STEROIDOGENESIS IN MOUSE PREIMPLANTATION EMBRYOS

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The Faculty of the School of Sciences and Mathematics
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In Partial Fulfillment
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Master of Science in Biology

by
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Master's Committee:

Director of Thesis

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ABSTRACT

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Marcia E. Kendall
Morehead State University, 1979

Steroid hormone production (steroidogenesis) was studied in mouse preimplantation embryos (PIE's) from mice treated with gonadotropins. This study was designed to determine which hormones, if any, were synthesized de novo by PIE's in vitro. Results were obtained by the use of histochemical stains for specific enzymes (3β-HSD and 17β-HSD) and the conversion of a radioactive precursor.

It was concluded that PIE's contain both 3β-HSD and 17β-HSD, enzymes necessary for steroidogenesis, as evidenced by formazan deposition, but 3H-pregnenolone was not converted into steroid hormones. These in vitro experimentations suggest that even though the enzymes are present in PIE's, steroidogenesis does not occur in mouse PIE's.

Accepted by:  
Chairman
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>II. Materials and Methods</td>
<td>14</td>
</tr>
<tr>
<td>Animal Care</td>
<td>14</td>
</tr>
<tr>
<td>Glassware and Reagents</td>
<td>14</td>
</tr>
<tr>
<td>Ovulation Induction</td>
<td>14</td>
</tr>
<tr>
<td>Ova Recovery</td>
<td>15</td>
</tr>
<tr>
<td>Culture Medium</td>
<td>17</td>
</tr>
<tr>
<td>Ova Culture</td>
<td>18</td>
</tr>
<tr>
<td>Histochemical Enzyme Determinations</td>
<td>18</td>
</tr>
<tr>
<td>Steroidogenesis Determination</td>
<td>19</td>
</tr>
<tr>
<td>Thin Layer Chromatography</td>
<td>20</td>
</tr>
<tr>
<td>III. Results</td>
<td>22</td>
</tr>
<tr>
<td>IV. Discussion</td>
<td>30</td>
</tr>
<tr>
<td>V. Literature Cited</td>
<td>35</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Biosynthesis of estrogen from cholesterol as compiled from Tamaoki (1973), Savard (1973), and Dorfman and Ungar (1965)</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Enzyme reactions applied for histochemical detection of hydroxysteroid dehydrogenases as described by Tamaoki (1973)</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>Time sequence of hormone injections and PIE cleavages</td>
<td>16</td>
</tr>
<tr>
<td>4.</td>
<td>Conversion of $^3$H-pregnenolone to steroid hormones in 2 and 4-cell PIE's</td>
<td>28</td>
</tr>
<tr>
<td>5.</td>
<td>Conversion of $^3$H-pregnenolone to steroid hormones in 8-cell PIE's and morula</td>
<td>29</td>
</tr>
</tbody>
</table>
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 3β-HSD activity in PIE's as determined by a histochemical enzyme stain</td>
<td>23</td>
</tr>
<tr>
<td>2. 17β-HSD activity in PIE's as determined by a histochemical enzyme stain</td>
<td>25</td>
</tr>
<tr>
<td>3. Percentage of PIE's (of total PIE's) showing 3β-HSD and 17β-HSD deposition</td>
<td>26</td>
</tr>
</tbody>
</table>
INTRODUCTION

It is well established that early pregnancy in mammals is maintained by substances of ovarian origin. This was first evidenced by the work of Frankel (1903) and Marshall and Jolly (1905), in which ovariectomy in rabbits during pregnancy led to abortion or resorption of the embryos. These "ovarian secretions" were later defined as the steroids progesterone and estrogen (Corner, 1928; Doisy, Veler, and Thayer, 1930). It was widely accepted that the ovaries were the only source of estrogen and progesterone required for the maintenance of early pregnancy (Robson, 1938; Hall and Newton, 1947). Recently, a hypothesis was proposed by Dickmann and Dey (1973) which suggested the preimplantation embryo (PIE) as a second source of steroid hormones involved in early pregnancy. This new concept proposed that the PIE had the capacity to synthesize steroid hormones, which might serve as a regulator for one or more of the following phenomena: 1) morula to blastocyst transformation, 2) shedding and dissolution of the zona pellucida, 3) implantation of the blastocyst, and 4) a variety of undefined metabolic activities in morula and blastocysts (Dickmann, 1975; Dickmann, Dey, and SenGupta, 1976). Presently, the only non-tumorous organs in mammals known to synthesize
steroid hormones are the ovary, testis, adrenal, and placenta.

Uncertainties in the reproductive behavior of mice have made it difficult to carry out experiments involving the production of PIE's. Since the original studies by Engle (1927) and Smith and Engle (1927), many investigators have attempted to induce both estrus and ovulation in mature rats and mice. Methods for the induction of estrus and superovulation in the female by injection of gonadotropins have come into general use, both as a means for controlling the erratic nature of the estrus cycle of the mouse and as a technique for increasing the yield of embryos per mouse. (Fowler and Edwards, 1957). More importantly, the stages of maturation division, ovulation, and the first and subsequent cleavage divisions can be ascertained following treatment with gonadotropins (Spears and Walker, 1968).

Hammond (1949) was the first to culture 8-cell mouse ova to blastocysts in vitro, utilizing a medium containing egg white. His work has since been extensively improved (Whitten, 1956, 1957). Whitten demonstrated that 8-cell mouse ova developed to blastocysts when cultured in Krebs-Ringer bicarbonate containing glucose and crystalline bovine plasma albumin (BPA) (Whitten, 1956). He also
found that energy sources such as glucose, mannose, lactate, pyruvate, and malate supported normal development of the 8-cell mouse embryo (Whitten, 1957). In 1966, Brinster and Thomson found that the 8-cell mouse embryo required only an energy source or a fixed nitrogen source for normal development to blastocyst in vitro. Brinster (1965b) reported that glucose in concentrations between 0.5mg/ml and 5.0mg/ml supported development of 4-cell ova into blastocysts, provided pyruvate was also present at low concentrations as an energy source.

In 1963 Brinster improved the method of in vitro cultivation of mouse ova by using a medium consisting of a balanced salt solution, BPA as a nitrogen source, sodium lactate as an energy source, and penicillin and streptomycin. Later, he clearly established optimal requirements for the 2-cell embryo developing in vitro in terms of pH, osmolarity, energy source, and fixed nitrogen source (Brinster, 1965a,b,c). The energy sources which supported development of the 2-cell embryos were those within one metabolic reaction of pyruvic acid, the best results obtained with a combination of pyruvate and lactate (Brinster, 1965d). It is of interest that the 2-cell embryo could not use glucose as an energy source, a substrate which could not be utilized until the 8-cell
stage (Brinster and Thomson, 1966). The culture of mammalian PIE's in vitro was usually performed using an atmosphere of 5% CO₂ in air (Brinster, 1963).

The metabolic pathways leading to steroid hormone synthesis have become increasingly well defined (Dorfman and Ungar, 1954; Dorfman, 1956). It is well established that the major route of steroid formation in all steroidogenic cells is via the enzymatic conversion of cholesterol to the steroid pregnenolone (3β-hydroxy-Δ⁵-pregnen-20-one) (Dorfman and Ungar, 1965). Pregnenolone serves as the biochemical precursor for steroid hormones in adrenal tissue (Hayano, Saba, Dorfman, and Hechter, 1956), placental tissue (Nissim and Robson, 1952; Pearlman, Cerceo, and Thomas, 1954), testicular tissue (Tamaoki, Inano, and Nakano, 1969), as well as in the steroidogenic tissues of the ovary (Savard, Marsh, and Rice, 1965).

Free cholesterol becomes available to the cell: 1) by direct transport of preformed cholesterol from serum, 2) after enzymatic hydrolysis of cholesterol esters from intracellular deposits, and 3) biosynthesis de novo from acetate by joint action of the endoplasmic reticulum and cytosol. The pathways involved in steroid biosynthesis are depicted in Figure 1. In steroid forming cells, cholesterol is carried to the inner mitochondrial membrane where the side chain cleavage enzymes are located.
Figure 1. Biosynthesis of estrogen from cholesterol as compiled from Tamaoki (1973), Savard (1973), and Dorfman and Ungar (1965).

ENZYMES:
1. Cholesterol 20α-hydroxylase
2. 3β-HSD
3. Δ⁵-Δ⁷ isomerase
4. C₁₇-C₂₀ lyase
5. 17β-HSD
6. 16α-hydroxylase
7. 19α-hydroxylase
8. Aromatizing enzyme system
As a result, pregnenolone is synthesized on the inner mitochondrial membrane. Pregnenolone reenters the cytoplasm where it is converted to progesterone by $\Delta^5$-3$\beta$-hydroxysteroid dehydrogenase, an enzyme which catalyzes the oxidation of the hydroxyl group at the 3$\beta$ carbon, and by $\Delta^5$-$\Delta^4$ isomerase, which converts the double bond from $\Delta^5$ to $\Delta^4$ (Tamaoki, 1973). These enzymes are located on the agranular endoplasmic reticulum.

The enzymes which are associated with androgen formation consist of a 17$\alpha$hydroxylase and a C-17,20 lyase. The former enzyme hydroxylates both pregnenolone and progesterone at carbon 17, and the latter removes the 2-carbon side chain from both 17$\alpha$hydroxy pregnenolone and 17$\alpha$hydroxyprogesterone to give rise to dehydroepiandrosterone (DHEA) and 4-androstenedione, respectively (see Figure 1). The enzyme system associated with the formation of estrogens from androgens consists of a 19$\alpha$hydroxylase-aromatase which selectively oxidizes and removes the angular carbon 19 from the androgen and leads to the formation of the phenolic ring A of the estrogen molecule. The enzyme 17$\beta$-HSD (hydroxysteroid dehydrogenase) catalyzes the interconversion of estrone and estradiol.

All of the enzymes in Figure 1 are present, at least in a qualitative sense, in virtually all gonadal tissues.
of all species examined (Dorfman and Ungar, 1965; Savard, 1970). All enzymes, excluding the cholesterol side chain cleavage complex, are found in the endoplasmic reticulum (Samuels and Eik-Nes, 1968; Tamaoki, Inano, and Nakano, 1969).

Since 3β-HSD (hydroxysteroid dehydrogenase) is a key enzyme in the synthesis of steroid hormones, its presence in a tissue constitutes strong evidence for steroidogenesis (Weist and Kidwell, 1969). Subsequent to the formation of pregnenolone, the 3β-hydroxy group is oxidized by steroid 3β-HSD (Samuels, Helmreich, Lasater, and Reich, 1951; Rubin and Dorfman, 1959; Rubin, Leipsner, and Deane, 1961). Nicotinamide adenine dinucleotide (NAD) serves as a coenzyme for this reaction (Beyer and Samuels, 1956; Samuels, et al., 1951). Enzymatic reactions in which NAD serves as a coenzyme can be coupled via NADH-diaphorase with tetrazolium salt reduction (Brodie and Gots, 1951; Kun, 1951). These reactions and the basis for the histochemical stain are shown in Figure 2. The hydroxysteroid dehydrogenase enzymes, 3β-HSD and 17β-HSD, can be demonstrated histochemically by established techniques for the presence of oxidative enzymes by tetrazolium salts (Farber, Sternberg, and Dunlap, 1954, 1956; Wattenberg, 1958; Levy, Deane, and Rubin, 1959; Allen, 1960; Nieme and Ikonen, 1961). This histochemical method is of value in that the enzyme can
Figure 2. Enzyme reactions applied for histochemical detection of hydroxysteroid dehydrogenases as described by Tamaoki (1973).
be demonstrated in a very small amount of tissue, and the exact location of the enzyme's activity can be determined (Dickmann, et al., 1976). The reactions in this histochemical stain are the reduction of NAD from the steroid substrate by 3β-HSD or 17β-HSD, followed by the transfer of electrons from the reduced NAD to the tetrazole (Levy, et al., 1959).

Formazan (reduced tetrazole) deposition develops at the site where enzymatic dehydrogenation enzymes are located (Baillee, Ferguson, and Hart, 1966). A positive formazan reaction indicates 3β-HSD activity which suggests synthesis of steroid hormones (Dorfman and Ungar, 1965; Weist and Kidwell, 1969).

Dey and Dickmann (1974) studied 3β-HSD activity in mouse PIE's and concluded that weak enzyme activity was already seen in 30% of 2-cell embryos. However, the activity declined, and disappeared shortly after implantation. Using the same histochemical technique, 3β-HSD was also found in rat embryos incubated in dehydroepiandrosterone (Dickmann and Dey, 1974a,b), in rabbit embryos (Dickmann, Dey, and SenGupta, 1975), and in hamster embryos (Dickmann and SenGupta, 1974).

The enzyme 17β-HSD catalyzes the interconversion of estrone and estradiol (see Figure 1). The enzyme's
activity in mouse PIE's was confined to the same developmental stages as was 3β-HSD (Dickmann, et al., 1976). The presence of 17β-HSD was also identified in vitro in rat PIE's (Dey and Dickmann, 1974) and in hamster PIE's (Dey and Dickmann, 1974) when 17β-estradiol was the substrate. Evidence has also been obtained for the presence or the aromatase, 17-20 desmolase, and 3-sulphatase enzyme systems within the pig blastocyst (Perry, Heap, and Amoroso, 1973; Flood, 1974).

It is assumed that quantitative changes in HSD activity reflect changes in synthesis of steroid hormones. Therefore, Dey and Dickmann (1974) have postulated that steroid hormones produced by the PIE's play a key role in the phenomena of morula to blastocyst transformation and implantation of the blastocyst.

Huff and Eik-Nes (1966) showed that rabbit blastocysts cultured in vitro could synthesize cholesterol and pregnenolone from acetate; moreover, when progesterone, pregnenolone, 17αhydroxypregnenolone, and androstenedione were used as substrates, they were converted to other steroids by the blastocysts (Dickmann, et al., 1975). By using a competitive protein binding assay, Seamark and Lutwak-Mann (1972) found that day-5 and day-6 rabbit blastocysts contain progesterone, 20αhydroxypregn-4-en-3-one, and 17αhydroxyprogesterone. This was repeated and
confirmed by Fuchs and Beling (1974). Dickmann, Dey, and SenGupta (1976) radioimmunoassayed for progesterone in rat and mouse blastocysts and found that each contained about 0.1 pg. Radioimmunoassays have also shown that day-6 rabbit blastocysts contain 17β-estradiol (Dickmann, et al., 1975). Recently, Dickmann, SenGupta, and Dey (1977) demonstrated that blastocyst estrogen played a key role in initiating the local increase in capillary permeability which is a prerequisite for implantation.

The activity of the 3β-HSD enzyme is essential in the early biosynthetic pathway of almost all the biologically active steroid hormones (Samuels, 1960), and the 17β-HSD enzyme is essential for estrogen synthesis (Dorfman and Ungar, 1954). Therefore, inhibition of either enzyme could implicate a more precise function of PIE steroids. A paucity of information exists on the effects of specific hydroxysteroid enzyme inhibitors on PIE development. However, an inhibitor of estradiol binding to its receptor protein, CI-628, implicates estrogen in PIE development (Callantine, Clemens, and Shih, 1969). Bhatt and Bullock (1974) found that the cytoplasm of blastocysts contained receptors for estrogen. SenGupta, Dey, and Dickmann (1977) reported that CI-628 did interfere with the "PIE estrogen" and blocked the development of the
mouse PIE's grown in vitro by inhibiting the transformation of morula to blastocyst. If this interpretation is correct, then the results demonstrate the significance of "PIE estrogen" for early embryonic development. The results also support the postulate that "PIE steroids" are necessary for morula to blastocyst transformation. Therefore, "PIE estrogen" is a crucial factor for the development of the PIE.

Despite these evidences of steroidogenic capabilities of the PIE, Sherman and Atienza (1977) were unable to detect conversion of pregnenolone to progesterone by mouse embryos prior to implantation by using radioimmunoassays. After trophoblast outgrowth, however, progesterone was produced. This suggests that the activities of enzymes, such as 3ß-HSD, which are involved in the metabolism of progestins and androgens, are not acquired or active until after implantation. Grube, Gwazdauskas, Lineweaver, and Vinson (1978) used radioimmunoassays for 17ß-estradiol and progesterone and did not detect the synthesis of these two steroids by mouse PIE's in a defined culture system.

This study will attempt to clarify the presence of conflicting results regarding PIE steroidogenesis. Specifically, the following study proposes to determine:

1) if mouse PIE's contain 3ß-HSD and 17ß-HSD by the use of
a histochemical enzyme stain, and 2) if PIE's are capable of synthesizing steroid hormones by using a radioactive precursor.
MATERIALS AND METHODS

Animal Care

Female Swiss Webster mice (Bio-Lab Corp.), 8-10 weeks of age, were kept in an animal room with a lighting regimen of 12 hours of light and 12 hours of dark. They were maintained on Purina Mouse Chow (Ralston-Purina Co.) ad libitum. An antibiotic drinking solution (Sulmet Drinking Solution, American Cyanamid Co.) was added to their drinking water at a dosage of 30 ml/3.8 liters water. This solution was given alternately with tap water at every other watering period.

Glassware and Reagents

All glassware used for embryo collection and culture was siliconized with Silicolad (Clay-Adams), rinsed in distilled water, and dried in an oven at 60°C. Triple distilled water and redistilled organic solvents were used throughout the experiments.

Ovulation Induction

Ova were recovered from hormone-treated females according to the methods of Fowler and Edwards (1957). Mice were induced to ovulate by an intraperitoneal (IP) injection of 10 international units (IU) of pregnant mares serum (PMS, Sigma Chemical Co.), followed 45 hours
later by an IP injection of 10 IU of human chorionic gonadotropin (HCG, Sigma Chemical Co.). Immediately after the HCG injection, the females were placed with a fertile male (see Figure 3). The presence of vaginal plugs in the females the following morning was used to indicate that mating had taken place. This was considered to be day one of pregnancy.

**Ova Recovery**

The techniques employed for ova recovery were those of Spears and Walker (1968). Approximately 48 hours post-HCG injection, the mice were sacrificed by cervical separation. The reproductive tract was exposed with a mid-ventral incision, and the oviducts were excised by cutting the uterotubal junction and the bursa at the ovarian end. The oviducts were rinsed in an embryological watch glass (A.H. Thomas) containing culture medium (see below). Ova were recovered by inserting a blunted 30-gauge needle, attached to a 2.5 ml syringe containing culture medium, into the fimbrial end of the oviduct and flushing the contents into another embryological watch glass.

The ova were manipulated by using a drawn out capillary tube inserted into a 15-gauge needle, which was attached to a 1 ml Hamilton Thumb Wheel Microsyringe
10 IU PMS

\[ \text{45 h} \]

\[ \rightarrow \]

10 IU HCG \( \text{PLACED WITH MALE} \)

\[ \text{12 h} \]

\[ \rightarrow \]

OVULATION

\[ \text{37 h} \]

\[ \rightarrow \]

2-CELL STAGE

\[ \text{12 h} \]

\[ \rightarrow \]

4-CELL STAGE

\[ \text{10 h} \]

\[ \rightarrow \]

8-CELL STAGE

\[ \text{5 h} \]

\[ \rightarrow \]

MORULA (16 AND 32-CELL STAGES)

\[ \text{10 h} \]

\[ \rightarrow \]

BLASTOCYST

Figure 3. Time sequence of hormone injections and PIE cleavages.
All manipulations and observations were made with the aid of a stereomicroscope at 20X magnification.

Culture Medium

The culture medium was prepared according to the methods of Spears and Walker (1968) from modifications of Brinster (1963).

A stock culture medium, which was prepared by adding 10 ml of Earle's Balanced Salt Solution (EBSS, Grand Island Biological Co.) diluted to 100 ml with water, was supplemented with 0.2 ml of 60% sodium lactate (Fisher Scientific Co.), 1.0 ml of a 0.3% solution of sodium pyruvate (Grand Island Biological Co.) prepared in a 10-fold dilution of EBSS in water, 100 mg bovine plasma albumin (BPA, Grand Island Biological Co.), 10 mg penicillin-G (Sigma Chemical Co.), and 5 mg streptomycin sulfate (Nutritional Biochemical Corp.). Prior to use, 0.85 ml of a 1.3% solution of sodium bicarbonate (Grand Island Biological Co.) was added to 10 ml of the stock medium and the pH was brought within pH 6.8-7.2 by gassing with 5% CO₂ in air (Brinster, 1963).
Ova Culture

Two milliliters of light weight paraffin oil (Saybolt Viscosity 125/135, Fisher Scientific Co.) were added to the center well of an organ culture dish (15x60, Falcon Plastics) and stored approximately 1 hour in a CO₂ incubator (#528, Hotpack CO₂ Refrigerated Incubator). Thirty minutes before use, 0.1 ml of the culture medium was placed in the paraffin oil to allow equilibration.

Within 10 minutes after the ova were recovered from the oviducts, they were handled individually using a Hamilton Thumb Wheel Microsyringe, and deposited into the culture medium, under the paraffin oil in the organ culture dish, and placed in the incubator. The incubator had a plexiglass chamber that allowed for retention and re-equilibration of the CO₂ and air when the door was opened. The flow of CO₂ and air was controlled by standard flow meters on the incubator.

The ova were removed from the incubator shortly after their respective cleavage times (see Figure 3) and utilized for various experiments.

Histochemical Enzyme Determinations

The determination of Δ⁵-3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) activity was made according to the methods of
Deane, Rubin, Driks, Lobel, and Leipsner (1962). Ova were transferred to 0.5 ml of freshly made stain medium, which was prepared as follows: 1.8 mg dehydroepiandrosterone (DHEA, Sigma Chemical Co.) dissolved in 0.25 ml of acetone (Fisher Scientific Co.) and later removed by evaporation, 4.0 mg nicotinamide adenine dinucleotide (NAD, Sigma Chemical Co.), 2.0 mg Nitro-Blue Tetrazolium (Nitro-BT, Sigma Chemical Co.), and 9.6 ml of 0.1 M phosphate buffer (pH 7.5). The DHEA was eliminated for controls. The ova were then incubated for 3 hours to allow for formazan deposition in cells in which the enzyme was present. Following the incubation, the ova were examined under a compound microscope using bright light for the best visualization of the formazan granules.

The same methods were employed for 17β-HSD as were for 3β-HSD except 17β-estradiol (Sigma Chemical Co.) was used in place of DHEA.

Steroidogenesis Determination

Five lambda (λ) of pregnenolone [7-3H(N)] (New England Nuclear, Specific Activity 17.2 Ci/mmmole) in absolute methanol was added to the ova in a 25 ml Erlenmeyer flask (Kimble Products) in order to determine the steroidogenic activity of the PIE's. Culture media (0.5 ml) was added to the flask containing ova. The ova
were then incubated for 2 hours in a Dubnoff Metabolic Shaking Incubator (GCA Precision Scientific) at 37°C under an atmosphere of 95% O₂ and 5% CO₂, with 40 shaking oscillations per minute. One milliliter of dichloromethane (Fisher Scientific Co.) was added to the flask in order to terminate the incubation and extract the steroids. The entire flask contents were poured into a 12-ml conical centrifuge tube (Kimble Products), which contained 50 μl of testosterone, estradiol, pregnenolone, and progesterone. The flask was again rinsed with 1 ml of dichloromethane and this rinse was added to the contents of the centrifuge tube. The centrifuge tube was gently vortexed (Vortex-Genie, Scientific Industries, Inc.) for 30 seconds and centrifuged for 10 minutes at 1820 rpm at room temperature in a clinical centrifuge (Precision Scientific). The aqueous layer was aspirated and discarded. The centrifuge tube was placed in a water bath and the organic phase was dried down under a stream of air.

Thin Layer Chromatography

Thin layer chromatography (TLC) plates were coated with Silica Gel according to Stahl (type 60, EM Laboratories, Inc.) by using 35 g of Silica Gel to 70 ml of water. These plates were allowed to air dry at least
24 hours prior to their use. The plates were heat activated in an oven at 50°C for 1 hour before spotting.

To each centrifuge tube containing the dried dichloromethane, steroids were taken up in 3 drops of acetone and were spotted on TLC plates. This step was repeated twice with an additional 3 drops of acetone. Pooled steroid standards, 50μl of testosterone and estradiol and 100μl of pregnenolone and progesterone (1 mg/1 ml acetone) were spotted in lanes parallel to the samples. The plates were chromatographed twice in an organic system containing dichloromethane:ether (98:2). The plates were sprayed with 50% H₂SO₄ and the steroids were visualized after being heated for 15 minutes at 100°C. An area representing each steroid was scraped with a razor blade into a scintillation vial (Fisher Scientific Co.). To each vial was added 10 ml of scintillation fluid containing 19.0 g 2,5-diphenyloxazole (PPO-scintanalyzed, Fisher Scientific Co.) and 1.9 g 1,4-bis (5-phenyloxazolyl) benzene (POPOP-scintanalyzed, Fisher Scientific Co.) added to 3.8 liters of toluene. The vials were counted by using a scintillation counter (Model 2009, Packard).
RESULTS

The recovery of mouse PIE's from hormone injected female mice produced from 10-80 viable embryos per mouse. The PIE's were either used for experiments immediately after recovery, or cultured in vitro until the specific embryonic stage was reached for experimental studies.

The results of 3β-HSD formazan deposition in PIE's as determined by the histochemical enzyme stain reaction, are summarized in Table 1. Concurrent with the above experiments, control PIE's were incubated in substrate-free media. The controls are also indicated in Table 1. In PIE's, formazan deposition was scored as follows: no deposition (0), and three increasing grades of positive (blue) deposition, (+, ++, +++). In all cases, positive reactions were confined to the blastomeres, i.e., there were no formazan granules in either the perivitelline space or the zona pellucida. It can be seen in Table 1 that 3β-HSD activity was present in PIE's as early as the 2-cell stage. The enzyme 3β-HSD was histochemically detectable at all the PIE cell stages, from 2-cell to blastocyst, with variations in intensity of deposition. The enzyme's activity appeared strongest at the morula stage, and weakest at the blastocyst stage, with only weak to moderate deposition at 2, 4, and 8-cell stages.
Table 1. 3β-HSD activity in PIE's as determined by a histochemical enzyme stain.
The results of the histochemical enzyme determination of 17β-HSD deposition are presented in Table 2. Formazan deposition was scored the same in 17β-HSD PIE's as it was in 3β-HSD PIE's. The evidence presented in Table 2 indicates that 17β-HSD was also weakly present in PIE's as early as the 2-cell stage, and that all stages showed formazan deposition. The presence of 17β-HSD induced formazan deposition appeared to be the most intense at the morula stage, and the least intense at the blastocyst stage, results directly comparable to 3β-HSD data. Again, 4 and 8-cell PIE deposition was only weak to moderate.

Table 3 shows the percentage of PIE's, of the total observed, showing 3β-HSD and 17β-HSD deposition at each level of deposition. These data reflect moderate PIE deposition at the 2-cell stage for 3β-HSD (85%), compared to weak deposition for 17β-HSD (98%). The most intense deposition occurred at the morula stage, with 78% of the PIE's showing moderate to strong deposition for 3β-HSD and 71% showing moderate to strong deposition for 17β-HSD. Deposition declined at the blastocyst stage, as evidenced by 92% of the 3β-HSD blastocysts, and 96% of the 17β-HSD blastocysts, being either negative or only showing weak enzymatic deposition. Weak to moderate
### Formazan Deposition

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<td>0</td>
<td>28</td>
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<tr>
<td>Control</td>
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<td>0</td>
<td>0</td>
<td>24</td>
</tr>
</tbody>
</table>

*aControl had substrate excluded from stain media.

0 = no deposition  
+ = weak blue coloration  
++ = moderate blue coloration  
+++ = strong blue coloration

**Table 2.** 17β-HSD activity in PIE's as determined by a histochemical enzyme stain.
### 3β-HSD Formazan Deposition

<table>
<thead>
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<th>++</th>
<th>+++</th>
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<td>48</td>
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<td>4</td>
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### 17β-HSD Formazan Deposition

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</tbody>
</table>

Table 3. Percentage of PIE's (of the total PIE's) showing 3β-HSD and 17β-HSD deposition.
3β-HSD deposition occurred in 82% of 4-cell PIE's and 79% of 8-cell PIE's. These data can be compared to 79% of 4-cell PIE's and 68% of 8-cell PIE's showing weak to moderate deposition of 17β-HSD.

To determine if mouse PIE's were capable of synthesizing steroid hormones from a radioactively labelled precursor, 3H-pregnenolone was incubated with embryos at various stages. The results of the conversion of 3H-pregnenolone into steroid hormones are presented in Figures 4 and 5. It can be seen from Figure 4 that 2 and 4-cell PIE's demonstrate a lower percentage of total cpm (counts per minute) for estrogen (17% and 20%) than for the controls (26%). These results indicate that there is no synthesis of estrogen by the 2 and 4-cell PIE's. The results of the conversion of 3H-pregnenolone to steroid hormones in 8-cell PIE's and morula are presented in Figure 5, along with the controls. There is no significant synthesis of estrogen in 8-cell PIE's or morula when compared to the controls. In all the experimental cell stages there was either negligible or no synthesis of progesterone and testosterone.
Figure 4. Conversion of $^{3}$H-pregnenolone to steroid hormones in 2 and 4-cell PIE's.
Figure 5. Conversion of $^3$H-pregnenolone to steroid hormones in 8-cell PIE's and morula.
DISCUSSION

The maintenance of early mammalian pregnancy is not completely understood. It is widely accepted that the ovaries are a source of estrogen and progesterone required for the maintenance of early pregnancy (Robson, 1938; Hall and Newton, 1947). A possible second source of steroid hormones involved in early pregnancy is the preimplantation embryo (PIE) (Dickmann and Dey, 1973). Whether or not the PIE's do synthesize steroid hormones is still to be determined.

Since 3β-HSD is a key enzyme in the metabolism of steroid hormones (see Figure 1), its presence in cells constitutes strong evidence for steroidogenesis (Weist and Kidwell, 1969). In this study, histochemical enzyme deposition was found to begin at the 2-cell stage and gradually increase until the morula stage, and then slightly decline at the blastocyst stage (see Table 1). The histochemical findings of this study are interpreted to indicate that in mouse PIE's, the key enzyme for steroid hormone synthesis is present, suggesting steroid hormone production.

Dey and Dickmann (1974) studied 3β-HSD activity in mouse PIE's and concluded that weak enzyme deposition was already seen in 30% of 2-cell embryos. However, the
presence of 3β-HSD declined slightly at the blastocyst stage, then sharply declined, and disappeared shortly after implantation. The present study indicates similar data, but at a much higher percentage, 85% deposition at the 2-cell stage. Formazan deposition peaked at the morula stage and declined at the blastocyst stage, results similar to Dey and Dickmann (1974). Using the same histochemical technique with dehydroepiandrosterone as the substrate, 3β-HSD was also found in rat embryos (Dickmann and Dey, 1974a,b), in rabbit embryos (Dickmann, et al., 1975), and in hamster embryos (Dickmann and SenGupta, 1974). Accordingly, the results presented in Table 1, add support to Dickmann's hypothesis that PIE's possess the key enzymes for steroid synthesis.

The interconversion of estrone and estradiol is catalyzed by the enzyme 17β-HSD (see Figure 1). This interconversion can occur during either the degradation or synthesis of estrogen. Since 17β-HSD was present in conjunction with 3β-HSD, Dickmann, et al., (1975) suggested that estrogen synthesis was occurring in the PIE's. Dickmann, et al., (1976) found that the presence of 17β-HSD in mouse PIE's was confined to the same developmental stages as was 3β-HSD. The presence of 17β-HSD was also identified in vitro in rat PIE's (Dey and Dickmann, 1974) and in hamster PIE's (Dey and Dickmann,
1974) when 17β-estradiol was the substrate. Evidence has also been obtained for the presence of the aromatase, 17-20 desmolase, and 3-sulphatase enzyme systems within the pig blastocyst (Perry, Heap, and Amoroso, 1973; Flood, 1974). Data presented in this study show 17β-HSD deposition to be confined to the same embryonic cell stages as 3β-HSD (see Table 2).

Seamark and Lutwak-Mann (1972) have shown that blastocysts contain progesterone, 20α-hydroxypregn-4-en-3-one, and 17α-hydroxyprogesterone. Huff and Eik-Nes (1966) showed that rabbit blastocysts cultured in vitro can synthesize cholesterol and pregnenolone from acetate; moreover, when progesterone, pregnenolone, 17α-hydroxy-pregnenolone and androstenedione were used as substrates, they were converted by the blastocysts to other steroids, namely 5β-pregnanedione and 20α-dihydroprogesterone.

Despite these evidences of steroidogenesis in PIE's, Sherman and Atienza (1977) were unable to detect conversion of pregnenolone to progesterone by radioimmuno-assays (RIA's) in mouse embryos prior to implantation. Grube, et al., (1978) used RIA's for 17β-estradiol and progesterone and did not detect synthesis of these two steroids by mouse PIE's in a defined culture system. Investigations in this lab involving the conversion of
a radioactive precursor ($^3$H-pregnenolone) to steroid hormones indicates that steroidogenesis does not occur in mouse PIE's cultured in vitro (see Figures 4 and 5).

It can be concluded from the data presented in this paper that the enzymes necessary for steroidogenesis are present in the mouse PIE's, but the PIE's are not actively synthesizing steroid hormones from $^3$H-pregnenolone. However, the possibility exists that steroid hormones could have been synthesized, but were beyond the levels detectable by this assay system. Additionally, it is possible that $^3$H-pregnenolone did not enter into the blastomeres of the PIE's, therefore it could not be converted into other steroid hormones. Another possible reason conversion of $^3$H-pregnenolone did not occur could be that a specific factor or cofactor required in vivo was not present in vitro. It may also be possible that conversion would occur if other precursors were used. Consequently, further experimentation is necessary in order to determine the possible steroidogenic capabilities of mouse PIE's.

Experimental studies in the future could possibly include: 1) radioimmunoassays of pregnenolone, 2) use of additional radioactive precursors, such as progesterone, 17α-hydroxypregnenolone, 17α-hydroxyprogesterone,
dehydroepiandrosterone, androstenedione, or testosterone, and 3) incubation of PIE's with specific enzyme inhibitors, i.e., trilostane, cyanoketone, or danazol.
LITERATURE CITED


Frankel, L. 1903. Archives für Gynäkologie. 68, 438.


