
</tbody>
</table>
TABLE XII. Conversion of Progesterone-$4\text{-C}^{14}$ (1.282 x $10^7$ dpm) to Androstenedione, Testosterone, Estrone, and Estradiol by 10 Tortoise Mutant Mice Adrenal Glands after TLC.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Activity in dpm</th>
<th>% Conversion</th>
<th>% Conversion per g Wet Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androstenedione</td>
<td>1441</td>
<td>1.124</td>
<td>2.530 x $10^2$</td>
</tr>
<tr>
<td>Testosterone</td>
<td>427</td>
<td>0.333</td>
<td>0.749 x $10^2$</td>
</tr>
<tr>
<td>Estrone</td>
<td>14.1</td>
<td>0.00011</td>
<td>----</td>
</tr>
<tr>
<td>Estradiol</td>
<td>-3.94</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Total</td>
<td>1852</td>
<td>1.4571</td>
<td>3.279 x $10^2$</td>
</tr>
</tbody>
</table>
TABLE XIII. Recrystallization of Androstenedione & Testosterone, Resulting from *in vitro* Biosynthesis by Normal and Tortoise Mice Adrenal Glands.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Sample</th>
<th>Recrystallization Number</th>
<th>$\text{dpm/mg} \frac{H^3}{C^{14}}$</th>
<th>$\text{H}^3/C^{14}$'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>Androstenedione</td>
<td>1</td>
<td>60735</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>61179</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>60324</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>1</td>
<td>62396</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>57745</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>57234</td>
<td>52</td>
</tr>
<tr>
<td>Tortoise</td>
<td>Androstenedione</td>
<td>1</td>
<td>57046</td>
<td>289</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>56805</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>53073</td>
<td>118</td>
</tr>
<tr>
<td></td>
<td>Testosterone</td>
<td>1</td>
<td>62557</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>62298</td>
<td>113</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>69245</td>
<td>108</td>
</tr>
</tbody>
</table>
Appendix

A Typical Calculation (39, 40)

I. Single label

1. For $^{14}$C calculation

Sample number: The crystals of testosterone fraction after second crystallization in trial II.

Sample net weight: 1.02 mg

Average counts per minute (cpm):

<table>
<thead>
<tr>
<th>Channel</th>
<th>Sample</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red channel</td>
<td>36</td>
<td>24</td>
</tr>
<tr>
<td>Green channel</td>
<td>29</td>
<td>18</td>
</tr>
</tbody>
</table>

\[
\text{Channel ratio} = \frac{\text{cpm in green channel} - \text{blank}}{\text{cpm in red channel} - \text{blank}}
\]

\[
\text{Channel ratio} = \frac{29 - 18}{36 - 24} = 0.92
\]

% Efficiency: 82%

\[
\frac{\text{Disintegration (dpm)}}{\text{% efficiency}} = \frac{\text{cpm in red channel} - \text{blank}}{\text{cpm in green channel} - \text{blank}}
\]

\[
\text{Disintegration (dpm)} = \frac{12}{0.82} = 14.4
\]

Specific Activity (dpm/mg) = \[
\frac{14.4}{1.02} = 14.1
\]

2. For $^3$H calculation

Channel ratio = \[
\frac{\text{cpm in red channel} - \text{blank}}{\text{cpm in green channel} - \text{blank}}
\]
II. Double label

Sample number: The crystal of testosterone fraction after first crystallization in trial III.

Sample net weight: 1.003 mg

Average cpm:

<table>
<thead>
<tr>
<th>Sample</th>
<th>C(^{14}) Standard</th>
<th>H(^3) Standard</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>148</td>
<td>19125</td>
<td>40</td>
</tr>
<tr>
<td>Green</td>
<td>79459</td>
<td>2597</td>
<td>19646</td>
</tr>
</tbody>
</table>

\[
m = \frac{\text{cpm of green channel (C}\(^{14}\)\text{ standard) - blank}}{\text{cpm of red channel (C}\(^{14}\)\text{ standard) - blank}}
\]

\[
n = \frac{\text{cpm of red channel (H}\(^3\)\text{ standard) - blank}}{\text{cpm of green channel (H}\(^3\)\text{ standard) - blank}}
\]

\[
H^3 = (\text{green channel - blank) - m(red channel - blank)}
\]

\[
C^{14} = (\text{red channel - blank) - n(green channel - blank)}
\]

Calculation:

\[
m = \frac{2597 - 15}{19125 - 15} = 0.135
\]

\[
n = \frac{40 - 15}{19646 - 15} = 0.00127
\]

\[
H^3 = (79459 - 15) - 0.135(148 - 15)
\]

\[
= 79435 \text{ dpm}
\]

\[
C^{14} = (148 - 15) - 0.00127(79459 - 15)
\]

\[
= 32 \text{ dpm}
\]

Specific Activity (dpm/mg) = \[
\frac{32}{1.003} = 31.7
\]
LITERATURE CITED


