IN VITRO CHARACTERIZATION OF AROMATASE, ESTRONE SULFOTRANSFERASE AND ESTRONE SULFATASE ACTIVITIES IN THE PORCINE PLACENTA AND ENDOMETRIUM AT 30, 60 AND 90 DAYS OF GESTATION

by

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IN VITRO CHARACTERIZATION OF AROMATASE, ESTRONE SULFOTRANSFERASE AND ESTRONE SULFATASE ACTIVITES IN THE PORCINE PLACENTA AND ENDOMETRIUM AT 30, 60 AND 90 DAYS OF GESTATION

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(ABSTRACT)

The purpose of this investigation was to characterize the activities of three enzymes, aromatase, estrone (E_1) sulfotransferase and E_1 sulfatase, in the porcine placenta and endometrium on d 30, 60 and 90 of gestation. These enzymes play key roles in determining in utero concentrations of estrogens. Days 30, 60 and 90 were chosen because previous investigations had determined that these were times of substantial changes in in vitro estrogen production by the porcine placenta and endometrium.

Each enzyme assay was performed in separate tissue culture systems, and the level of enzyme was determined by measuring product formation from tritium-labelled (^3H) substrates provided in the incubation medium. Aromatase activity was determined by the formation of ^3H_2O and estradiol from ^3H-testosterone (T). E_1 sulfotransferase activity was determined by the formation of ^3H-estrone sulfate (E_1SO_4) from ^3H-E_1. Estrone sulfatase activity was

determined by the formation of $^{3}H\text{-}E_1$ from $^{3}H\text{-}E_1SO_4$. Concentrations of T, E_1SO_4 and E_1 in plasma collected from the anterior vena cava (AVC), uterine artery (UA) and uterine vein (UV) were determined by radioimmunoassay.

There were no significant differences in mean enzyme activity of the placenta or the endometrium among the three days of gestation examined. Endometrial aromatase activity was twice that of placental aromatase on all three days that were examined. The ratio of placental to endometrial sulfotransferase activity underwent a reversal from 1:1.3 on d 30 to 2.3:1 on d 60 and 90. The ratio of placental to endometrial sulfatase activity changed from 1:1.6 on d 30 to 1:2.2 on d 60 and 1:3.0 on d 90. There were significant differences in response of enzyme activity to extended periods (3 h) in incubation which varied with tissue type, gestational age and enzyme of interest. Overall, placental enzymes did not respond consistently to increased time in incubation with increased product formation. Endometrial aromatase activity on all three days and endometrial sulfatase activity on d 90 responded to increased time in incubation with increased product formation. In vivo, the uterus appears to be involved in T, E_1 and E_1SO_4 production since UV steroid concentrations were greater than those of the UA. The E_1SO_4 to E_1 ratio of the UV decreased from 15:1 on d 30 to 4:1 on d 60 and 1:2 on d 90.

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INTRODUCTION

A major interest of agricultural sciences is to increase production without sacrificing economic efficiency. In order to apply the rapid advances in scientific technology to the animal sciences, it becomes increasingly important to define the basic physiological parameters of the animal system in question. As researchers undertake the quest to discover methods for increasing litter size at term in swine, it becomes imperative that the fundamentals of gestational physiology be elucidated. The system under scrutiny includes the endometrium, the placenta and the fetus. Each of these components plays a critical role in the development and maintenance of the other two. It remains a puzzle as to why, within any given swine pregnancy , some conceptus units will develop and function synergistically to yield healthy, viable fetuses at the end of the 114 d gestation period while other conceptus units fail and fetal death results. However, pieces of this complex puzzle are falling into place as more information is uncovered on the conceptus and how it functions with the endometrium as a system.

Estrogens, particularly estrone and estradiol, are influential throughout gestation. Their role in maternal recognition of pregnancy and placental expansion has been firmly established. Their roles in other aspects of placental and endometrial function and fetal development are being intensively investigated. Because ovariectomy of the pig at any stage of gestation results in loss of the whole litter, it was believed that progesterone of corpora lutea origin was necessary, not only for maintenance of pregnancy but also as a precursor for estrogen synthesis. Investigations of porcine placental and endometrial tissue, in vitro, revealed that

these tissues were capable of de novo synthesis of progesterone and estrogen (Kukoly, 1984; Jeantet, 1985). The capacity of the blastocyst to produce estrogens has been firmly established (Perry et al., 1973; Flint et al., 1979; Gadsby et al., 1980; Heap et al., 1981ab).

Thus, it is documented that estrogens play many important roles throughout gestation and that local, in utero, estrogen production is possible. The mechanisms of in utero estrogen pruduction now require elucidation. Robertson and King (1974) reported that estrone sulfate was the primary estrogen in plasma during early gestation. Estrone becomes the primary estrogen during the latter third of gestation in swine. Estrone sulfate is formed by sulfotransferase, an enzyme which conjugates estrone with a sulfate group. Estrone sulfate is well known as an excretable, water-soluble form of estrogen; however, it also forms a biological reservoir of estrogen in the blood stream. Estrone sulfate has a longer half-life than estrone and will not bind to estrogen receptors. Estrone sulfate must lose the sulfate group via hydrolysis by the enzyme sulfatase before it can exert an effect on estrogen-sensitive target tissues. Two enzymes, sulfotransferase and sulfatase, control the supply of biologically active estrogens by regulating the concentrations of sulfoconjugated estrogens relative to free estrogens. Aromatase is the enzyme system involved in generating estrogens from androgens. It is these three enzymes: aromatase, sulfotransferase and sulfatase, which play a major role in determining estrogen concentrations available to the endometrium, placenta and fetus.

The purpose of this project was to characterize the in vutro activities of aromatase, sulfotransferase and sulfatse of the porcine placenta and endometrium on

d 30, 60 and 90 of gestation. These three days of gestation were chosen because previous investigations have shown them to be times of substantial changes in in vitro estrogen production by the porcine placenta (Kukoly, 1984; Jeantet, 1985) and endometrium (Jeantet, 1985). Plasma concentrations of testosterone, estrone and estrone sulfate of the anterior vena cava, uterine artery and uterine vein were determined to provide some basis for comparison of vitro enzyme activity to the in vivo condition.

REVIEW OF LITERATURE

Porcine Placental Development and Function

Placental Development

The porcine placenta is classified as a diffuse epitheliochorial type (Grosser, 1909). The epitheliochorial placenta constitutes the most complete morphological barrier, consisting of six tissue layers separating maternal and fetal circulatory systems. From the maternal to the fetal system, these six layers are: 1) endometrial capillary epithelium; 2) endometrial connective tissue; 3) endometrial epithelium; 4) chorioallantoic epithelium; 5) fetal connective tissue and 6) chorioallantoic capillary epithelium. In comparison to placentae of other domestic species, the porcine placenta contains a larger amount of Wharton's Jelly, which is an edematous, gelatinous mesenchyme found between trophoblastic ectoderm and endoderm of the placenta through which allantoic vessels course (Marrable, 1968) and found surrounding the umbilical cord. Wharton's Jelly is most prominent in early gestation (Mossman, 1987). Hyaluronic acid is a major component of Wharton's Jelly which decreases the barrier offered by cellular and other membranes to the diffusion of material across the placenta. In sheep, it has been demonstrated that dye-linked albumin passes freely along the umbilical cord in Wharton's Jelly toward the abdomen of the fetus, constituting an alternate pathway of substrate transport from placenta to fetus (Barcroft et al., 1944). Atrophic ends, or necrotic tips on each conceptus unit, are another unique feature of the porcine placenta (Mossman, 1987) which will be discussed in greater detail in a subsequent

section on placental morphology.

Prior to development of a functional placenta, the zygote and early blastocyst are dependent upon the yolk sac to provide energy and substrates for continued cell growth and division (Patten and Carlson, 1974). In the pig, as in all higher mammals, virtually no yolk is accumulated in the ovum; however, the yolk sac is lined with an abundance of functional blood vessels which are in close proximity to the maternal endometrium. These blood vessels absorb components of histotrophe and make them available to the embryo (Patten and Carlson, 1974). Histotrophe, which is secreted by endometrial glands, thereby plays a significant role in conceptus nourishment, not only during blastocyst formation, but throughout gestation (Bazer et al., 1979). Components of histotrophe include: uteroferrin, a carrier protein for iron having acid phosphate activity (Roberts and Bazer, 1976; Knight, 1974; Bazer, 1975; Chen, 1975); a retinal-carrier protein (Adams et al., 1981); proteolytic enzymes such as leucine aminopeptidase and cathepsin B, D and E (Roberts et al., 1976); bacterial enzymes, such as lysozyme; and enzymes involved in carbohydrate metabolism, such as phosphohexose isomerase (Bazer, et al., 1977).

Placentation in the pig involves the development of an interface between maternal and fetal circulatory systems allowing for efficient exchange of blood borne nutrients, gases and hormones while maintaining a physical barrier between the two systems. Placentation begins with the initial attachment of the trophoblast layer of the blastocyst to the maternal endometrium on approximately d 13 of a 114 d gestation period. Substantial regions of the trophoblast can be seen lying very close to and following the contours of the maternal epithelium (Crombie, 1970). These

contours or folds of the endometrium are known as rugae. By d 18, the epitheliochorial placenta has formed by interlocking microvilli of the trophoblast and the endometrium (Crombie, 1970; Perry, 1981). As pregnancy advances, the opposing surfaces of the placenta and the endometrium are thrown in primary and secondary interlocking folds which, together with the microvilli, are sufficient to keep the two surfaces attached (Crombie, 1970). Fetal blood pressure in conjunction with tissue lymph may be important in maintaining placental apposition in the pig by distention of the extremely vascular folds (Amoroso, 1952). Placentation in the pig is noninvasive under normal, in utero conditions (Amoroso, 1952; Crombie, 1972). Evidence exists suggesting some factor within the porcine uterus may determine the degree of invasiveness of the trophoblast through an inhibitory influence. If the pig embryo is transferred to an ectopic site, it forms syncytial masses which actively invade surrounding tissues by cytosis and phagocytosis (Samuel and Perry, 1972).

Placental development is closely associated with blastocyst development; therefore, in order to describe placental development, it is necessary to begin with blastocyst development. Embryos enter the uterine horn from the oviduct approximately 48 h after ovulation at about the four-cell stage (Dzuik, 1985). The blastocysts hatch from a non-cellular coat, the zona pellucida, on approximately d 6 to 8 of gestation and begin their migration throughout both uterine horns (Dzuik, 1985). The blastocyst cells at this time can be characterized as being within one of two regions (Heuser and Streeter, 1929). The inner cell mass of the blastocyst develops into the embryo proper, the amnion, the allantois and the mesodermal lining of the chorion. The trophoblast, comprising two-thirds of the total cell number of

the blastocyst, develops into the chorion (Steven and Morris, 1975). The blastocysts undergo dramatic and well documented morphological changes between d 10 and 16 of gestation (Heuser and Streeter, 1929; Perry and Rowlands, 1962; Crombie, 1972; Anderson, 1978; Geisert et al., 1982b). The most accelerated period of blastocyst elongation is between d 10 and 12 when the blastocyst changes from a spherical form of 3 to 10 mm in diameter to a tubular form of 10 to 50 mm in length to a filamentous form of greater than 100 mm in length. Following the initiation of blastocyst elongation and placentation at approximately d 13, the blastocyst can no longer migrate (Dzuik, 1985). By d 16 of gestation, the blastocyst may be 700 to 1000 mm in length (Perry and Rowlands, 1962; Anderson, 1978). The rapid increase in size of the spherical blastocysts is the result of cellular hyperplasia (Geisert et al., 1982b). The morphological changes of the blastocyst from a spherical to a filamentous form are due to cellular remodelling and not hyperplasia (Geisert et al., 1982b). Blastocyst elongation involves surface and ultrastructural changes of both the trophectoderm and endoderm, occuring by migration or condensation of trophoblastic cells into the region of the embryonic pole. This migration forms what is known as the elongation zone. Once the elongation zone is established, when the blastocyst is approximately 10 mm in diameter, alterations in microfilaments and junctional complexes of trophoblast cells and extension of filapodia from endodermal cells allow for the reduction in diameter and rapid increase in length of the blastocyst as elongation proceeds (Geisert et al., 1982b). The elongation of the trophoblast enables the embryo to acquire a larger area of uterine space and endometrial surface contact resulting in more surface area through

which the blastocyst can absorb histotrophe before and after placentation is complete.

The porcine placenta is composed of three tissue layers: the amnion, the allantois, and the chorion (figure 1). The allantois appears on approximately d 14, within the filamentous blastocyst, as a diverticulum of the fetal hind gut. By d 17, the allantois is as long as the embryo (Friess et al., 1980). The allantois remains attached to the fetal bladder by way of the urachus, which runs through the umbilical cord, for most of fetal life (Renegar et al., 1982). The allantois is composed of embryonic mesoderm, also know as splanchnopleure. The allantois acquires an early abundant blood supply via large branches of the caudal aorta. These branches further branch into a plexus of fenestrated capillaries within the allantoic wall. The allantoic circulatory system becomes more developed and replaces the yolk sac circulatory system as the primary source of nutrients for the developing embryo (Patten, 1948).

The amnion is the inner most layer of the placenta, surrounding the embryo proper. The amnion arises as a layer of somatopleure which enfolds the developing embryo. Briefly, it develops following settling of the embryonic disk into the blastocyst, as folds of the extra-embryonic somatopleure become more voluminous and fuse above the mid-dorsal region of the embryo (Patten, 1948). Formation of the amnion is complete by d 18 of gestation (Friess et al., 1980).

On d 19 of gestation, the allantois makes physical contact with the trophoblast, which is now known as the chorion. By d 30, the chorion has become extensively vascularized by the blood vessels of the allantois (Steven and Morris, 1975). The

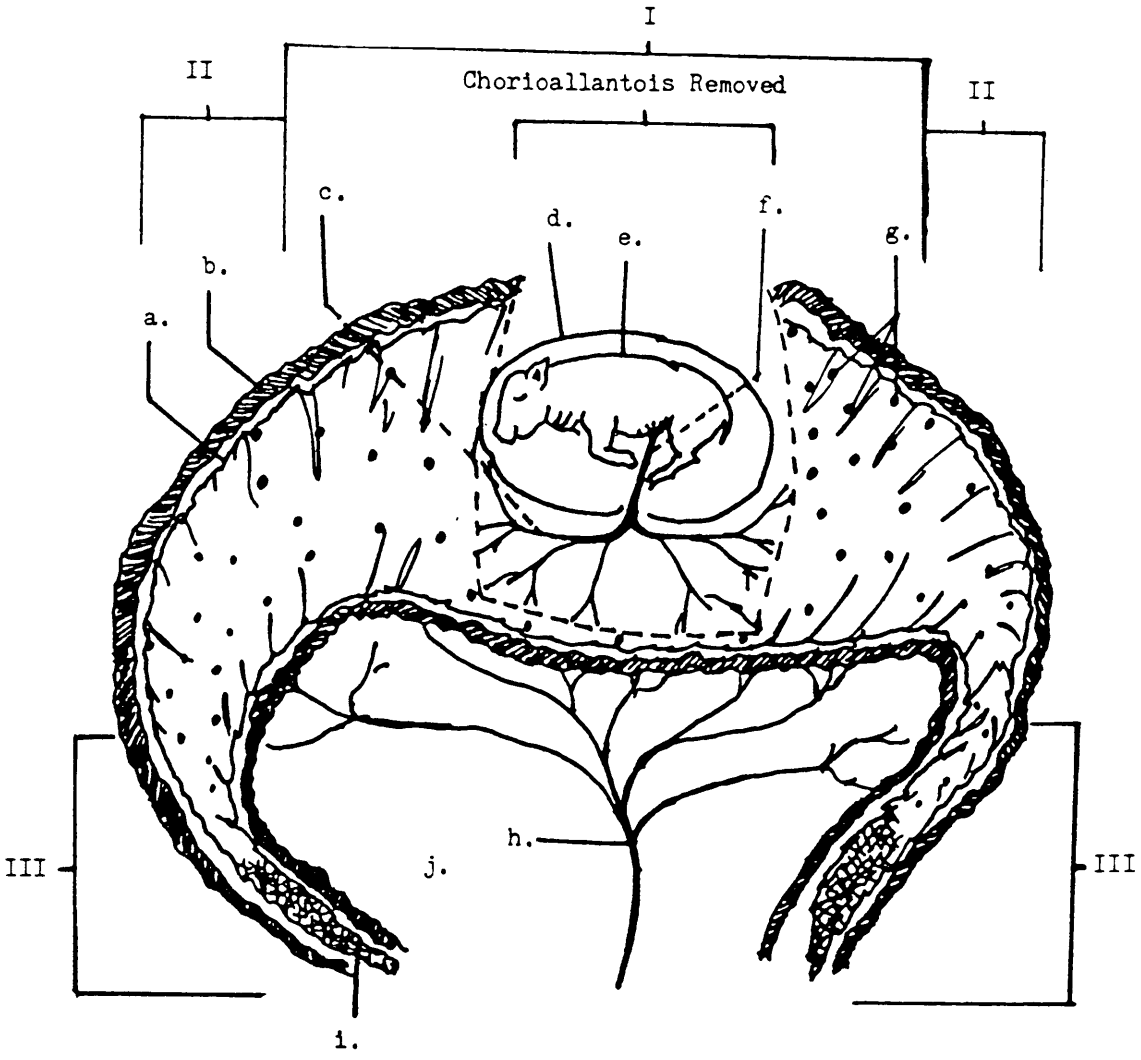

Figure 1. Fetal Membranes of the Pig.

a. Chorioallantois
b. Endometrium
c. Fetal arteries and veins
d. Amnion
e. Fetus
f. Umbilicus
g. Areolae
h. Maternal arteries and veins
i. Necrotic tip
j. Broad ligament

I. Placental zone
II. Paraplacental zone
III. Avascular extremeties

allantois does not extend to the extremities of the chorion, and these areas degenerate, becoming necrotic tips (Steven and Morris, 1975). The chorionic and allantoic layers of the placenta, considered to be fused at this time, are referred to as the chorioallantois. The chorioallantois becomes the area of placenta forming the interface for metabolic exchange between the maternal and fetal systems for the remainder of gestation (Patten, 1948). Between d 30 and 40 of gestation, the terminal branches of the allantoic vessels regress, decreasing blood flow to the extremities of the chorion (Steven and Morris, 1975). By d 40, the chorioallantois can be divided, morphologically, into three distinct areas: the avascular extremities, two paraplacental zones and the centrally-located, large placental zone (Steven and Morris, 1975; figure 1).

The two necrotic tips, ischaemic zones or "diverticular allantoidis" which form the extremities of the conceptus are well formed by mid-gestation (d 50 to 60) (Ashdown and Marrable, 1967). At this time, the chorion requires a greater vascular supply than was necessary earlier in gestation, to support the metabolic activity associated with fetal growth (Amoroso, 1952). The surfaces of the placental and paraplacental zones and the necrotic tips meet abruptly at a transverse annular constriction beyond which the allantoic vessels are no longer functional (Marrable, 1968). The walls of the necrotic tips are composed of superimposed collagenous lamellae (Marrable, 1968). It is possible, for a time at least, that the necrotic tips prevent the malformative dangers which are known to arise from interfetal vascular anastomoses (Hughes, 1929). Since ischaemia develops at the ends of the sacs nearest the ovaries, it probably does not arise by mutual induction between the

neighboring membranes, but is either an intrinsic property of each conceptus or the result of interaction with the uterine lining.

On d 30 of gestation, minute white disks become morphologically apparent on the chorioallantois (Brambell, 1933). In cross-section, they appear as dome-shaped formations covering the mouths of endometrial glands (Friess et al., 1981). These specialized structures of the chorioallantois are known as areolae; their function is absorption of secretions (histotrophe) of the endometrial glands (Chen, 1975; Knight et al., 1977; Friess et al., 1980; Roberts and Bazer 1980; Friess et al., 1981). Areolae are initially found in greatest concentration in the large placental zone of the chorioallantois. As gestation progresses, more areolae develop in the paraplacental zones. By d 50, there is no significant difference in areolae concentration in these areas (Brambell, 1933; Knight et al., 1977). The number of areolae increases until approximately d 50 of gestation and remains constant until term; total areolar surface area increases rapidlly until d 50, then increases slowly until d 100 (Knight et al., 1977).

By d 30 of gestation, the chorioallantois and the amnion, placental components which will support the fetus for the remainder of gestation, have been established. However, the placenta is a dynamic organ and, with the endometrium will adjust functionally and morphologically to the changing needs of the developing fetus as gestation progresses. By d 58 of gestation the folds of the chorioallantois, important for greatly increasing placental surface area, are well developed and will remain so throughout gestation (Friess et al., 1981). The porcine chorioallantois can be defined for histological purposes as being composed of two regions, the areolar and

interareolar regions. The interareolar regions can further be defined as being composed of two areas with different histological characteristics: the troughs or bases of the chorioallantoic folds, and the lateral walls and summits of the chorioallantoic folds (Friess et al., 1980). The troughs of the chorioallantoic folds are in apposition to the summits of the endometrial rugae or folds; likewise, the summits of the chorioallantoic folds are in apposition to the troughs of the rugae (Friess et al., 1980). The thickness of the porcine placenta decreases from d 30 to 110 of gestation due to alterations in the degree of indentation of both chorioallantoic and endometrial epithelia by their respective capillary networks (Crombie, 1970; Friess et al., 1980). This indentation is limited to the lateral walls and summits of the chorioallantoic folds. The bases of the chorioallantoic folds as well as the endometrial epithelium remain high columnar throughout gestation (Friess et al., 1980). In the region of the summits and lateral walls of the chorioallantois, the effective placental barrier between maternal and fetal circulatory systems is approximately 40 um on d 30 of gestation. At term, the effective placental barrier has been reduced to 2 um or less (Friess et al., 1980). Decreased placental thickness along with increased intrauterine blood flow are adaptations promoting efficient substrate transport to a rapidly growing fetus with increased substrate requirements.

A brief description of the histological changes occurring within the chorioallantois and the endometrium as gestation progresses has been given by Friess et al. (1980,1981) and is summarized here in order to give a measure of histological basis for changes in placental and endometrial functional capacities

occurring as gestation progresses. On d 30 of gestation, the chorioallantoic epithelium is high simple columnar ranging from 20 um on the lateral walls and summits to 40 um in the troughs of the folds. Endometrial cell height is relatively static throughout gestation at approximately 20 um. The apical surface of the chorioallantoic epithelial cells has numerous long microvilli which interdigitate with those of the endometrial epithelial cells. The chorioallantoic epithelial cells possess basal nuclei rich in euchromatin associated with many short strands of rough endoplasmic reticulum and dense bodies of varying size. These cells contain elongated mitochondria with lamellar cristae, a number of small vesicles between the mitochondria, and an irregularly shaped vacuole beneath the nucleus. Neighboring cells share well-developed tight junctions on their apical lateral borders and show a high degree of interdigitation between their lateral membranes. Capillaries of the chorioallantois are separated from the chorioallantoic epithelial cells by surrounding mesoderm and by the well-developed basal lamina of the epithelial cells. Day-30 endometrial epithelial cells frequently have pinocytic invaginations and numerous small vesicles beneath their microvilli. Like those of the chorioallantois, these cells contain many mitochondria with lamellar cristae distributed throughout the cell. They also contain a well-developed rough endoplasmic reticulum, a small golgi apparatus and a few lysosomes of varying sizes; their nucleus by contains evenly-dispersed chromatin. Finger-like processes project from neighboring cells into intercellular channels which form between the lateral and basal membranes of neighboring cells. Maternal capillaries are in close proximity to the epithelial layer, separated by a thin layer of connective tissue.

On d 58 of gestation, the fetal capillaries deeply indent the chorioallantoic epithelium on the summits and lateral walls of the folds; the basal lamina of the capillaries and epithelia usually fuse forming a single lamina. Overall, epithelial cell height has decreased . In areas where capillaries protrude into the epithelium, cell height is reduced to 2 um or less. In contrast to d 30, the d-58 chorioallantoic epithelial cells contain many mitochondria with transverse cristae, and the rough endoplasmic reticulum is concentrated lateral to the nuclei in parallel arrays. These cells contain vacuoles of varying sizes, many electron dense granules and many pinocytic vesicles beneath the microvilli. Endometrial epithelium on d 58 exhibits more variation in cell height, which remains approximately 20 um. A characteristic feature of these cells is a well- developed endoplasmic reticulum consisting of short-rough as well as smooth profiles and numerous small vesicles, and a distinct golgi apparatus. Mitochondria are smaller than those of the chorioallantoic epithelial cells.

During the last third of gestation, the indentation of chorioallantoic blood vessels continues,and the height of the chorioallantoic epithelium overlying these vessels is further reduced. On the maternal side, capillaries of the endometrium project between the endometrial epithelial cells. The organelles of the chorioallantoic epithelial cells, including: the nuclei, large mitochondria, cisternae of rough endoplasmic reticulum, a small golgi apparatus and many small vesicles are generally confined to the cytoplasm protruding between neighboring fetal capillaries. The endometrial epithelial cells are similar between d 58 and d 100 to 110. A well-developed golgi apparatus persists as does rough endoplasmic reticulum. On d

100 to 110, an abundance of free ribosomes is present. Intercellular channels from the basal lamina to the tight junctions of the endometrial epithelium remain prominent.

The cytological appearance of areolae does not change significantly as gestation progresses (Friess et al., 1981). Like the epithelium of the interareolar region of the placenta, these cells have apical tight junctions and a high degree of lateral cell-to-cell interdigitation. The apical cell surface posesses long microvilli over-lying pinocytotic vesicles and tubules. The cells of the areolae contain many small mitochondria, short strands of rough endoplasmic reticulum and a well-developed golgi apparatus (Friess et al., 1981). The cells contain several droplets on d 30 of gestation which become larger, but less numerous, as gestation progresses (Friess et al, 1981). The fetal capillary network with its highly-fenestrated epithelium is in intimate contact with the areolar epithelium, but never protrudes into the epithelial layer (Friess et al,. 1981).

Along with the reduction in the thickness of the placental barrier as gestation progresses, placental size (both length and surface area) increases, resulting in a greater area for substrate acquisition by the fetus. It is generally accepted that placental size is the main factor which is ultimately responsible for fetal growth (Wrathall, 1971). Potential placental size is limited by uterine accomodation as well as number of embryos present (Wrathall, 1971). Intrauterine overcrowding due to an increased number of blastocysts, which is often associated with uneven blastocyst spacing within the uterus, results in decreased endometrial surface area available for placentation (Wrathall, 1971). Blastocysts with inadequate attachment

areas fail to develop normally in the period following placentation and often die (Wrathall, 1971). Uterine length increases rapidly through d 18 of gestation; elongation is particularly rapid from d 2 to 6 with length increasing as much as 50% over that of the nonpregnant uterus (Perry and Rowlands, 1962). There is no increase in uterine length following the third to fourth week of gestation (Pomeroy, 1960). Placental length increases rapidly between d 20 and 30 of gestation, then increases at a slower rate until d 60. There is little change in placental length from d 60 to term (Pomeroy, 1960; Knight et al., 1977). Placental weight increases steadily until d 70 of gestation, then changes very little until term (Knight et al., 1977).

It has been proposed that the allantoic membrane accumulates fluid for the purpose of expanding the chorioallantois (Knight et al., 1977). Allantoic fluid volume rapidly increases between d 20 and 30 of gestation temporally corresponding to the rapid increase in placental length; there is a decrease in volume to d 40 followed by a second increase to d 60 and subsequent decrease to d 100 (Knight et al., 1977; Goldstein, 1977). Estrogens, which have been shown to increase cell permeability to water and alter electrolyte movement, may affect water accumulation in the allantoic sac (Goldstein et al., 1976). The early expansion of the chorioallantois is due to the increase in allantoic fluid volume. Allantoic fluid volume plays a crucial role in fetal development by increasing placental surface area available for contact with the endometrium and for absorption of the secretions of the endometrial glands (Knight et al., 1977).

Placental Function

In addition to chorioallantoic expansion, allantoic fluid maintains an osmotic gradient that prevents fluid loss to the maternal circulation and aids in dilation of the cervix by fluid pressure at term (Hafez, 1987). Secretory activity of the allantois is the major source of allantoic fluid in early gestation; whereas fetal urine becomes the major constituent of allantoic fluid later in gestation (Hafez, 1987). The amniotic layer of the placenta keeps the fetus suspended in a medium of amniotic fluid that is suitable for symmetrical development of the fetus, free from adhesions to surrounding tissues (Patten, 1948; Bazer et al., 1979). Both amniotic and allantoic fluid protect the fetus from mechanical injury and act as a reservoir for water and water-soluble nutrients for fetal nutrition (Amoros, 1952). Despite the early development of the amnion, measurable amounts of amniotic fluid are not present before d 30 of gestation (Knight et al.,1977). Amniotic fluid volume increases to d 70, then decreases to term (Wislocki, 1935; Knight et al., 1977).

Just as allantoic and amniotic fluids are important in fetal support and development, the chorioallantoic layer of the placenta is important in fetal nutrition. The areolae, the troughs and lateral walls and summits of chorioallantoic folds separate the maternal and fetal circulatory systems while allowing nutrient and substrate exchange between the two systems. Each of these three areas of the porcine placenta is believed to be somewhat specialized for a particular role in maternal-fetal exchange. The main transfer mechanisms involved in moving substances between maternal and fetal circulation across the placental barrier are: simple diffusion, facilitated diffusion, active transport and transport via

micropinocytic processes (Sperhake, 1971). The transport mechanism involved depends upon the molecular weight and chemical composition of the substance to be transported. Electrolytes, water and respiratory gases cross the placental barrier via rapid simple diffusion; steroids cross via slow simple diffusion; amino acids, sugars and most water-soluble vitamins cross primarily by active transport systems; and plasma proteins, antibodies and whole cells cross via pinocytosis or leakage through pores in the chorioallantois (Hafez, 1987). Transfer of diffusible solutes depends upon the rate of uterine and umbilical blood flow and the permeability characteristics of the barrier between the maternal and fetal circulatory systems (Michael et al., 1985).

Indenting capillaries characteristic of the lateral walls and summits of the chorioallantoic folds make this area of the porcine placenta particularly suitable for exchange of freely diffusible substances, especially gases (Friess et al., 1980). The high columnar cells of the troughs of the chorioallantoic folds which contain many different sized vacuoles, are involved in the transport and metabolism of less-freely-diffusible substances including blood-borne nutrients (Friess et al., 1980). The existence of a high number of intercellular channels between cells of the summits of the endometrial rugae and between cells of the chorioallantoic troughs, which are apparent on d 30 of gestation, morphologically supports the theory that this area is involved in some manner of active transport. Despite the lack of continuity between maternal and fetal channels, the large number of pinocytotic vesicles in this area would facilitate transport from mother to fetus (Friess et al., 1980). Intense sodium-and-potassium dependent adenosine triphosphatase

(sodium-potassium ATPase) activity associated with the areolae suggests the areolae are the main sites for fetally-directed active sodium transport across the placental barrier (Firth, 1984). No other porcine placental structures exhibit any sodium potassium ATPase activity; the endometrial glands exhibit moderate activity of this enzyme (Firth, 1984). The membrane potentials created by the activity of this enzyme are involved in electrochemical excitability, absorption, secretion and any other process of specialized cells requiring the electrochemical energy of an ion gradient (Sweadner and Goldin, 1980). In the areolae, the electrochemical energy of the ion gradient is used in the active transport of substances from the endometrium to the placenta and the fetus. Tracer studies with horse-radish peroxidase indicate that the direction of transport in the chorioallantois of the porcine placenta is from the maternal to the fetal system (Friess et al., 1980). In vitro, the d 95 to 105 porcine placenta generates a membrane potential difference and a short circuit current with the fetal side positive indicating net maternal to fetal system ion flux which appears to be stimulated by the involvement of fetally-facing ß-adrenergic receptors (Boyd, et al., 1984). Adrenaline applied to the fetal side of the chorioallantois causes a significant increase in the short circuit current towards the fetus indicating that adrenaline has a stimulatory effect on the transport of sodium and possibly other ions across the placenta (Boyd et al., 1985).

Estrogens

The basic steroid structure is a perhydrocylopentanephenathrene ring. Most naturally occurring and active steroids are relatively flat with substituent groups lying above and below the plane of the ring (Speroff et al., 1978). Steroids

consisting of 21 carbons are classified as corticoids or progestins; those of 19 carbons are classified as androgens and those of 18 carbons are classified as estrogens (E). The pathway of steroid biosynthesis is illustrated in figure 2. In the human placenta, progesterone (P_4) is synthesized mainly from cholesterol taken-up via lipoproteins from maternal blood (Diczfalusy, 1969; Rabe et al., 1979). The rate-limiting step in steroid biosynthesis is the conversion of cholesterol to pregnenolone by cholesterol side chain cleavage enzymes associated with cytochrome P-450 which is strictly located in the mitochondria (Speroff et al., 1978; Rabe et al, 1983). During steroidogenesis, the number of carbons in a steroid can be decreased, but never increased; therefore in the course of steroidogenesis, progestins are converted to androgens which are subsequently converted to E (Speroff et al., 1978). Immediately following synthesis, steroids are secreted into the bloodstream where the majority are bound by a protein carrier, a beta-globulin. Binding aids efficiency of transport in the bloodstream; it may also limit biological activity and/or prevent rapid metabolism, thus ensuring time for the steroid to exert its biological effect (Speroff et al., 1978). There is some discrepancy concerning the amount of steroid bound by a given protein. Speroff et al. (1978) reported that steroid hormone binding globulin (SHBG), a beta-globulin, carries approximately 80% of the circulatory estradiol (E_2) and testosterone (T); another 19% is loosely bound to albumin and the final 1% of the circulating steroids are unbound in the general circulation. Gorrill and Marshall (1986) reported that 38% of E_2 is bound to SHBG, 60% is bound to albumin and 2% is unbound. In either case, the unbound

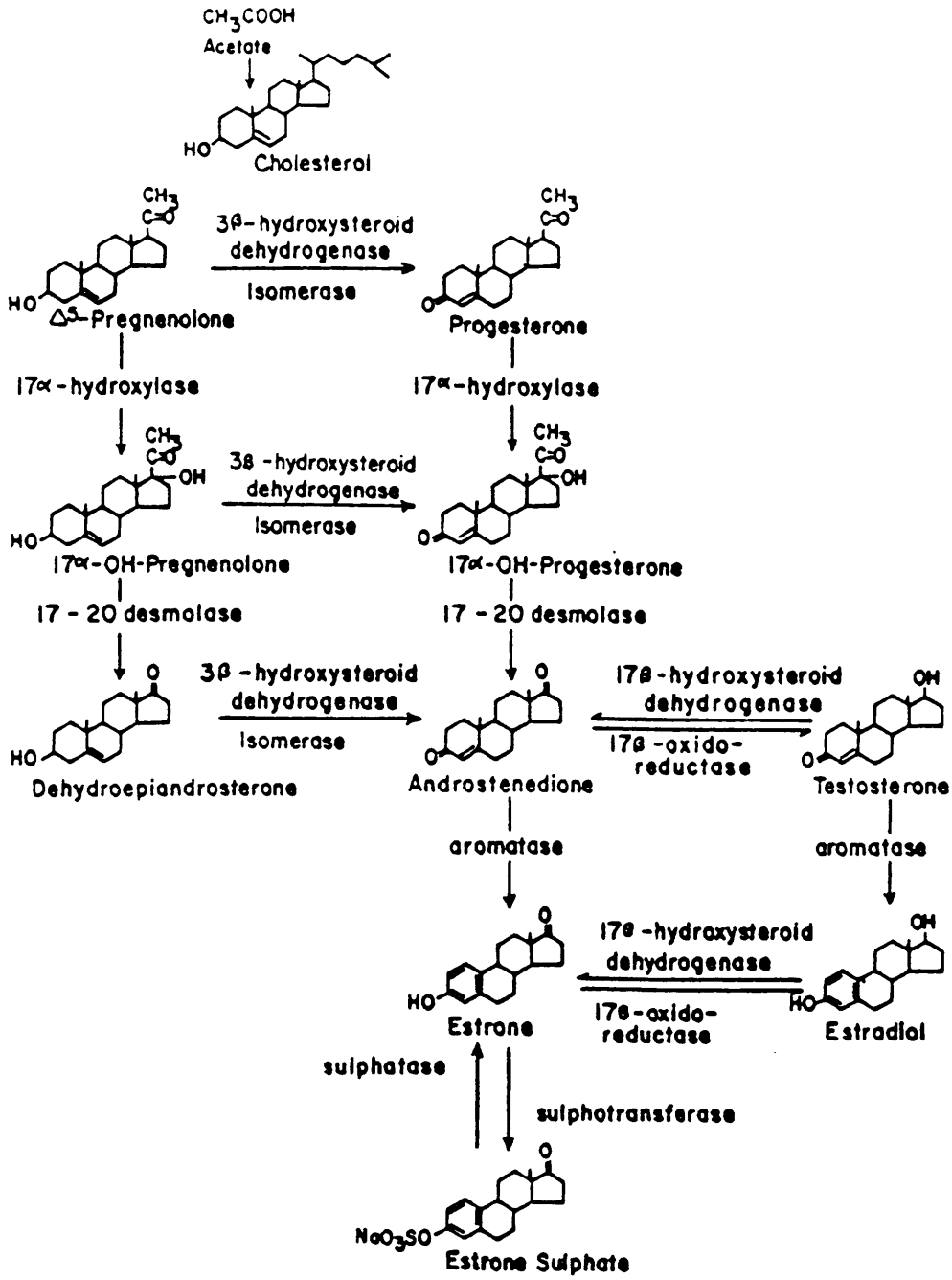

Figure 2. Steroidogenic Pathway in the Pig.

portion is very small relative to the total concentration of circulating steroids. Estrone (E_1) is not strongly bound to plasma proteins and has a much greater metabolic clearance rate than E_2 (Gorrill and Marshall, 1986). Sulfoconjugated E circulate bound to proteins, particularly albumin and alpha-globulin. Approximately 90% of the circulating estrone sulfate (E_1SO_4) is bound to albumin ensuring the low metabolic and renal clearance rate of this compound (Gorrill and Marshall, 1986).

The biologic and metabolic effects of a hormone are determined by the ability of the target cell to receive and retain that hormone (Speroff et al, 1978). These effects are determined by the unbound (free) portion of a steroid in the general circulation. Deviations from normal levels of binding globulins do not affect the efficacy of cortisol, T, or E_2 indicating that free circulating steroid levels are maintained (Speroff et al., 1978). The mechanism of steroid hormone action within the cell is as follows. The steroid enters the cell by simple diffusion. The dissociation constant of the steriod-receptor complex is lower than that of the transport complex; therefore, steroids will accumulate intracellularly in the absence of an active transport mechanism. The concentration of steroids in non-target tissue is in free equilibrium with that of the blood (Karlson et al., 1975). The receptor is involved in retention of the steroid, not uptake or transport into the cell (Karlson et al., 1975). In an older model of steroid action, the steroid was thought to be bound to a cytoplasmic receptor; this complex was then translocated to

the nucleus where it caused increased transcription of DNA to mRNA either by removal of a repressor protein or by positive cooperation of the hormone receptor complex with RNA polymerase to start transcription at a specific operon (Jensen et al., 1968 and Gorski et al., 1968). In a newer model, proposed and supported by investigations with E, the receptor appears to be nuclear at all times, whether or not E is bound, and is considered to be immobilized by its association with some structural element of the nucleus. Upon E binding, the receptor undergoes conformational changes, but remains immobilized at its nuclear site. Activation or transformation of the receptor may alter the nature of this nuclear interaction or may lead to interactions with additional nuclear components (Gorski et al., 1984).

The biological actions of E are multi-fold. The following list was compiled by Heftman (1970). Estrogens stimulate growth and development of the female reproductive organs and secondary sex characteristics. They induce proliferation of the epithelium in the oviducts, uterus, cervix and vagina. In rats, rabbits and guinea pigs, E stimulates increased water content and subsequent weight gain of the uterus. During proestrus, E stimulation results in increased quantity and decreased viscosity of the cervical mucus. In rodents, they cause the proliferation and cornification of the superficial layers of the vagina. In primates, E increases glycogen and mucopolysaccharide deposition in the uterus. During pregnancy, E acts with progestins to aid in placentation, maintain pregnancy and facilitate parturition. Estrogens affect basal metabolic rate which may explain the increase in body temperature at ovulation; they affect salt and water retention (Szego and Sloan, 1961), thereby raising blood pressure, and they decrease clotting time. Estrogens

favor a positive calcium balance, affect lipid metabolism and increase protein anabolism and feed efficiency. Estrogens alter protein production by the liver and affect bone maturation and epiphyseal closure and mineral metabolism (Gorrill and Marshall, 1986). Estradiol in the uterus stimulates vasodilation and water uptake from the general circulation mediated by the liberation of histamine and serotonin; this action could be due to intracullular action of E_2 or its action on the cell membrane (Karlson et al., 1975). Estriol (E_3) also causes vasodilation but has no effect on RNA metabolism (Karlson et al., 1975). Estrogens of fetal origin may promote the release of uterine secretions (histotrophe) from secretory granules of the endometrial glands during early gestation in swine (Raub et al., 1985) and increase blood flow to the gravid uterus (Resnik et al., 1974).

Estrogens may act locally at the site of placentation in concert with P_4 and possibly other hormones such as placental lactogen, resulting in luteostasis; increased uterine blood flow; enhanced water, electrolyte and substrate (such as carbohydrate and amino acid) transport across the placenta; and modulation of histotrophe synthesis and secretion (Bazer et al., 1979). Estrogen production by pig blastocysts is assumed to be the signal for maternal recognition of pregnancy (Bazer and Thatcher, 1977; Flint et al., 1979). Quantitative and qualitative changes in the protein content and secretion of histotrophe are induced and maintained by P_4 (Knight et al., 1973) and P_4 and E in a positive dose-response relationship (Knight et al., 1974; Bazer et al., 1979). A P_4 to E_1 ratio of 2000:1 is optimal for

promoting uterine protein secretion (Knight et al., 1973). The same ratio has also been demonstrated to increase litter size when treatment is initiated at least 14 d into gestation (Wildt et al., 1976), a period associated with maximum uterine protein production in the cyclic gilt (Murray et al., 1972), or when administered for 10 d during the third and fourth weeks of gestation (Reddy et al., 1958). Estrogens are believed to play a major role in fluid accumulation within the allantoic sac which is in turn responsible for placental expansion between d 20 and 30 of gestation in swine (Goldstein et al., 1976; Knight et al., 1979). Administration of exogenous P_4 and E_1 in a ratio of 2000:1 from either d 20 to 30 or from d 25 to d 30 of gestation augments placental development seen on d 50 of gestation (Dalton and Knight, 1983).

Estrone conjugates, particularly sulfates, are rapidly hydrolyzed following oral and intravenous administration making it difficult to associate effects exclusively with sulfoconjugated E (Bernstein et al., 1970). Keeping this fact in mind, the following effects have been attributed to sulfoconjugated E. In vitro, sulfoconjugated E influence red blood cell uptake of uric acid and inhibit a variety of enzymes which require pyridoxal phosphate (Bernstein and Solomon, 1970). In vivo, intravenous administration of sulfoconjugated E results in increased transcortin, ß-glucuronidase, ceroplasmin, circulating triglyceride and nonspecific hyaluronidase inhibitor concentrations in the plasma. They decrease pulmonary diffusing capacity and decrease capillary strength (Bernstein and Solomon, 1970).

Certain areas of the brain, such as the circumventricular organs, presumably

lack the normal blood-brain barrier and may serve as a portal of entry for E_1SO_4 into the central nervous system. Two of these regions, the median eminence and the organum vasculosum lamina terminals of the hypothalamus, are highly vascularized and contain specialized cells which are postulated to transport large molecular weight substances that normally would not cross the blood-brain barrier (Platia et al., 1984). Estrone sulfate may enter the brain and exert a biological effect after its in situ hydrolysis and subsequent binding to E receptors (Platia et al.,1984). The hydrolysis of E_1SO_4 is a prerequisite for exerting effects via E receptors; sulfoconjugated E do not bind to E receptors (Brooks et al., 1978). In vivo studies with rats indicate that circulating E_1SO_4 can enter the brain and be hydrolyzed as well as transformed to other compounds (Kishimoto, 1973). There is further evidence for the hydrolysis and subsequent direct conversion of E conjugates to other conjugate forms without entry of the free steroid into the general pool. Metabolism of the steroid conjugate within the nucleus may occur without hydrolysis (Hobkirk, 1979b).

Bioassays of the effects of E on vaginal smears and uterine weight of rats and mice indicate E_2 is more potent than E_1 which is more potent that E_3 when administered subcutaneously. When administered orally, E_3 is more potent than E_2 which has greater potency than E_1 (Heftman, 1970). Estradiol is probably re-released to the general circulation after initiating a cell response; therefore, it may "act" several times before clearance from the bloodstream by metabolism. Many

E_2 molecules are metabolized without having exerted an effect (Speroff et al., 1978). Estrone formation has been indicated as the first step in E_2 inactivation (Gorrill and Marshall, 1986). In most animal species, E_1 and E_2 are interconvertible; however, the equilibrium is displaced in favor of E_1 (Heftman, 1970). Cells clearing E_2 from the general circulation, convert it to E_1, which is moderately effective as an E and E_3 which is a weak E, or conjugate it with sulfate and glucuronate groups rendering it water soluble (Speroff et al., 1978). Testosterone is metabolized within the cell in which it has elicited an effect and is re-released into the general circulation as an inactive compound (Speroff et al., 1978).

Estrogen metabolites, such as catechol- E, epiestriols, and E_3 as well as E_1 and E_2 are conjugated with sulfuric and glucosiduronic acid in the liver and kidney rendering them water soluble; they are then excreted in the urine. Estrogen metabolites can also be excreted in the feces via the enterohepatic system (Gorrill and Marshall, 1986). The liver is the most active organ in steroid conjugation in most species (Hobkirk, 1979b; Singer, 1982). Estrone sulfate, initially discovered by Schachter and Marrian (1938) in urine of pregnant mares, was originally believed to be a water soluble detoxification mechanism protecting the body from accumulating harmful levels of steroid hormones and was therefore an end product of metabolism (France, 1979). Numerous investigations in recent years have made it evident that steroid sulfates are not merely inactive end products of metabolism; they

may play an important role in steroid interconversions, transport and activity (Purdy et al., 1961; Pack and Brooks, 1970; Brooks and Horn, 1971; Pack and Brooks, 1974). Conjugation with glucosuronide, sulfate or phosphate render steroids water soluble; methyl and acetyl derivatives are more lipid soluble (Hobkirk, 1979b). Additional physiological roles for sulfoconjugated steroids are suggested by the existence of the sulfatase enzyme system, which removes the sulfate group, and by the adrenal secretion of sulfated steroids, especially dehydroepiandrosterone sulfate (DHEAS) (France, 1979). Sulfoconjugated E are of interest as important metabolites of steroid hormone homeostasis and activity (Brooks et al., 1978).

High circulating levels of E_1SO_4 can be a reservoir for active E providing E_1SO_4 can reach the intracellular compartment and be selectively hydrolyzed by hydrolases present (Noel et al., 1983; Platia et al., 1984). The metabolic clearance rate for E_1SO_4 is significantly lower than for E_1, supporting the hypothesis that sulfoconjugation serves as a method for storage of steroids in the blood (Hobkirk, 1979b). In humans, the half-life of sulfoconjugated E is approximately 7.5 h; the half-life of free E_2 is 0.5 h (Ruder et al., 1972). In sheep near term, the half-life of tritiated E_1SO_4 administered intravenously is 8 min; this is approximately three times greater than the plasma E_1 half-life in sheep (Tsang and Hackett, 1979). The shorter half-life values in sheep may be due to the rapid reduction of E_1SO_4 to

E_2-3-SO_4 in sheep, a product which has not been observed in humans (Tsang and Hackett, 1979). Postulated capillary and cellular membrane barriers to steroid sulfates may prevent direct uptake of these blood-borne compounds by most tissues. These barriers may be one of the factors responsible for the drastic reduction in metabolic clearance rate of steroids upon sulfation (Holinka and Gurpide, 1980). Although extensive metabolism of E_1SO_4 and DHEAS has been demonstrated in humans, these particular sulfoconjugated steroids appear to be hydrolyzed mainly in organs where capillary barriers are easily crossed, such as the placenta and the liver (Holinka and Gurpide, 1980).

Physiological regulation of circulatory and intercellular E concentrations is of vast importance, especially during pregnancy. Three of the enzymes involved in modulating E concentrations are aromatase, sulfotransferase and sulfatase. Aromatase is involved in the conversion of androgens, particularly androstenedione and T, to E: E_1 and E_2 respectively. Sulfotransferase conjugates E with a sulfate group and sulfatase hydrolyzes the sulfate-estrogen bond yielding free E.

Studies based on human placental insufficiency reveal that some cases of intrauterine fetal growth retardation are associated with slowed aromatization of DHEAS (Thoumisin et al., 1982), suggesting normal levels of E production are necessary for normal fetal growth patterns. The importance of sulfoconjugated steroids and the role of sulfatase in liberating these steroids is demonstrated in cases of human placental sulfatase deficiency. Placental sulfatase deficiency, apparently an X-linked recessive disorder, is associated with greatly reduced E production. Fetal

growth and development may be normal; however, excessively low E concentrations can result in fetal death. Failure of parturition mechanisms is characteristic of placental sulfatase deficiency (France, 1979).

Aromatase

The aromatase enzyme system is the key enzyme system for E synthesis, converting 19-carbon Δ^4-3 ketosteroids to aromatic A-ring E (Rabe et al., 1982). Aromatase activity is found in the ovary, subcutaneous adipose tissue and placenta (Rabe et al., 1982), endometrium (Tseng, 1984) and preimplantation porcine blastocyst (Gadsby et al., 1980). Aromatase is a microsomal enzyme (Ryan, 1959). Three successive hydroxylations catalyzed by mixed function oxidase(s) are required during androgen aromatization (Thompson and Siiteri, 1974). Aromatization involves hydroxylation of the 19-methyl group followed by oxidation with a loss of the 19-methyl group followed by oxidation with a loss of the 19-methyl group as formaldehyde; the final step is A ring aromatization or dehydrogenation (Speroff et al., 1978; figure 3). The work of Thompson and Siiteri (1974) suggests the final hydroxylation is the rate-limiting step in the aromatization of androstenedione. Nicotinamide adenine dinucleotide phosphate in the reduced form (NADPH) and oxygen are required for the aromatase reaction (Ryan, 1959; Thompson and Siiteri, 1974). Therefore, aromatase is a multi-step, possibly multienzyme, pathway in which 19-hydroxy-androstenedione and 19-oxoandrostendeione are obligatory intermediates in the formation of E_1 and

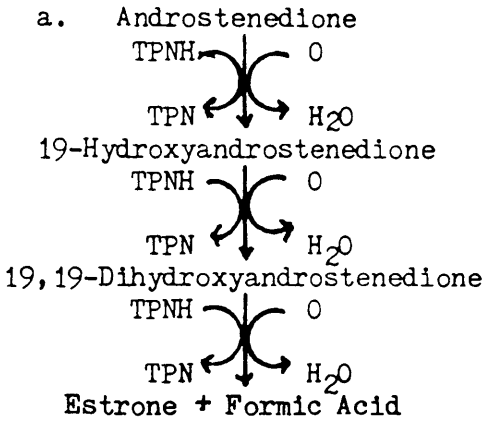

Figure 3. Aromatase Reaction by Thompson and Siiteri (1976) (a.) and Kelly et al. (1977) (b.).

formate from androstenedione (Thompson and Siiteri, 1974; Siiteri and Thompson, 1975). Results of Kelly et al (1974) suggest that aromatization is a concerted process occurring at a single catalytic site. The three oxidative attacks occur in the same region of the steroid molecule, so that little if any reorientation of the enzyme-bound steroid with respect to the direction of attack is necessary. The multi-step pathway requires three enzymes together with an electron transport system, all of which are associated with microsomal membranes; however, no separation of the individual enzymatic activities of the proposed multienzyme pathway has been reported. The proposal of Kelly et al. (1977) is supported by the research of Hollander (1962) who found that 19-hydroxyandrostenedione was not an obligatory intermediate in the aromatase pathway.

In the human placenta and endometrium at term, NADPH-cytochome C reductase is a required component of the microsomal aromatase (Tseng and Bellino, 1985). Aromatase is able to metabolize both androstenedione and T in one high-affinity site (Reed and Ohno, 1976). The aromatization of androstenedione yields E_1 as the major E product and formate (Thompson and Siiteri, 1974; Gibb and Lavoie, 1980). Estradiol accounts for up to 30% of the E formed from androstenedione and is the major E product produced by the aromatization of T (Gibb and Lavoie, 1980). The Michaelis-Menton constant (K_M), which is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity, reported for aromatase covers a wide range of values due to the variation in assays used to measure the reaction. The lower the K_M, the higher the affinity of the

enzyme for that substrate. The K_M for aromatase of human placental microsomes has been reported as 0.5 uM androstenedione and 0.1 uM T (Bellino and Osawa, 1974); 0.1 uM androstenedione (Canick and Ryan, 1976; Reed and Ohno, 1976); 5.0 uM androstenedione (Thompson and Siiteri, 1976); 0.04 uM androstenedione (Kelly et al.,1977) and 14 nM for androstenedione and 41 nM for T (Gibb and Lavoie, 1980). The K_M for aromatase of the porcine ovary has been reported as 4.4 uM androstenedione (Kautsky and Hagerman, 1976) and 1.0 uM androstenedione (Kautsky and Hagerman, 1980).

There is no evidence of product inhibition or non-hyperbolic kinetics on the aromatase enzyme using substrate concentrations which are five- to ten-fold greater and less than the apparent K_M values (Gibb and Lavoie, 1980). Androstenedione and T are competitive inhibitors (Reed and Ohno, 1976). Nicotinamide adenine dinucleotide phosphate (NADP) is a potent inhibitor of aromatization of androstenedione (Thompson and Siiteri, 1974).

In porcine granulosa cells, aromatase activity is stimulated by follicle stimulating hormone (FSH) (Speroff et al., 1978; Chan and Tan, 1986). Aromatizable androgens act as substrates and induce aromatase activity in rat granulosa cells in vitro (Hillier and DeZwart, 1981). Induced aromatase activity by FSH in prepuberal porcine granulosa cells in culture is reported to be inhibited by 5 αdihydrotestosterone (DHT) which was found not to be a substrate for granulosa cell aromatase (Chan and Tan, 1986). However, other researchers have found aromatase in the rat ovary will use DHT as a substrate, although it is a less effective substrate

than androstenedione or T (Daniel and Armstrong, 1980, 1983; Hillier and DeZwart, 1981). Pregnenolone and P_4 significantly reduced FSH-induced aromatization in vitro (Chan and Tan, 1986).

By analogy with human studies, the overall mechanism by which a 19-carbon substrate is aromatized appears to be essentially the same in those placentae in which aromatization has been demonstrated, irrespective of the type of placentation (Ainsworth and Ryan, 1966); therefore, information on aromatase acitivity in the placentae of other species can be applied to the porcine placenta. Aromatase activity of the placenta and ovary are similar in many respects, but there are important quantitative differences between them. Ovarian aromatase has a larger Michaelis-Menton constant, lower maximal velocities, greater facility of intermediate exchange and greater carbon monoxide sensitivity; differences in catalytic properties suggest that the aromatase enzymes of these two organs possess significant structural differences in or near their binding sites (Kautsky and Hagerman, 1980).

In vitro aromatase activity, which is minimal on d 10 of gestation (Perry et al., 1973), is prominent in the preimplantation or d 14 to 18 trophoblast of the pig (Gadsby et al., 1980). The blastocysts are capable of aromatizing tritiated androstenedione, dehydroepiandrosterone (DHEA) and T to E_1 and E_2 (Perry et al., 1973; Flint et al., 1979; Gadsby et al., 1980). The amount of precursor converted to E was greater with DHEA than with androstenedione although the only significant difference was in the amount of E_1 formed (Perry et al., 1973; Gadsby et al., 1980).

Porcine endometrial tissue exhibits negligible aromatase activity at this stage of gestation (Perry et al,. 1973). Aside from activity of the early trophoblast, research on porcine placental aromatase activity is limited to late gestation. Ainsworth and Ryan (1966) using the placenta of one sow between 109 and 112 d of gestational age, reported microsomal aromatization of tritiated DHEA to be approximately 17% and of androstenedione to be approximately 5%. Craig (1982) reported porcine placental microsomal aromatization of androstenedione to be 44% on d 100 (n=4), 49% on d 106 (n=3) and 74% on d 114 (n=4). Craig (1982) concluded that the apparent activity of placental aromatase along with 17 -hydroxylase and C-17-20-lyase increases between d 100 and term concurrent with increasing maternal plasma E concentration. At term in the human, aromatase activity of the placenta is greater than that of the endometrium (Tseng and Bellino, 1985). Based on in vitro studies with human endometrium, a large degree of variation exists between aromatase activity of stromal cells and that of endometrial gland cells at various stages of the menstrual cycle (Tseng, 1984). In general, stromal cell aromatase activity is 2.5 fold greater than that of the endometrial glands (Tseng, 1984). Estradiol has no effect on in vitro endometrial aromatase activity; P_4 increased stromal cell aromatase 8-to 40-fold over controls, but had no effect on glandular activity, and a combination of E_2 and P_4 increased stromal activity 20-to 100-fold and glandular activity 3-fold over controls (Tseng, 1984). Aromatase activity of human decidual tissue from the first trimester is greater than that of nonpregnant endometrial tissue, which lends further support for the role of P_4 in

stimulating aromatase activity. The response to P_4 is probably potentiated by E_2 under physiological as well as in vitro conditions (Tseng, 1984).

Sulfotransferase

There are two sulfotransferase enzymes recognized by the Commission of Biochemical Nomenclature. One is estrone 3'-sulfotransferase (E.C.2.8.2.4) which is specific for the transfer of activated sulfate to the 3-hydroxyl group of E (Adams and Poulos, 1967; Hobkirk, 1979a). The other is 3ß-hydroxysteroid sulfotranferase (E.C.2.8.2.2) which is specific for the transfer of activated sulfate to the 3ß-hydroxyl group of 19-carbon or 18-carbon steroids (Hobkirk, 1979a). Sulfotransferase activity is found in significant amounts in the mammalian liver, kidney, lung, mammary tissue, adrenal glands, jejunal mucosa, testes, ovaries and feto-placental unit; however, there is a good deal of species variation (Bernstein and Solomon, 1970; Hobkirk et al., 1970a). Muscle, adrenal gland, ovary, testes and endometrium incubated in vitro are capable of steroid conjugation (Hobkirk, 1979a). In adults of some species, ovarian sulfotransferase plays a major role in steroid metabolism; in others, such as cattle and humans, ovarian sulfation of 18- and 19-carbon steroids is minimal (Hobkirk et al., 1979a). Human and rat fetal levels of steroid sulfotransferase are less than those of adult tissues with adrenal glands and kidney tissue being exceptions (Wengle, 1966; Bernstein and Solomon, 1970). Estrone sulfotransferase is widespread in the fetus, present in all tissues except the cerebrum; this distribution suggests the importance of the enzyme to fetal life

(Wengle, 1966).

Sulfotransferase occurs in the soluble cell fraction (Hobkirk, 1979a). The biosynthesis of a sulfoconjugated steroid by sulfotransferase involves two distinct reactions. First is the formation of an activated sulfate, 3'-phosphoadenosine-5' phosphosulfate (PAPS) by the sulfate activating system. The sulfate activating system is found in microorganisms, plants and animals. In mammals, it is found in most tissues including endocrine glands. This system is inhibited by disodium ethylenediamine tetraacetate (EDTA) which causes the removal of Mg^{2+} ions which are necessary as cofactors (Bernstein and Solomon, 1970). The formation of PAPS is a two step process occuring in the cell cytoplasm. In the first step, sulfate adenylyl transferase (ATP-sulfurylase; E.C.2.7.7.4) activity results in the formation of andenosine 5' phosphosulfate (APS) from adenosine 5'-triphosphate (ATP) and sulfate (SO_4). This is a highly specific, SH- activated enzyme with a pH of optima 7 to 9.5. The reaction is reversible; however, equilibrium favors the reactants. The reaction is as follows:

$$ATP + SO_4^{2-} \xrightarrow[Mg^{2+}]{\text{ATP Sulfurylase}} APS + PPi$$

PP_i = inorganic pyrophosphate

In the second step of the sulfate activating system adenylyl sulfate kinase (E.C.2.7.1.25.) catalyzes the following reaction:

$$APS + ATP \xrightleftharpoons{Mg^{2+}} PAPS + ADP$$

This reaction requires Mg^{2+} as a cofactor and has a pH optima of 8.5 to 9. This

reaction which is essentially irreversible, is very efficient at trapping low APS concentrations formed in step one and allows the accumulation of significant amounts of PAPS (Bernstein and Solomon, 1970). The second reaction of sulfoconjugated steroid formation involves the transfer of activated sulfate to the steroid molecule by sulfotransferase in the following, essentially irreversible, reaction, where R is the steroid:

$$PAPS + R\text{-}OH \longrightarrow PAP + R\text{-}OSO^{-}_{3}$$

In general, hydroxyl groups at the 3, 17 and 21 positions of the steroid molecule are readily sulfated (Bernstein et al., 1970).

Human placental E sulfotransferase is extremely unstable. The reported molecular weight of 76,500 g/mol is similar to that of human adrenal DHEA sulfotransferase (Tseng et al., 1985). Estrogen sulfotransferase purified from bovine adrenal, ovary and placental tissue is a monomer with a molecular weight of 74,000 g/mol (Adams and Low, 1974). The Michaelis-Menton constant for E_1 sulfotransferase of bovine tissue is approximately 15 uM and for human endometrium is 10 nM (Tseng and Gurpide, 1975). In the bovine adrenal, the pH optima for E_1 sulfotransferase activity is between 8 and 10 (Singer, 1982). The isoelectric point of E sulfotransferase is 5.8 in human placenta and endometrium, bovine adrenal glands, guinea pig uterus and placenta but is 6.1 in porcine endometrium (Tseng et al., 1985). Kinetic studies indicate that E_2 is the preferred substrate for E sulfotransferase of human placenta and endometrium (Tseng et al.,

1985). Estrogen sulfotransferase of the bovine adrenal and placenta exhibited no substrate preference among E_1, E_2 and E_3 (Adams and Low, 1974). These findings reveal that substrate binding sites as well as enzyme structure may be modified in different species (Tseng et al., 1985). Estrone sulfotransferase is difficult to study due to complex kinetics (Adams and Low, 1974). Specific characterization of E_1 sulfotransferase activity in tissue homogenates is virtually impossible due to the high level of E sulfatase in many tissues (Tseng et al., 1985). Studies of bovine adrenal E_1 sulfotransferase suggest nonsubstrate steroids such as 11-deoxycorticosterone and T are noncompetitive inhibitors of E sulfation (Rhozin et al., 1977).

Despite lower tissue levels of sulfotransferase activity in the fetus, the concentration of sulfoconjugated steroids in humans is greater in fetal circulation than in maternal circulation (Hobkirk, 1979a). Studies of human fetal adrenals indicate that their major function is the formation of sulfoconjugated steroids which are transferred via cord blood to the placenta where they are hydrolyzed to free steroids and further metabolized to E (Hobkirk, 1979a).

The uterine epithelium contains 17 ß-hydroxysteroid dehydrogenase (HSD) and sulfotransferase activity (Pack and Brooks, 1974) which could convert free E produced by the ovary to E_1SO_4 as it moves through the endometrium (Geisert et al., 1982a). Conjugated E content in uterine flushings of pregnant gilts between d 11 and 16 increased concurrently with the increase in free E content, which could reflect

endometrial sulfation of free E of blastocyst origin (Geisert et al., 1982a).

Porcine uterine E sulfotransferase activity appears only after puberty (Brooks et al., 1978). Prepuberal porcine uteri exhibit no 17 ß-HSD activity, sulfotransferase activity or microsomal peptide synthesis; puberal uteri which have had E but no P_4 stimulation still do not exhibit activity of the above enzymes. They do exhibit microsomal peptide synthesis at a level comparable to that of mature porcine uteri (Brooks et al., 1972). Therefore, the appearance of sulfotransferase activity as well as 17 ß-HSD activity is not related to the initial stimulation of uterine protein synthesis by E (Brooks et al., 1972). After puberty, E sulfotransferase activity in the porcine uterus fluctuates in response to the hormonal milieu of the animal and relates inversely to the nuclear concentration of E receptors in the cycling uterus (Pack and Brooks, 1974). Cycling of E sulfotransferase activity can be directly related to plasma P_4 levels in porcine as well as in human uteri (Brooks et al., 1978).

In vitro, the porcine uterus exhibits the greatest amount of E metabolism both sulfotransferase and 17 ß-HSD activity during the secretory phase, d 5 to 13, of the estrous cycle (Pack and Brooks, 1974). In vitro sulfotransferase activity rapidly increases until d 9 when E_1SO_4 represents 80% of E present. Estrogen sulfotransferase activity steadily decreases with a small surge on d 16 corresponding temporally to the initiation of a new follicular phase in the ovary (Pack and Brooks, 1974). Thereafter, E sulfotransferase activity decreases sharply. There is no apparent E sulfotransferase during estrus, d 1 and 2 of the estrous cycle (Pack and

Brooks, 1974). Unlike E sulfotransferase, 17ß-HSD is present at all stages of the estrous cycle (Brooks et al., 1978). The extent of E sulfation noted by Pack and Brooks (1974) is a result of both E sulfotransferase and E sulfatase activities occurring simultaneously within the uterine tissue during the 2 h incubation period. Progestins, such as P_4 and medroxyprogesterone acetate (MPA) stimulate human endometrial E sulfotransferase and 17ß-HSD activities (Tseng and Gurpide, 1975; Tseng, 1978; Tseng and Gurpide, 1979; Tseng and Liu, 1981). The increased production of sulfo-conjugated E in human proliferative endometrial gland tissue caused by MPA is derived from an increase in E sulfotransferase activity rather than a decrease in sulfatase activity (Tseng and Liu, 1981). Their assays measured cytosol activity; hydrolysis does not occur as sulfatase is membrane bound to various particulate fractions (Tseng and Liu, 1981). The influential role of P_4 on E sulfotransferase activity is seen in the pregnant pig where the drop in plasma P_4 from d 14 to 28 (Robertson and King, 1974) is followed by a decline in endometrial E sulfotransferase activity after d 30 of gestation (Dwyer and Robertson, 1980). The observations of Dwyer and Robertson (1980) concerning sulfotransferase activity are influenced by coincident sulfatase activity.

As a result of the complex kinetics associated with E_1 sulfotransferase, it has been proposed that this enzyme plays a regulatory role in E metabolism (Adams and Low, 1974). Brooks et al. (1978) suggest that E sulfotransferase present in E target

tissues such as the placenta and endometrium may be involved in E metabolism by limiting it's nuclear activity; since sulfoconjugated E does not bind to E receptors.

Sulfatase

Steroid sulfatases liberate steroids from their sulfoconjugated form (Bernstein and Solomon, 1970). The actual number of enzymes responsible for the cleavage of sulfoconjugated steroids is uncertain (Noel et al., 1983). There are two steroid sulfatases recognized by the Commission on Biochemical Nomenclature; one is and the other may be membrane bound, like aromatase but unlike sulfotransferase. Estrone sulfate sulfhydrolase (E.C.3.1.6.1) also known as arylsulfatase or E_1 sulfatase is generally thought to be membrane bound in mammalian cells and is relatively nonspecific with respect to the phenolic part of the substrate (Bernstein and Solomon, 1970; Hobkirk, 1979a; Moutaouakkil et al., 1984). Three types of E_1 or arylsulfatase have been reported to hydrolyze aromatic sulfate esters in human tissue. Types A and B have been reported to be soluble enzymes occurring within lysosomes; these types are at normal levels in sulfatase deficient pregnancies(France, 1979). These two types are also known collectively as Type II arylsulfatase (E.C.3.1.6.1). However, Type II arylsulfatase has been reported to be membrane bound within the lysosomal fraction of the cell (Webb and Morrow, 1959). Type II arylsulfatase is powerfully inhibited by sulfate and phosphate ions and is specific for the phenol group of the steroid molecule (Bernstein and Solomon, 1970). Sulfatase C or Type I arylsulfatase is an insoluble, microsomal membrane

bound enzyme (France, 1979) and is the most important in the hydrolysis of E_1SO_4 (Pulkkinen and Paunio, 1963). Type C is the enzyme that is missing in sulfatase deficient pregnancies of humans (France, 979). Sulfatase C is highly active in the human placenta using E_1SO_4 as a substrate (French and Warren, 1967). Estrone sulfatase has been reported to be insensitive to inhibition by sulfate and phosphate ions and to have a bimodal optimal pH for the hydrolysis of E_1SO_4 of 6.5 and 8.0 (French and Warren, 1967). The Michaelis-Menton constant for E_1 sulfatase has been reported as 0.1 mM E_1SO_4 (French and Warren, 1967) and 2uM E_1SO_4 (Tseng and Liu, 1981).

The other recognized sulfatase is steroid sulfate sulfhydrolase or steroid sulfatase (E.C.3.1.6.2) which is specific for 3 ß-yl steroid sulfates; DHEAS, pregnenolone sulfate, cholesterol and E_1SO_4 are substrates for this enzyme (Notation, 1969; Noel et al., 1983; Moutaouakkil et al., 1984). This sulfatase, when found in human placental tissue, has a bimodal optimal pH of 5.0 and 7.5 (Noel et al.,1983). The Km for E_1SO_4 is .02 mM (Notation, 1975). The molecular weight of this sulfatase, in human placental tissue, is 72,000; much higher values have been reported which are probably due to polymerization producing various active forms (Noel et al., 1983). An essential problem concerning E_1SO_4 hydrolysis is knowledge of whether or not E_1 sulfatase (E.C.3.1.6.1) is identical to steroid sulfatase (E.C.3.1.6.2) (Moutaouakkil et al., 1984). Studies of the effects of pH,

buffers, temperature and noncompetitive inhibittors on enzyme activity in guinea pig uterine and liver tissue suggest the existence of two distinct sulfatases; however, the two enzymes have not been physically separated (Moutaouakkil, et al., 1984).

The sulfatase reaction is irreversible as follows, where R is the steroid:

$$R\text{-}OSO^{-}_{3} + H_2O \longrightarrow R\text{-}OH + SO_4{}^{2-} + H^{+}$$

(Bernstein and Solomon, 1970).

Estrone sulfatase activity has been located in the many tissues including the human adult hypothalamus, frontal cortex, pituitary, lung, liver, placenta, endometrium, myometrium, skin, adipose tissue; fetal hypothalamus, frontal cortex and pituitary (Platia et al., 1984), fetal liver (French and Warren, 1966), porcine and ovine endometrium (Dwyer and Robertson, 1980) and porcine blastocyst (Perry et al., 1973). In vitro, the human nonpregnant endometrium and lung hydrolyze E_1SO_4 to E_1 and then convert E_1 to E_2 (Tseng et al., 1972; Milewich et al., 1983). There is no measurable metabolism of E_1 following E_1SO_4 hydrolysis in any nonpregnant human tissue except the endometrium (Platia et al., 1984). The placenta is the richest source of human steroid sulfatase activity (French and Warren, 1966). Quantitative in vitro studies of human fetal and adult brain and pituitary by Platia et al. (1984) reveal that not all regions have equivalent E_1 sulfatase activity levels. Their findings may not be representative of the general population, since the adults were elderly and diseased; however, adult hypothalamus and frontal cortex were shown to have greater activity than fetal hypothalamus,

frontal cortex and pituitary which exhibited greater activity than adult pituitary.

In the rat uterus, E_1 sulfatase activity is inhibited by E (Utaaker and Stoa, 1980). Alternative substrates are the most important group of potential endogenous sulfatase inhibitors (Townsley, 1973). In the human placenta in vitro concentrations of endogenous free steroids of placental and umbilical cord blood regulate placental E production from conjugated precursors due to the inhibitory effect of free steroids on steroid sulfatase (Townsley et al., 1970). This may explain why sulfatase instead of aromatase appears to be rate-limiting during placental perfusions (Townsley et al., 1970). The concentration of steroids required to demonstrate appreciable inhibition are within physiological ranges with DHEA being most potent. Other inhibitors, in decreasing order of effectiveness, are: 16 hydroxydehydroepiandrosterone, androstenediol, T, androstenedione, E_2, E_1 and E_3 (Townsley et al., 1970). The endoplasmic reticulum, where the steroid-metabolizing enzymes are located, has the ability to concentrate certain steroids selectively, which could result in higher local concentrations and more extensive inhibition than would be expected on the basis of mean placental or umbilical cord plasma steroid concentrations (Townsley et al., 1970). In guinea pigs, where E_1 sulfatase activity is lower in the liver than the uterus, a soluble, intracellular effector of hepatic cytosol origin has been discovered by Adessi and Moutaoukkil (1986). This effector has a molecular weight of 7,600 and acts as a non-competitive inhibitor of E_1 sulfatase (E.C.3.1.6.1) of uterine and hepatic

microsomes. Changing intracellular concentrations of the inhibitor could play a role in controlling the expression of E_1 sulfatase activity (Adessi and Moutaouakkil, 1986).

Despite the fact that free endogenous steroids can inhibit sulfatase activity, E_1 sulfatase and steroid sulfatase activities in the human endometrium do not vary during the menstrual cycle and are not correlated to P_4 and E_2 concentrations (Prost and Adessi, 1983). In the rat and hamster reproductive tracts, sulfatase activity is under hormonal control, increasing at the time of estrus and reaching a maximum during early metestrous (Legault et al., 1981).

Steroidogenic and Enzymatic Profile of the Pig During Gestation

Due to the sulfatase enzymes within the trophoblast of porcine blastocysts, E sulfates are more efficiently converted to free E than is androstenedione (Perry et al., 1973, 1976). The pig endometrium is known to have 5α- reductase activity which reduces progestins and androgens (Henricks and Tindall, 1971), which subsequently cannot be aromatized to E (Wilson, 1972). This is another reason why free androgens may have little significance, in vitro, when compared to sulfoconjugated E as precursors for free E production by the trophoblast (Bazer et al., 1977).

The conceptus plays an important role in endometrial activity in the pig. For example, 3ß-and17ß-HSD enzymes appear in the porcine endometrium after there is endometrial- conceptus contact (Christie, 1968; Dufour and Raeside, 1969; Flood,

1974). Furthermore, although the qualitative nature of uterine secretions are the consequence of maternal regulation alone, the endometrium underlying the cenceptus tissue is quantitavely more active than that from unoccupied regions of the uterine horn. This suggests that the local modulating effects may be due to conceptus-produced factors such as steroids or polypeptide hormones (Basha et al., 1980).

In humans, the ovaries are not necessary throughout gestation, and the placenta is dependent upon externally supplied precursors for E synthesis (Townsley, 1973). Maternal adrenal DHEA is the primary precursor for E synthesis in early gestation; by mid gestation, precursors are primarily of fetal origin (Siiteri and MacDonald, 1966; France, 1979). Fetal adrenal DHEA and 16_{α}-hydroxydehydroepiandrosterone sulfate produced from acetate and some maternal cholesterol, are transferred to the placenta and converted to E_1 and E_2 or E_3, respectively (France, 1979). Fetal involvement in placental endocrine activity in the pig is demonstrated by the increased conversion of fetally-administered 19-carbon steroids to E_1 appearing in the maternal urine (Fevre, 1970).

The porcine blastocyst exhibits steroidogenic capability early in gestation. Biochemical evidence of aromatase, 17-20 desmolase and 3-sulfatase, within blastocyst tissue, suggests that the presence of unconjugated E and P_4 in blastocyst tissue is the result of local production, not diffusion from material circulation (Perry, et al., 1973). Blastocysts initiate E synthesis on approximately d 11 of gestation (Perry et al., 1973; Flint et al., 1979; Gadsby et al., 1980; Heap et al.,

1981ab). The onset of blastocyst E production coincides with the initiation of the sequence of events leading to maternal recognition of pregnancy (Dhindsa and Dzuik, 1968), blastocyst elongation (Perry and Rowlands, 1962; Heap et al., 1979; Anderson, 1978; Geisert et al., 1982ab) and the sequestering of histotrophe within the uterine lumen (Bazer and Thatcher, 1977; Zavy et al., 1980; Geisert et al., 1982b). The maternal recoginition of pregnancy involves a mechanism whereby the developing conceptus signals its presence to the mother resulting in the arrest of the normal ovarian cycle and the prolonged activity of the corpora lutea (Perry et al., 1973). Estrogens of fetal origin act as the luteotrophic signal by altering the direction of luteolytic prostaglandin $F_{2\alpha}$ secretion by the endometrial glands from an endocrine to an exocrine direction (Frank et al., 1977; Moeljono et al., 1977; Thatcher and Bazer, 1977; Zavy et al., 1980). Significant increases in E_1SO_4 concentration in the utero-ovarian vein plasma occur between d 11 and 12, which coincides with the initiation of blastocyst E production, then E_1SO_4 concentration declines until d 16 (Stoner et al., 1981). Conversion of E by sulfoconjugation to a biologically-inactive form before entering the circulation suggests that blastocyst E may exert a local effect on the uterine endometrium (Geisert et al., 1982). Neither the maternal ovaries nor the pituitary is required for the production of E_1SO_4 in the pig (Fevre et al., 1970). Estrogen may be secreted by the blatocyst as E_1SO_4 with the subsequent conversion of part to biologically- active, unbound, E by the endometrium as suggested by the existence of high concentrations of E_1SO_4 in the

allantoic fluid, but not ovarian venous blood, between d 20 and 35 of gestation (Lunaas et al., 1973; Robertson and King, 1974). Furthermore, this suggests that maternal plasma E_1SO_4 is derived from the conceptus (Robertson and King, 1974). Local uterine effects of E could be ellicited by the diffusion of unconjugated E from the blastocyst to the uterus, with subsequent sulfation of the E within the endometrium, as suggested by the findings of Perry et al. (1973) that in vitro d 16 blastocysts do not produce conjugated E. However, detectable increases in unconjugated and conjugated E in peripheral circulation of pregnant pigs occur by d 16 of gestation (Robertson and King, 1974, 1978).

The fact that early maternal plasma E_1SO_4 is of embryonic origin raises the possibility of a relationship between plasma E_1SO_4 concentrations and litter size (Robertson et al., 1978; Hattersley et al., 1980). There is a positive association between maternal plasma E_1SO_4 concentrations at d 30 of gestation and litter size at parturition in swine; therefore, early characterization of maternal plasma E_1SO_4 concentrations can be useful in reproductive management to identify non-pregnant animals and those with small litters (Horne et al., 1983; Stoner et al., 1986).

Potential steroid precursor(s) available to the trophoblast for E synthesis appear to be produced through endometrial conversion of P_4 to unconjugated androgens (androstenedione and T) and conjugated E (E_1SO_4 and E_2SO_4) (Bazer et al., 1979). Enzymes involved in this conversion are aromatase 3-HSD-isomerase

and sulfatase (Hobkirk, 1979a). The sow is dependent upon P_4 of the corpora lutea for maintenance of pregnancy (du Mesnil du Boisson and Dauzier, 1957). Plasma P_4 concentrations in the pregnant gilt drop from d 14 to 28 of gestation; decrease steadily until term; then drop sharply on the day of parturition (Robertson and King, 1974). The absence of a rise in maternal plasma P_4 in the pregnant sow suggests that the conceptus does not contribute materially to the maternal pool of P_4 (Robertson and King, 1974). The 30 to 70% decrease in plasma P_4 between d 14 and 30 of gestation (Guthrie, 1972; Robertson and King, 1974) could be due to either partial regression of the corpora lutea or rapid P_4 matabolism by the pregnant uterus (Bazer et al., 1977).

There is significantly more plasma P_4 entering the uterus via the uterine artery, going into the uterus, than leaving the uterus via the uterine vein at all stages of gestation, indicating that P_4 is being taken up and possibly metabolized by some element(s) of the pregnant uterus (Knight et al., 19771, Kukoly, 1984; Jeantet, 1985). The presence of high levels of P_4 in fetal circulation supports the role of the porcine placenta in P_4 synthesis (Barnes et al., 1974; MacDonald et al., 1980). In vitro, there is substantial placental P_4 production throughout gestation in the pig (Kukoly, 1984; Jeantet, 1985). Progesterone accumulation in vitro increased steadily from d 25 to 100. These increases could be due either to

increased availability of endogenous precursors or to maturation of the enzyme complexes of the placenta (Kukoly, 1984). Kukoly (1984) reported no placental P_4 production after 1 h of incubation, suggesting that the de novo synthesis of P_4 was from endogenous pregnenolone, the supply of which was exhausted as time in incubation increased. However, Jeantet (1985) reported continued placental P_4 production at 30, 60 and 90 d throughout the 2 h incubation period, suggesting that either endogenous pregnenolone or cholesterol in the incubation media could be utilized by placental tissue in P_4 production. Progesterone synthesis by porcine tissue at 30, 60 and 90 d of gestation was two-times greater when pregnenolone was added to the incubation medium as a precursor for P_4 synthesis (Jeantet, 1985). The steroidogenic capacity of the placenta to produce P_4 from pregnenolone is supported by histochemical evidence of $\Delta^5 3\beta$-HSD, the enzyme responsible for this conversion along with 3-ketosteroid isomerase in placental tissue from approximately d 28 to 101 of gestation (Christie, 1968; Dufour and Raeside, 1969). At d 60 and 90 of gestation, the addition of cAMP and pregnenolone increased in vitro placental P_4 production over the addition of pregnenolone alone (Jeantet, 1985). In vitro endometrial tissue has a limited capacity for P_4 production on d 30, 60 and 90 of gestation, although the necessary enzymes are present. The addition of pregnenolone or pregnenolone plus cAMP to the incubation medium significantly increased endometrial P_4 production (Jeantet, 1985). Lower in vitro endometrial

P_4 production, in comparison to in vitro placental P_4 production may be due to: 1) limited precursor availability, 2) metabolism of P_4 to less active derivatives such a pregnanolone and pregnanediols (Henricks and Tindall, 1971), or 3) steroidogenic dependence of the endometrium on the conceptus (Jeantet, 1985). Allantoic fluid P_4 increases as gestation progresses; whereas, amniotic fluid P_4 concentration is relatively static throughout gestation (Knight et al.,1977; Goldstein, 1977; Kukoly, 1984; Jeantet, 1985).

The concentration of E_1 is significantly greater than that of E_2 in porcine plasma throughout gestation; therefore, E_1 is the major unconjugated E in the pregnant pig. In vivo E synthesis is unaffected when the ovarian source of P_4 is surgically removed and pregnancy is maintained with MPA, a non-aromatizable progesterone (Heap et al., 1981b) indicating that ovarian P_4 is not essential for trophoblast E synthesis. The placenta has been implicated as the primary E source (Velle, 1960; Raeside, 1963; Molokwu and Wagner, 1973; Choong and Raeside, 1974; Robertson and King, 1974; Perry et al., 1976; Knight et al., 1977). Plasma concentrations of E_1 and E_2 are significantly greater in the uterine vein than in the uterine artery, indicating that some element(s) of the pregnant porcine uterus are the source of these E (Knight et al., 1977; Kukoly, 1984; Jeantet, 1985). The extremely high concentrations of E_1 and E_2 in allantoic fluid compared to relatively low plasma concentrations support the role of the porcine placenta as the primary source of E

(Knight et al., 1977; Kukoly, 1984; Jeantet, 1985). Ainsworth and Ryan (1966) demonstrated the conversion of DHEA and androstenedione, with E_1 being the major E formed by porcine placental tissue in late gestation.

Plasma E_1 and E_2 are first present in measurable quantities on d 80 of gestation and increase to a peak just before parturition. Following parturition, plasma E levels rapidly decrease (Robertson and King, 1974). Plasma E_1SO_4 concentrations, measurable on d 16 of gestation, increase dramatically to a peak between d 23 and 30; levels decline rapidly until approximately d 46 then increase again to a peak just before parturition; plasma E_1SO_4 concentrations decreases rapidly following parturition (Robertson and King, 1974). Between d 23 and 30, the ratio of plasma E_1SO_4 to E_1 is extremely high at approximately 200:1, approaches unity around d 70 and finally, at peak concentration, just before parturition, is approximately 1:2 (Robertson and King, 1974).

Perry et al., 1976 attributed the early increase in maternal plasma E concentration to an increase in placental mass rather than a change in the level of steroidogenic activity. However, Kukoly (1984) and Jeantet (1985) demonstrated a dynamic change in E_1 production by similar quantities of placental tissue in vitro as gestation progressed. Placental E_1 production exhibits a distinct biphasic pattern on d 30 and d 90 to term with exponential increases in E_1 concentration occurring during the latter half of gestation (Kukoly, 1984; Jeantet, 1985). Addition of

pregnenolone or pregnenolone and cAMP to the incubation medium results in greater placental E_1 production at d 60 and 90 of gestation (Jeantet, 1985). Cyclic AMP has an additive effect above that produced by pregnenolone alone suggesting that cAMP either increases available P_4 for conversion to androgens and subsequent aromatization to E, or that cAMP increases aromatase activity (Jeantet, 1985). Because there was no change in E_1 production over time in incubation despite available P_4, Kukoly (1984) concluded that conversion of P_4 to E did not occur in placental tissue in vitro . This result agrees with earlier findings that while blastocysts have the ability to synthesize E from P_4 (Perry et al., 1973; Heap et al., 1979), porcine placentae of late gestation do not (Ainsworth and Ryan, 1966). Porcine placental microsomes of late gestation demonstrate aromatase and 17ß-HSD activity, but no 17,20-desmolase activity; therefore, it cannot produce E from the P4 but must start from androgens (Ainsworth and Ryan, 1966). Aromatase converts androgens to E; 17ß-HSD interconverts to E_1 and E_2 and 17, 20-desomolase converts 17_α-hydroxypregnenolone to androstenedione. Subsequent in vitro investigations of porcine tissue have demonstrated that both endometrial and placental tissue can actively metabolize steroids of the Δ^4 pathway; however, only the placenta possesses all the enzymes necessary for the synthesis of E from pregnenolone by the Δ^4 pathway (Craig, 1982). Estrone concentration of amniotic and allantoic fluid reflect changes in in vitro placental E_1 production suggesting that

the in vitro results resemble in vivo placental production (Kukoly, 1984).

Endometrial P_4 metabolism in pregnant females has been proposed as a source of precursors for E production. Porcine endometrium from pregnant sows is capable of synthesizing androgens and conjugated E from 21-carbon steroids on d 18 and 25 of gestation (Deuben et al., 1977; Bazer et al., 1979). These could then serve as precursors for E production. In vitro endometrial estrone production is similar to that of the placenta on d 30 and 60 of gestation; however, on d 90, endometrial production is greater than on d 30 and 60, but is one-fourth that of the placenta (Jeantet, 1985). In vitro endometrial E_1 production is enhanced in the presence of pregnenolone (Jeantet, 1985) indicating that the endometrium is capable of synthesizing E_1 from pregnenolone as well as from endogenous precursors on d 30, 60 and 90 of gestation. The lack of T in the media following incubation of placental tissue suggests rapid aromatization to E_1 and E_2 (Kukoly, 1984; Jeantet, 1985). The conversion of 19-carbon steroids to E has been well documented in the porcine placenta under in vitro and in vivo conditions (Ainsworth and Ryan, 1966; Fevre, 1970; Perry et al., 1973, 1976). Between d 18 and 25 of gestation there is no consistent change in serum androstenedione concentrations while serum E_1SO_4 concentrations, of fetal origin, exhibit a progressive increase. Maximum androstenedione concentrations are observed 1 to 2 d following the E_1SO_4 maxima between d 28 and 30, when E_1SO_4 concentrations are already declining (Hattersley

et al., 1980). The reasons behind these relative changes are not known; however they suggest that if the androstenedione is also of embryonic origin, the drop in serum E_1SO_4 concentrations after d 30 is not primarily due to a lack of E_1 precursor (Hattersley et al., 1980).

The available reports on sulfatase and sulfotransferase activity in the sow have examined in vitro systems where sulfotransferase and sulfatase activities are co-existent. In in vitro pregnant porcine endometrium, sulfatase activity is low, hydrolyzing approximately 20% of the available tritium labelled E_1SO_4 until d 30 of gestation (Dwyer and Robertson, 1980). Sulfotransferase activity is elevated between d 12 and 28 of gestation, sulfating approximately 78% of the available tritium-labelled E_1 (Pack et al., 1979; Dwyer and Robertson, 1980). After d 30, sulfotransferase levels decline to 20% sulfation on d 60 (Dwyer and Robertson, 1980). In contrast, sulfatase activity increases sharply after d 30 to 80% hydrolysis between d 60 and 70 (Dwyer and Robertson, 1980). Examining ratios of percent sulfation to percent hydrolysis, Dwyer and Robertson (1980) reported that sulfotransferase activity predominates in the porcine endometrium during early pregnancy; however, after d 30, there is a gradual change in net activity to favor sulfatase. Circulating plasma steroid concentrations are in agreement with these reported enzyme activity levels. During early gestation, E_1SO_4 is the predominent plasma E. At mid-gestation, plasma E_1SO_4 and E_1 levels are equal, and near term, the predominent plasma E is E_1 (Robertson and King, 1974). It appears then that the

activities of sulfotransferase and sulfatase in the endometrium may play a role in determining the concentrations of unconjugated and sulfoconjugated E present in maternal and fetal fluids during pregnancy in the pig (Dwyer and Robertson, 1980).

MATERIALS AND METHODS

Twelve sexually mature crossbred gilts of similar age (11 to 14 mo), weight (100 to 140 kg) and genetic background were bred at estrus and randomly assigned to be bilaterally hysterectomized (Hyst-X) at either d 30, 60 or 90 of gestation. These days of gestation were chosen because earlier work in our laboratory (Kukoly, 1984)g which examined placental progesterone (P_4) and estrone (E_1) production at 12 stages of gestation between d 20 and 110 demonstrated that d 30, 60 and 90 were times of substantial and dynamic changes in steroid production. Four gilts were assigned to each day. The gilts, kept in outdoor lots of 6 to 12 animals, were checked daily for estrus at approximately 7:30 a.m. and were bred after exhibiting two consecutive estrous cycles of normal duration (18 to 22 d). Mature boars were used to aid in estrus detection. The gilts were bred immediately upon detection of standing heat using natural service and were serviced three additional times at 12, 24 and 36 h following first detection of standing heat. No gilt was bred by the same boar for two consecutive services. The first day of estrus was designated as d 0 of gestation. After being bred, the gilts were kept in groups of up to six pregnant animals housed in sheltered concrete pens. These gilts continued to be monitored for estrus exhibition; those animals failing to maintain pregnancy were culled and replaced.

Tissue Collection Procedures

All gilts were withheld from feed and water for approximately 25 h prior to Hyst-X. At surgery the gilt was restrained by snaring her by the snout and initial

anesthesia was induced by injection of one to one-half grains of sodium thiopental solution (Abbott Laboratory, Inc., Chicago, Illionis) in a 5% solution, into an ear vein using a 20-gauge butterfly needle attached by polyethylene tubing to a 30-ml syringe. As the gilt succumbed to the initial anesthesia, she was guided into a dorsal recumbant position in a specially designed cradle. The gilt in the cradle was lifted onto the surgery table where anesthesia was maintained at a surgical plane with halothane (Halocarbon Laboratories, Inc., Hackensack, NJ) vapor in a nitrous oxide:oxygen (1:2, v/v) mixture administered to the animal via tubes fitted into her nostrils. As the gilt was settling under the anesthesia, a blood sample was taken from the anterior vena cava (AVC) with a 15- gauge needle on a 20-ml syringe containing 13.8% disodium ethylenediamine tetraacetate (EDTA)as an anticoagulent.

Using sterile techinque, the reproductive tract was exposed following midventral laparotomy. Blood samples were collected from the uterine artery (UA) and uterine vein (UV) using a 20-gauge needle and 20-ml syringe. The blood was decanted into polypropylene tubes containing 13.8% EDTA. The blood was centrifuged at 2000 x g for 10 min; 6 ml of the plasma was decanted into 16x67 mm polypropylene culture tubes and stored at -20 C. Following collection of the blood samples, the ovaries, oviducts, uterine horns, uterine body and anterior cervix were surgically removed from the peritoneal cavity as previously described by Knight et al. (1977) and Dalton (1980). Briefly, the blood supply to the portion of the tract to be removed was diverted by ligating the three main branches of the blood supply to this area: the ovarian, cranial uterine and middle uterine arteries and veins. Each of these branches was ligated with several strands of 1 cm umbilical tape (Ethicon,

Inc., Somerville, NJ) to ensure minimal internal bleeding following removal of the reproductive tract. The incision in the body wall was closed in 3 layers. The linea alba was sutured with continuous interlocking pattern of size 1 chromic gut (Ethicon, Inc., Somerville, NJ). The subcutaneous fat was sutured with a continuous lockstitch pattern, also with size 1 chromic gut. The dermis was sutured in a interrupted horizontal mattress pattern of size 3 chromic gut. The suture line was then treated with Furox (Smithkline Animal Health Laboratories, Philadelphia, PA), followed by Wound-Kote (Morton, Inc., Memphis, TN). The animal was removed from anesthesia and following a 24 h recovery period was placed into a lot of cull animals. These gilts were kept for at least 4 wk following surgery before leaving the Virginia Tech Swine Center.

The excised uterus was bathed in tepid tap water and dissected free of the ovaries, oviducts and supporting broad ligament. The uterine horns were opened at the cranial ends by an incision along the border of the previous site of broad ligament attachment. The fetal membranes, the chorioallantois and amnion, and viable fetuses were manually separated from the endometrium, one fetal unit at a time. The amnion containing the fetus was then separated from the chorioallantois. Random samples of the chorioallantois and random samples of the endometrium which had been in direct apposition to viable placentas and had been separated from the myometrium, were immediately placed, by tissue, into sterile 500 ml beakers containing approximately 200 ml Medium 199 (M199, Gibco Laboratories, Grand Island, NY; Appendix A) at 37 C. Tissue was collected from more than one fetus and on d 60 and 90 when visual determination of sex was possible, tissue was collected from at least one male and one

female. Following tissue collection, the placental and endometrial tissue samples were transported to the laboratory for incubation. (See Appendix J for suggestions on imporving procedures).

Tissue Incubation Procedures

In the laboratory, the placenta and endometrium samples were manually minced into approximately 2 mm^3 pieces using sterile razor blades. This tissue preparation occurred within 1 h of tissue collection. The minced tissues of each pig were pooled by tissue type into two pairs of sterile 100 ml beakers containing approximately 50 ml of M199. One pair of beakers containing placenta and endometrium respectively was microwaved at full power for 1.5 min to kill the tissue. The use of dead tissue in the incubation to generate control or blank values was suggested by the procedure of Hoversland et al. (1982) which used enzyme activity of boiled embryos as control values. Tissue samples were weighed on a Torsion balance; 300 mg samples were incubated in duplicate in 16x100 mm polypropylene culture tubes at 37 C in a Dubnoff shaking water bath under an atmosphere of 95% O_2: 5% CO_2 for either 0.5, 1, 2 or 3 h. A control group of culture tubes was placed immediately into an ice bath to stop enzymatic activity.

The tissue samples were incubated with tritium (^3H)- labelled steroids (New England Nuclear Division of DuPont, Boston, MA) as markers of enzymatic activity (Appendix B). Three variations of incubation media were used. In order to evaluate aromatase activity, tissue was incubated using a modification of the procedure of Hoversland, et al. (1982), in culture tubes containing 0.5 uCi [1 ß, 2ß-$^3H(N)$]-T

(specific activity = 41.6 Ci/mmol) and 3 ml M199 plus T (.5 ug/ml). A total of eight tubes were run at each time period, duplicates of each tissue type: live placenta, live endometrium,microwaved placenta and microwaved endometrium. In order to evaluate E_1 sulfotransferase activity, 1 uCi [2,4,6,7-^{3}H(N)]-E_1 (specific activity = 88.5 and 87.5 Ci/mmol) was added to each of four tubes at each time period. In order to evaluate E_1 sulfatase activity, 1 uCi [6,7-^{3}H(N)]-E_1SO_4 (specific activity = 52.5 and 40.0 Ci/mmol) was added to four different culture tubes at each time period containing duplicate placenta and endometrium samples. This is a modification of the procedure of Dwyer and Robertson (1980). Three ml M199 was added to each tube containing either ^{3}H-E_1 or ^{3}H-E_1SO_4. Duplicate tubes containing either ^{3}H-E_1 or ^{3}H-E_1SO_4 and 3 ml M199 but no tissue were incubated with the rest of the samples for either 0 or 3 h. These tubes served as control tubes indicating degree of spontaneous sulfation or hydrolysis of the tritiated steroid markers. Following incubation, the culture tubes were capped, placed into an icebath and subsequently stored at -20 C.

Assay of Aromatase Activity

Aromatase activity of the placenta and endometrium was determined using an assay developed by T. Rabe, et al. (1982). This assay depends upon the release of tritiated water (^3H_2O) with the aromatization of ^{3}H-T. Tritiated water content is determined by a two-phase scintillation technique dependent upon the limited emulsifying capacity of a dioxane-based scintillation solution (Appendix C). Following

centrifugation at 3500 rpm (Damon International Equipment Co. IEC DPR-6000 refrigerated centrifuge) or 2800 rpm (International Equipment Co. IEC Centra-7R refrigerated centrifuge) for 30 min, the incubation media was decanted off the protein sample into 12 x 75 mm polypropylene culture tubes. Fifty ul of media was suspended in 5 ml of the dioxane based scintillation cocktail. Samples were counted for 10 min each on a liquid scintillation counter (LKB Wallac Rackbeta; ^{3}H counting efficienty = 69%) programmed by an internally loaded quench curve of ^{3}H-androstenedione (New England Nuclear Division of DuPont, Boston, MA) and methylene chloride (HPLC grade, Fischer Scientific Co., Fair Lawn, NJ) to give sample values in dpm; (Appendix D). Following scintillation counting, 1 ml distilled water was added to each vial. After addition of the water, Rabe reported 95% of the tritiated water is partitioned into the aqueous phase with the ^{3}H-T remaining suspended in the scintillation cocktail. The difference in dpm before and after the addition of the distilled water is equivalent to the amount of $^{3}H_2O$ in the media sample. Results for aromatase activity per sample are reported as pg T aromatized per sample.

The following sequence of formulas was used to determine this value:

$$2 \times \text{dpm } {}^{3}H_2O = \text{dpm } {}^{3}H\text{-T}*$$

*Rationale: Each $^{3}H_2O$ unit produced = one unit of ^{3}H-T which is aromatized. The numerator is doubled to account for the double label on the ^{3}H-T; only one of which is released as $^{3}H_2O$.

$$\text{dpm } {}^{3}H\text{-T}/(\text{dpm } {}^{3}H\text{-T}/\text{pmole } {}^{3}H\text{-T}) = \text{pmole } {}^{3}H\text{-T}$$

$$(\text{pmole } {}^{3}H\text{-T})(\text{pg } {}^{3}H\text{-T}/\text{pmole } {}^{3}H\text{-T}) = \text{pg } {}^{3}H\text{-T}$$

dpm ^{3}H-T/pmole = 9.235×10^{4}

pg ^{3}H-T/pmole = 292.44

Assay of Estrone Sulfotransferase and Estrone Sulfatase Activity

Estimation of relative levels of E_1 sulfotransferase and E_1 sulfatase activity was performed by determining conversion of ^{3}H-E_1 and ^{3}H-E_1SO_4 within the incubation media to ^{3}H-E_1SO_4 and ^{3}H-E_1, respectively. The procedure used in this assay was a modification of that given by Dwyer and Robertson (1980).

Following centrifugation at 2800 or 3500 rpm for 30 min, the incubation medium was decanted off the protein samples into 12 x 75 mm polypropylene culture tubes. The tritium- labelled steroids were extracted from 0.5 ml incubation medium with 3 ml tetrahydrofuran:ethyl acetate (1:1, v/v; HPLC grade, Fischer Scientific Co., Fair Lawn, New Jersey) in 16 x 100 mm borosilicate culture tubes. These were vortexed for 1 min. One ml of 0.5 M sodium bicarbonate saturated with sodium chloride was added to each tube following vortexing. The aqueous phase was frozen in solid CO_2 and ethanol; the organic phase containing the ^{3}H-E_1 and ^{3}H-E_1SO_4 was decanted into clean 16 x 100 mm borosilicate culture tubes. Following evaporation of the organic solvent, the sides of the tubes were rinsed with 1 ml methylene chloride:methanol (9:1 v/v) which was then evaporated to dryness under air. The steroid residue in the tubes was redissolved in 50 ul methanol, vortexed for 30 s and applied to 1 channel of a 19 channeled thin layer chromatography (TLC) plate (J.T. Baker Chemical Co., Phillipsburg, NJ). Tritium labelled E_1 and E_1SO_4 standards (3

ul) were run with each plate to monitor steroid migration patterns. After thorough sample drying, the plates were developed three times in a solvent of methylene chloride:diethyl ether:methanol (5:2:0.5, v/v) in a developing tank lined with filter paper. The filter paper ensured adequate vapor equilibration. The plates were permitted to dry thoroughly between the three developing times. A solvent volume sufficient to wet only the lower 3 mm of the preadsorbent area was used. Following plate development, the TLC plates were given time to dry thoroughly and were then covered with a thin layer of EN^{3}HANCE spray (New England Nuclear Division of DuPont, Boston, MA), a surface autoradiography enhancer for visualization of the low level beta-emitting tritium labelled E_1 and E_1SO_4. The TLC plates were placed into Kodak X-Ray Exposure Holders with 8 x 1 in Kodak X-Ray film (Eastman Kodak Co., Rochester, NY). The film was exposed to the TLC plate for 12 h at -70 C. The X-ray film was developed (Appendix E) and used as a template for detecting the location of ^3H-E_1 and ^3H-E_1SO_4 on the TLC plates.

For each sample on the TLC plate, the zones of ^3H-E_1 and ^3H-E_1SO_4, which appeared as darkened bands on the X-ray film, were removed from the TLC plates using a metal spatula. The steroid containing silica gel from the plates was aspirated as it was chiseled away into a pasteur pipette containing a glasswool filter which trapped the gel but permitted air to pass through into vacuum lines (Thompson and Siiteri, 1974). Each steroid was eluted from the pasteur pipette with 2 ml methanol into mini PolyQ scintillatlion vials (Beckman, Somerset, NJ). The methanol in the vials was allowed to evaporate to dryness under air leaving the hormone residue

behind, which was then suspended in 5 ml Recovery Scintillation Cocktail (Appendix C). The vials were counted for 5 min each on a liquid scintillation counter (^{3}H counting efficiency = 67.3%; Beckman LS 1800, Beckman Instruments Inc., Irvine, CA). Counts per minute were converted to dpm via a quench curve of ^{3}H androstenedione using methylene choride as a quenching agent (Appendix D). Total recovery of ^{3}H-E was 99.5% and of E_1SO_4 was 95.7%. Data was corrected for recovery rate as well as for spontaneous hydryolysis of ^{3}H-E_1SO_4 or sulfation of ^{3}H-E_1. The values of spontaneous activity for 0.5, 1 and 2 h were interpolated from the values determined at 0 and 3 h from the media samples incubated with the tritium labelled steroids but no tissue samples (Tables 1 and 2). In this experiment, gilts 1 through 9 were run on a different batch of hormones than gilts 10 through12. The tissue from gilts 1 and 2 was not incubated with the 0 and 3 h control tubes; therefore, the average correction values from gilts 3 through 9 were used for the data from these two gilts. For gilt 11, the average correction values of gilts 10 and 12 was used.

Percent enzyme activity per sample was calculated as follows:

$$\% \text{ Estrone sulfotransferase activity} = \left[\frac{\text{DPM } ^{3}H\text{-}E_1SO_4}{(\text{DPM } ^{3}H\text{-}E_1 + \text{DPM } ^{3}H\text{-}E_1SO_4)}\right] X\ 100$$

TABLE 1.

Spontaneous Sulfation of E_1 (%)

	Gilt Number										
Incubation Time (h)*	3	4	5	6	7	8	9	Mean' 3 to 9	10	12	Mean' 10&12
0	0.4	0.8	1.5	0.4	0.6	1.7	2.9	1.2	0.8	4.9	2.8
0.5	0.5	0.8	1.7	0.5	0.8	1.6	2.6	1.2	0.8	6.1	3.4
1	0.5	0.8	1.9	0.6	0.9	1.6	2.4	1.2	0.8	7.3	4.0
2	0.6	0.8	2.4	0.8	1.2	1.5	1.9	1.3	0.8	9.7	5.2
3	0.6	0.8	2.8	1.0	1.6	1.4	1.4	1.4	0.8	12.1	6.4

*Values for 0.5, 1 and 2 h were interpolated from those determined for 0 and 3h.

'The ^3H-E_1 used to assess sulfotransferase activity of tissue from gilts 1 through 9 was from a different batch than that used for gilts 10 through 12. Control tubes were not included in the tissue incubations for gilts 1, 2 and 11; therefore, the mean spontaneous sulfation (%) for the batch was used to correct the data generated by these three gilts.

TABLE 2.

Spontaneous Hydrolysis of E_1SO_4 (%)

Gilt Number

Incubation Time (h)*	3	4	5	6	7	8	9	Mean† 3 to 9	10	12	Mean† 10 &12
0	13.2	10.5	9.0	10.6	12.1	10.7	12.6	11.2	2.1	0.3	1.2
0.5	12.6	10.2	9.1	12.8	12.0	10.7	11.6	11.3	1.9	0.6	1.2
1	11.9	9.9	9.3	14.9	12.0	10.7	10.6	11.3	1.7	1.0	1.4
2	10.6	9.3	9.5	19.2	11.9	10.6	8.6	11.4	1.3	1.7	1.5
3	9.4	8.8	9.8	23.5	11.7	10.6	6.7	11.5	0.9	2.5	1.7

*Values for 0.5, 1 and 2 h were interpolated from those determined at 0 and 3 h.

†The ^3H-E_1SO_4 used to assess spontaneous sulfatase activity of tissue from gilts 1 through 9 was from a different batch than that used for gilts 10 through 12. Control tubes were not included in the tissue incubations for gilts 1, 2 and 11; therefore the mean spontaneous hydrolysis (%) for the batch was used to correct the data generated by these three gilts.

$$\% \text{ Estrone sulfatase activity} = \left[\frac{DPM\ {}^{3}H\text{-}E_1}{(DPM\ {}^{3}H\text{-}E_1 + DPM\ {}^{3}H\text{-}E_1SO_4)}\right] X\,100$$

Soluble Protein Determination

Soluble protein present in the placental or endometrial sample was determined following separation from the incubation medium. Tissue was stored prior to this in the original 16 x 100 mm culture tubes at -20 C. Tissue was homogenized for 1.5 min using a tissue homogenizer (Bankman Instruments). Placenta was suspended in 2 ml and endometrium in 4 ml of 0.9% saline prior to homogenization. Soluble protein was determined using the procedure of M.M. Bradford (1976) which is based on the observation that Coomassie Brilliant Blue G-250 dye (Appendix F) converts from the red form to the blue form upon protein-dye binding. The standard curve for this assay was made with Bovine Serum Albumin (BSA) in distilled water at 0, 10, 30, 50, 80 and 100 ug/ml. One hundred ul of standard or sample was placed into 16 x 100 mm borosilicate glass culture tubes followed by 5 ml of the Coomassie Brilliant Blue. The tubes were then vortexed for 30 s. The absorbance at 595 nm on a Perkin-Elmer Lambda 3 spectrophotometer was measured after 2 min but within 1 h of adding the dye-binding protein reagent to the protein sample. Protein concentration per sample was calculated on a IBM personal computer using a program based on linear regression devised in our laboratory (G.L. Johnson, unpublished). Each sample was run in duplicate and the average reported as soluble protein concentration per sample.

Assays of Plasma Steroid Concentrations

Plasma T, E_1, and E_1SO_4 concentrations from the AVC, UA and UV were determined by radioimmunoassay (RIA) procedures modified from those of Chung-Hsui Wu et al. (1971; see Appendix G for crossreactivity of the T and E_1 antibodies (Ab) used in these assays. Testosterone Ab was provided by H.D. Hafs, Michigan State University (MSU #74). Estrone Ab was kindly provided by R.E. Staigmiller, USDA Agricultural Experimental Station, Miles City, MT). Both Abs were validated in our laboratory for use with bovine and porcine serum and M199.

Testosterone:

Testosterone was extracted from 200 ul samples of plasma with 5 ml hexane:ethyl acetate (1:1, v/v; Fischer Scientific Co., Fair Lawn, NJ) in 16 x 100 mm borosilicate culture tubes. The tubes were vortexed for 1 min then frozen in solid CO_2 and ethanol. The organic layer, containing T, was decanted into 12 x 75 mm borosilicate culture tubes. The organic layer was permitted to evaporate leaving the T residue behind in the tube. A standard curve containing T dissolved in ethanol in concentrations (ng/ml) of 00, 0, .005, .01, .025, .05, .1, .25, 1.0 and an excess dose (to saturate Ab binding sites) was run in duplicate with each assay. One hundred ul of each standard was pipetted into 12 x 75 mm borosilicate culture tubes in duplicate. One set of standards was run at the front andthe other at the back of the assay. One hundred ul of a 1:40,000 dilution of T Ab:Phosphate Buffered Saline (PBS-Ga; Appendix H) was added to each tube followed by 100 ul ^{3}H-T (10,000 cpm in PBS-Ga). The tubes were shaken and incubated at 4 C for 12 to 18 h.

Following the incubation period, the following steps were completed within a 10 min period: 1 ml cold deionized H_2O was added to the 00 tubes, which are indicative of the total binding potential of the T Ab for ^3H-T; 1 ml cold dextran coated charcoal solution (DCC; Appendix I) was added to the remaining tubes and all the tubes were placed into a refrigerated centrifuge The tubes were centrifuged for 10 min at 2800 rpm at 4 C. This step resulted in the precipitation of the DCC and the unbound ^3H-T. One -half ml of the supernatant containing T Ab bound to the ^3H-T and plasma-extracted-T was decanted into scintillation vials with 5 ml of Assay Scintillation Cocktail (Appendix C). Each vial was counted for 5 min on a Beckman LS 1800 scintillation counter. Counts per minute were converted to steroid concentration (ng/ml) using the standard curve generated with each individual assay by a personal computer program based on Logit-Log methods developed by D. Rodbard and modified in our laboratory (G.L. Johnson, unpublished).

Estrone and Estrone Sulfate:

Estrone and E_1SO_4 were extracted from the plasma using the procedure described by Hattersley et al. (1980). The same plasma sample was used to assay for the concentration of both of the above steroids. Duplicate 500 ul plasma samples were extracted with 5 ml diethyl ether and were then frozen in solid CO_2 and ethanol. The E_1-containing organic phase was decanted into 12 x 75 mm borosilicate culture tubes and the ether was permitted to evaporate to dryness under air. Following thawing and ether evaporation, the aqueous phase was saturated with sodium chloride

(200 mg/vial), then extracted with 5 ml tetrahydrofuran. These samples were vortexed for 1 min and frozen in solid CO_2 and ethanol. The organic phase, containing E_1SO_4 was decanted into 12 x 75 mm borosilicate culture tubes. The tetrahydrofuran was permitted to evaporate to dryness under air. Recovery of E_1SO_4 was 83.8%; recovery of E_1 in the tetrahydrofuran was 1.4%. The sides of the tubes were rinsed with 1 ml methylene chloride:methanol (1:1, v/v) which was then permitted to evaporate. Triethanolamine buffer (200 ul; 0.5 M, pH 7.3) containing 400 units of sulfatase (Helix pomatia Type H-1 #S-9626, Sigma Chemical Co., St. Louis, MO) was added to each E_1SO_4 residue-containing tube. The tubes were shaken, covered and incubated for 18 h at 37 C. This resulted in the hydrolysis of the E_1SO_4. Following incubation, 100 ul of the hydrolyzed mixture was pipetted into 12 x 75 mm borosilicate culture tubes. Five hundred ul of standard plasma samples known to have either high or low E_1 concentrations were extracted for E_1 and run with the standard curve of the RIA; duplicates of each plasma high and low standards were run at the front and at the back of the assay. These standards were run with each assay and the variation between the 4 samples of each standard was used to quantify intra-assay and inter-assay variation which was 11% and 20%, respectively. One hundred ul of a 1:8500 dilution E_1: PBS-Ga followed by 100 ul ^3H-E_1 (10,000 cpm in PBS-Ga) was added to each of the assay tubes which contained either: the E_1 residue, 100 ul of the hydrolyzed E_1SO_4

mixture, or the E_1-extracted high/low standards. These tubes were incubated at 4 C for 12 to 18 h. The assay was completed and steroid concentrations determined using the procedure given for the T RIA. The standard curve for E_1 and the hydrolyzed E_1SO_4 assays contained E_1 dissolved in ethanol in the following concentrations (ng/ml): 00, 0, .0025, .005, .01, .025, .05,. 1, .025, .5, 1.0 and an excess dose to saturate Ab binding sites. For the assay of the hydrolyzed E_1SO_4, 100 ul PBS-Ga was added to each tube of the standard curve and the high/low plasma samples to adjust the volume of these standards to that of the rest of the assay.

Statistical Analysis

The data for enzyme activity were analyzed using the General Linear Models (GLM) procedure of the Statistical Analysis System(SAS). Preliminary analyses were done by day of gestation, enzyme and tissue type and included tissue content as a continuous variable. These analyses indicated that enzyme activity was significantly influenced by protein content of the tissue. Activity per sample was adjusted to the mean protein content of each day of gestation and tissue type by enzyme assay using the following formulas:

Aromatase Activity per Sample:
Adjusted pg T aromatized = pg T - regression coefficient (protein (ug) - mean protein (ug).

Sulfotransferase or Sulfatase Activity per Sample:
Adjusted % activity = % activity - regression coefficient (protein (ug) - mean protein (ug).
(See Table 3 for regression coefficients and mean protein values.)

TABLE 3.

Regression Coefficients and Mean Protein content (ug)

Assay	Tissue		Regression Coefficient*	Mean Protein Content (ug)/ 300 mg tissue	n
Aromatase	D30	Placenta	- .0632	772.08	37
		Endometrium	- .1527	3489.21	37
		Micro.[a] Placenta	- .0035	1114.13	37
		Micro. Endometrium	.0139	1715.74	38
	D60	Placenta	.1923**	1080.32	34
		Endometrium	- .0405	3979.97	39
		Micro. Placenta	.1400	714.74	34
		Micro. Endometrium	.0073	1964.92	40
	D90	Placenta	.2030**	1518.47	38
		Endometrium	- .0621	3482.95	40
		Micro. Placenta	- .0188	514.03	36
		Micro. Endometrium	- .0130	1350.98	40
Sulfotransferase	D30	Placenta	- .0003	1083.70	30
		Endometrium	.0004	3337.84	37
	D60	Placenta	.0080	1077.71	35
		Endometrium	.0022	3895.59	37
	D90	Placenta	.0009	1578.50	39
		Endometrium	.0078	3601.98	39
Sulfatase	D30	Placenta	- .0120	986.16	32
		Endometrium	.0056	3488.84	38
	D60	Placenta	- .0031	986.70	33
		Endometrium	.0142	3959.00	38
	D90	Placenta	.0384**	1523.72	39
		Endometrium	- .0005	3706.21	38

*For aromatase activity, the regression coefficient is in pg T/ug protein.
For sulfotransferase and sulfatase activities, the regression coefficient is in % activity/ug protein.
[a]Micro. = Microwaved Tissue
** Indicates significance ($P \leq .01$).

The following statistical model was used to analyze enzyme activity per sample by day. of gestation, tissue type and enzyme and after adjustment for differences in protein content:

$$Y_{ijk} = \mu + (GILT)_i + (TIME)_j + (GILT * TIME)_{ij} + E_{ijk}$$

here μ is the mean enzyme activity; $(GILT)_i$ is the random effect of the i^{th} gilt; $(TIME)_i$ is the fixed effect of the j^{th} time in incubation (j = 0, 0.5, 1,2 and 3 h); $(GILT * TIME)_{ij}$ is the interaction of gilt and time in incubation, and $E_{(ij)k}$ is the random error associated with the i^{th} gilt and the j^{th} time in incubation. F-tests for TIME effects used the GILT*TIME interaction mean square. When time of incubation effects were significant (i.e., for endometrial aromatase activity on d 30, 60 and 90, placental aromatase activity on d 90, and placental and endometrial sulfatase activity on d 90), Student Newman-Kuells (SNK) tests were performed to test significant differences ($P \leq .05$) between the means of each incubation time. DAY effects were added to in the model to examine day of gestation effects, and the F-test was done using GILT(DAY) as the error term. TISSUE was included in the model to examine differences in tissue enzyme activity by day of gestation and by enzyme. The F-test for tissue used the GILT*TISSUE interaction as the error term.

Plasma steroid concentrations were analyzed for each steroid using GLM procedures of SAS. The following statistical model was used to analyze the data:

$$Y_{ijkl} = \mu + (DAY)_i + GILT(DAY)_{ij} + (VESSEL)_k + (DAY * VESSEL)_{ik} + E_{(ijk)l}$$

Where μ is the mean steroid concentration;$(DAY)_i$ is the day of gestation effect (i = 30, 60 and 90 d); GILT $(DAY)_{ij}$ is the effect of the j^{th} gilt (j = 1 to 4) nested in the effect of the j^{th} day of gestation; $(VESSEL)_k$ is the effect of the k^{th} blood vessel on steroid concentration (k = AVC, UA or UV); $(DAY*VESSEL)_{jk}$ is the effect of the blood vessel and day of gestation interaction and $E_{(ijk)l}$ is the random error associated with the k^{th} blood vessel of the j^{th} gilt within the i^{th} day of gestation. Orthogonal polynomial contrasts were performed in order to determine significant differences among days of gestation. Within day of gestation, SNK tests were performed to test significant differences among blood vessels. Orthogonal polynomial contrasts were performed within day of gestation and vessel to determine significant differences in concentrations among steroids.

RESULTS

Protein Content of Incubated Tissue Samples

Graphic illustration of the mean protein content of placental and endometrial tissue samples is presented by enzyme assay in figures 4,5, and 6. These figures illustrate changes in protein concentration by day of gestation. Mean protein content by tissue and by enzyme assay is presented in table 3. Protein content of placental tissue increased from d 30 and 60 to d 90. These results are consistent with those of Jeantet (1985) who examined placental activity on the same three days of gestation and with those of Kukoly (1984) who observed an increase in placental tissue protein content between d 25 and 100 of gestation. Protein content of placental tissue incubated in the aromatase assay increased in a linear manner ($P \leq .01$). Mean placental protein content was 772.08 ug on d 30, 1080.32 ug on d 60 and 1518.47 ug on d 90. There was no significant day of gestation effect on placental protein content in the assays of sulfotransferase and sulfatase activity. In the assay of sulfotransferase activity, mean placental protein content was approximately 1080 ug on d 30 and 60 and increased to approximately 1580 ug on d 90. In the assay of sulfatase activity, mean placental protein content remained constant between d 30 and 60 at approximately 986 ug and increased to approximately 1525 ug on d 90. Within a gilt, the pool of minced placental tissue used in each of the three enzyme assays was identical and separate tissue samples wereused for each time in incubation. Furthermore, in the assay of protein content per sample, samples were run in a random order. Therefore, the differences in mean placental content between

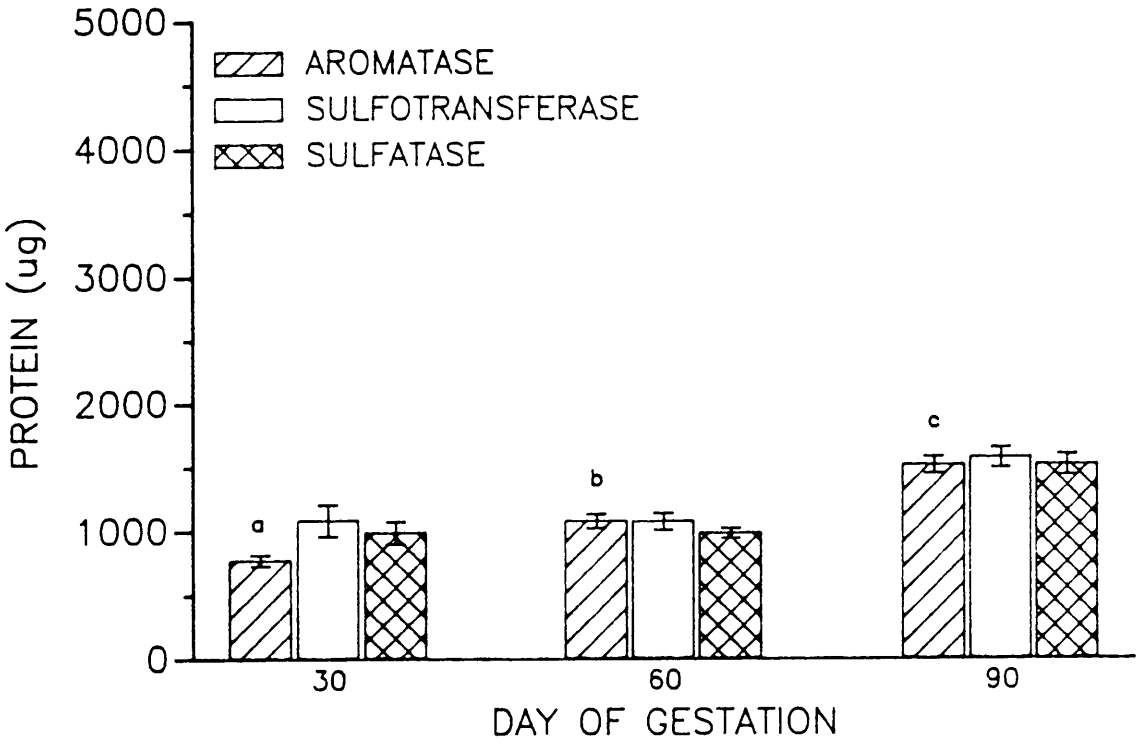

FIGURE 4. PROTEIN CONTENT (ug) OF PLACENTAL TISSUE USED TO ASSAY FOR AROMATASE, SULFOTRANSFERASE AND SULFATASE ACTIVITY ON D 30,60 AND 90 OF GESTATION.

^{abc} INDICATE SIGNIFICANT DIFFERENCES WITHIN ENZYME ASSAY ($P \leq .01$).

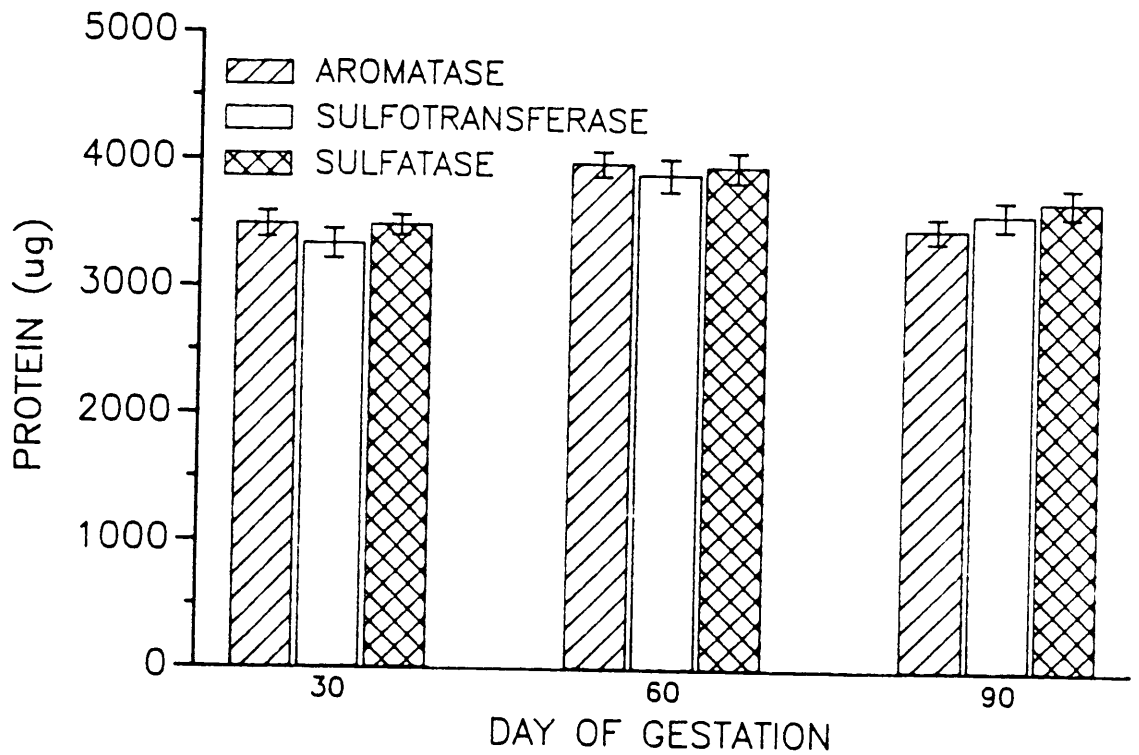

FIGURE 5. PROTEIN CONTENT (ug) OF ENDOMETRIAL TISSUE USED TO ASSAY FOR AROMATASE, SULFOTRANSFERASE AND SULFATASE ACTIVITY ON D 30,60 AND 90 OF GESTATION.

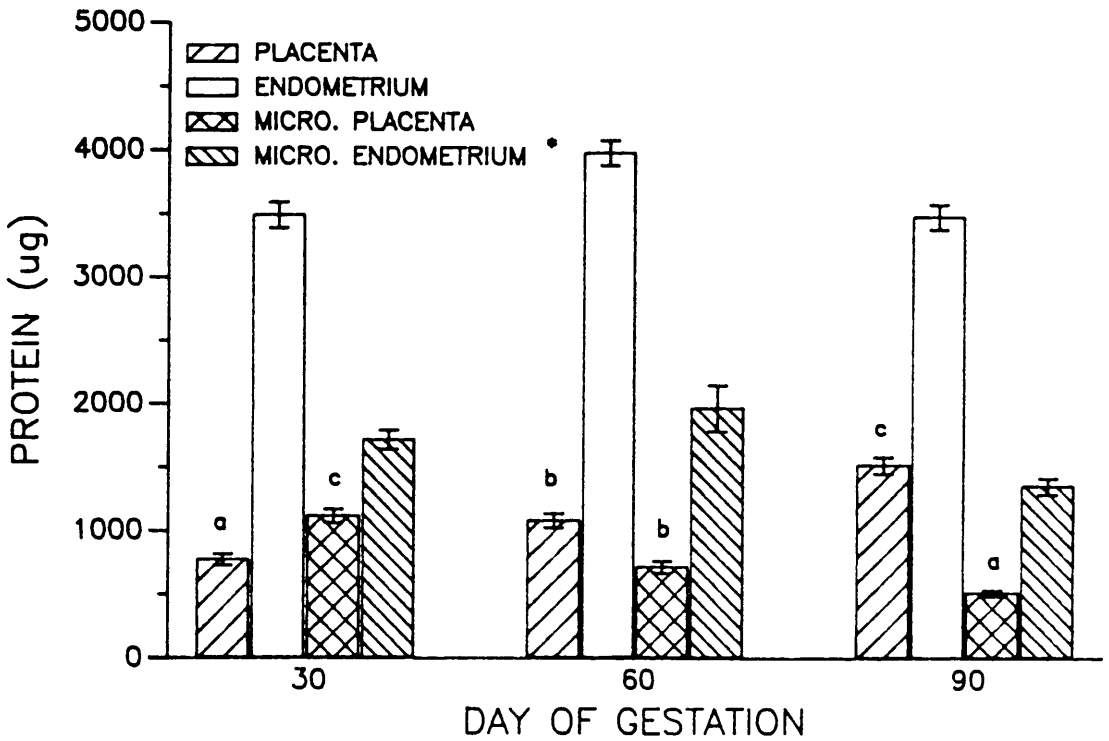

FIGURE 6. PROTEIN CONTENT OF TISSUES USED TO ASSAY FOR AROMATASE ACTIVITY ON D 30,60 AND 90 OF GESTATION.

abc INDICATE SIGNIFICANT DIFFERENCES WITHIN ENZYME ASSAY ($P<.05$) AND BETWEEN PLACENTA AND MICROWAVED PLACENTA ($P<.001$).

* PROTEIN CONTENT OF MICROWAVED ENDOMETRIUM IS SIGNIFICANTLY LESS THAN THAT OF ENDOMETRIUM($P<.05$).

the three enzyme assays are due to random variation. Likewise, differences in the pattern of change in mean placental protein content across day of gestation among the aromatase assay, where protein content exhibited a linear increase and the sulfotransferase and sulfatase assays, where protein content was the same on d 30 and 60 and then increased substantially on d 90, are due to random variation.

There was no significant effect of day of gestation effect on endometrial protein content. Protein content of endometrium was greater on d 60 than on d 30 or d 90, which is consistent with the observations of Jeantet (1985). Mean endometrial protein content in the assay of aromatase activity was approximately 3490 ug on d 30, 3980 ug on d 60 and 3480 ug on d 90. In the assay of sulfotransferase activity, mean endometrial protein content was approximately 3340 ug on d 30, 3895 ug on d 60 and 3600 ug on d 90. Finally, in the assay of sulfatase activity, mean endometrial protein content was approximately 3490 ug on d 30, 3960 ug on d 60 and 3700 ug on d 90. The differences in mean protein content of endometrial tissue among the three enzyme assays are due to random variation for the same reasons which were given for placental tissue. Protein content of endometrial tissue was greater than that of placental tissue on all three days of gestation examined.

Protein content of microwaved placental tissue, which was used only in the aromatase assay decreased from d 30 to d 90 ($P \leq .03$). Protein content of unaltered placental tissue was less than that of microwaved placental tissue on d 30 ($P \leq .0001$) and was greater on d 90 ($P \leq .0003$). The protein content of microwaved endometrial tissue followed the same pattern as unaltered endometrial tissue but was considerably less ($P \leq .05$).

In Vitro Aromatase Activity on d 30, 60 and 90 of Gestation

The total dpm recovered from media samples incubated with microwaved tissue were on the order of one-third to one-half that recovered from media samples incubated with unaltered tissue. This suggests a higher degree of affinity between ^{3}H-T and the microwaved tissue than between ^{3}H-T and unaltered tissue. In general, the calculated dpm of $^{3}H_2O$ was also lower in media samples incubated with microwaved tissue. This phenomenon could be explained by one or both of the following conditions: 1) lower aromatase activity in the microwaved tissue due either to loss of enzyme integrity or unavailability of ^{3}H-T substrate due to tissue affinity; and/or 2) a higher degree of affinity between $^{3}H_2O$ and microwaved tissue than with unaltered tissue. Aromatase activity in unaltered tissue was not corrected using values obtained from microwaved tissue since these values cannot be confidently classified as control values indicative of spontaneous ^{3}H loss to H_2O by ^{3}H-T. Mean values of T aromatized by microwaved tissue adjusted for sample protein content are given in tables 4,5 and 6. There were no significant effects of day of gestation tissue type, or time in incubation effects on aromatase activity in microwaved tissue (figure 7). Gilt effects on the activity in microwaved placenta were significant on d 60 ($P \leq .0001$) and gilt effects on the activity in microwaved endometrium were significant on d 30 ($P \leq .0063$) and 60 ($P \leq .0001$). Aromatase activity in microwaved tissue was substantially less than that of unaltered tissue on all days of gestation examined ($P \leq .002$; figure 7).

Mean values of T aromatized by unaltered tissue, adjusted for sample protein

TABLE 4.

Aromatization of Testosterone by Microwaved Placental Tissue Over a Three Hour Incubation Period at 30, 60, and 90 Days of Gestation

	Day of Gestation		
Incubation Time (h)	30	60	90
0	269.06 ± 28.58 (8)*	230.72 ± 43.62 (4)	210.63 ± 20.43 (7)
0.5	299.89 ± 28.58 (8)	349.08 ± 32.98 (7)	282.15 ± 20.43 (7)
1	252.72 ± 30.58 (7)	365.46 ± 32.98 (7)	218.11 ± 20.43 (7)
2	263.95 ± 33.02 (6)	388.89 ± 30.85 (8)	244.18 ± 20.43 (7)
3	305.26 ± 28.58 (8)	448.26 ± 30.85 (8)	278.31 ± 19.11 (8)

*Mean activity (pg T aromatized ; adjusted for protein content per sample) ± s.e.m. (n).

TABLE 5.

Aromatization of Testosterone by Microwaved Endometrial Tissue Over a Three Hour Incubation Period at 30,60 and 90 Days of Gestation

Incubation Time (h)	Day of Gestation 30	60	90
0	235.07±21.47(8)*	316.59±62.00(8)	226.53±13.46(8)
0.5	284.19±21.47(8)	354.48±62.00(8)	234.28±13.46(8)
1	219.95±21.47(8)	403.30±62.00(8)	219.13±13.46(8)
2	249.22±24.79(6)	402.72±62.00(8)	255.09±13.46(8)
3	232.68±21.47(8)	463.05±62.00(8)	259.94±13.46(8)

*Mean activity (pg T aromatized; adjusted for protein content per sample) ± s.e.m. (n).

TABLE 6.

Mean Aromatase Activity by Placental, Endometrial, Microwaved Placental and Microwaved Endometrial Tissue at 30, 60 and 90 Days of Gestation

	Day of Gestation		
Tissue	30	60	90
Placenta	462.13±18.43(37)*	507.71±19.22(34)	517.09±18.18(38)
Endometrium	1018.75±28.64(37)	1057.97±125.29(39)	1015.12±123.72(40)
Micro.Placenta	279.63±44.32(37)	371.23±46.24(34)	247.56±44.93(36)
Micro. Endometrium	243.96±76.94(38)	388.03±75.00(40)	238.99±75.00(40)

*Mean activity (pg T aromatized; adjusted for protein content per sample) ± s.e.m. (n).

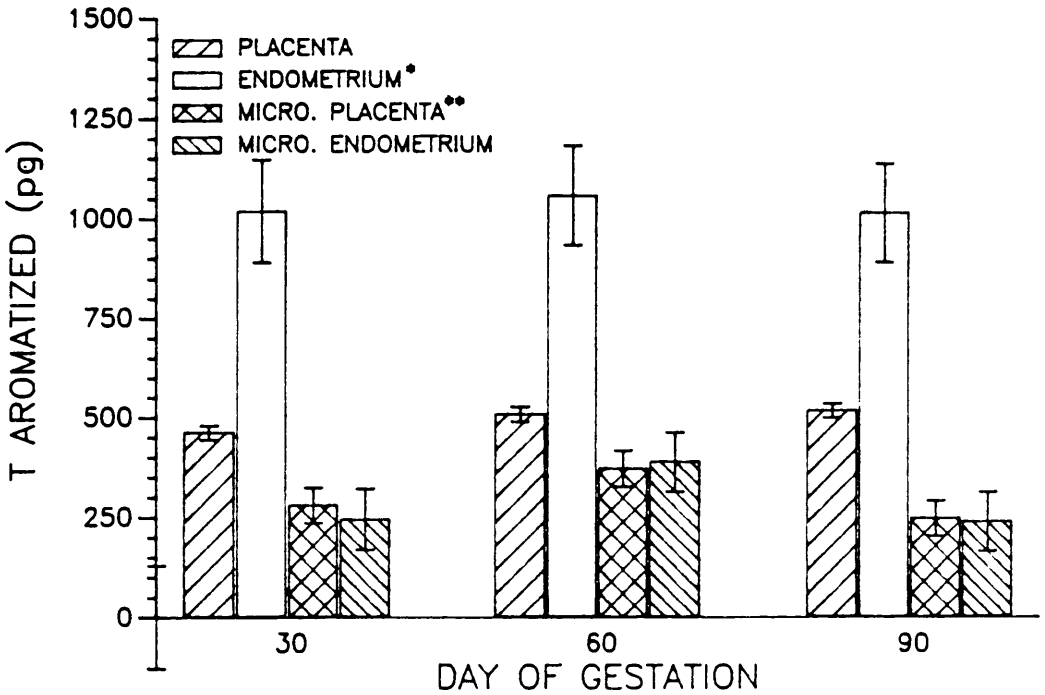

FIGURE 7. MEAN IN VITRO AROMATASE ACTIVITY ON D 30,60 AND 90 OF GESTATION.

* ENDOMETRIAL ACTIVITY WAS GREATER THAN PLACENTAL ACTIVITY ON ALL 3 DAYS($P<.002$).

** ACTIVITY IN MICROWAVED TISSUE WAS LESS THAN THAT OF UNALTERED TISSUE ($P<.002$).

content, are given in tables 7 and 8. There were no significant day of gestation effects on aromatase activity in either placental or endometrial tissue (figure 7). There were no significant gilt effects on placental aromatase activity. The gilt and time in incubation interaction had a significant effect on placental aromatase activity on d 30 ($P \leq .01$). On d 30 and 60 of gestation, in vitro placental aromatase activity was relatively stable over the 3 h incubation period. On both days, activity was slightly increased at 3 h compared to 0 h; however, this increase was not significant. In contrast to d 30 and 60, d 90 placental aromatase activity increased ($P \leq .0006$) from 390.58 pg T aromatized at 0 h to 733.84 pg T aromatized after 3 h in incubation (figure 8). Placental aromatase activity on d 90 was slightly lower at 0 h than that observed in placental tissue on d 30 and 60; however, d 90 placental tissue more than doubled its E production from T over the 3 h incubation period; while d 30 and 60 placental tissue exhibited little E production over the 3 h period. This suggests that by d 90 of gestation, porcine placental tissue has the enzymatic capability for prolonged conversion of T to E in vitro.

There were significant gilt effects ($P \leq .0002$) on endometrial aromatase activity on all three days of gestation examined. The gilt and time interaction had a significant effect on endometrial aromatase activity on d 30 ($P \leq .01$). Time in incubation significantly affected endometrial aromatase activity on d 30, 60 and 90 ($P \leq .0003$). Activity increased over the incubation period (figure 8). On d 30, in vitro endometrial aromatase activity increased in a linear fashion, aromatizing 440.81 pg T at 0 h and 1777.58 pg T after 3 h in incubation. On d 60, in vitro aromatase activity increased in an almost linear fashion; activity appeared to be

TABLE 7.

Aromatization of Testosterone by Placental Tissue Over a Three Hour Incubation Period at 30, 60 and 90 Days of Gestation

Time in Incubation(h)	Day of Gestation 30	60	90
0	458.55 ± 40.77 (7)*	444.10 ± 31.30 (6)	390.58 ± 40.34 (8)[a]
0.5	421.40 ± 44.04 (6)	544.76 ± 43.79 (6)	421.12 ± 26.37 (8)[a]
1	416.12 ± 38.14 (8)	481.01 ± 20.68 (8)	554.90 ± 29.13 (8)[b]
2	456.61 ± 38.14 (8)	540.16 ± 25.01 (7)	511.41 ± 34.01 (7)[ab]
3	547.30 ± 38.14 (8)	528.54 ± 32.27 (7)	733.84 ± 60.33 (7)[c]

* Mean activity (pg T aromatized; adjusted for protein content per sample) ± s.e.m. (n).

[abc] Superscripts within a column indicate significant differences ($P \leq .05$).

TABLE 8.

Aromatization of Testosterone by Endometrial Tissue Over a Three Hour Incubation Period at 30,60, and 90 Days of Gestation

Incubation Time (h)	Day of Gestation 30	60	90
0	440.81 ± 103.04 (8) *[a]	619.34 ± 78.22 (7)[a]	562.09 ± 99.10(8)[a]
0.5	740.74 ± 139.02 (7)[b]	844.32 ± 73.17 (8)[b]	871.00 ± 99.10 (8)[b]
1	876.90 ± 130.04 (8)[b]	1032.41 ± 73.17 (8)[b]	822.74 ± 99.10 (8)[b]
2	1360.54 ± 139.02 (7)[c]	1301.12 ± 73.17 (8)[c]	1366.66 ± 99.10 (8)[c]
3	1777.58 ± 139.02 (7)[d]	1437.84 ± 73.17 (8)[c]	1341.36 ± 99.10 (8)[c]

*Mean activity (pg T aromatized; adjusted for protein content per sample) ± s.e.m. (n).

abcd Superscripts within a column indicate significant differences ($P \leq .05$).

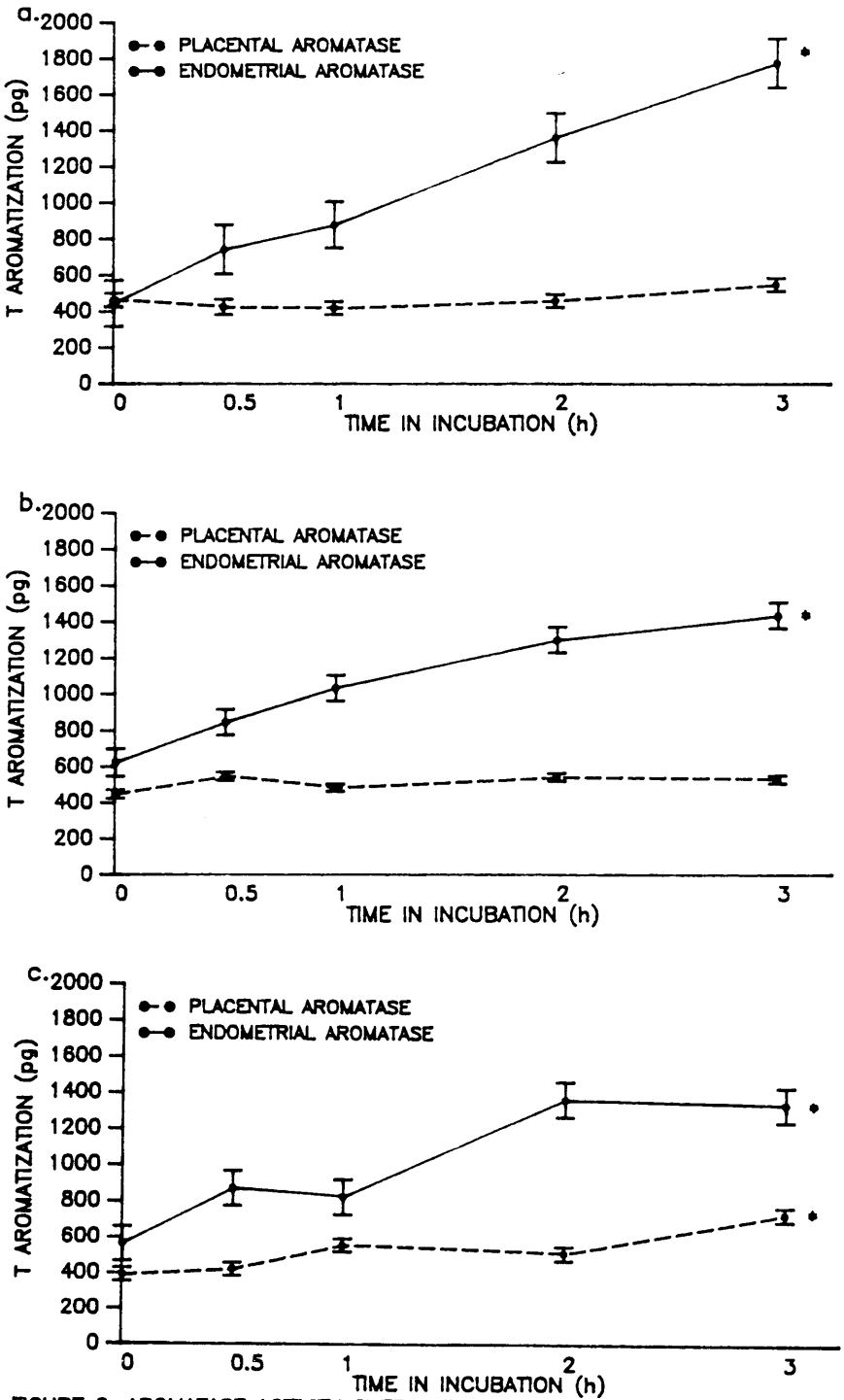

FIGURE 8. AROMATASE ACTIVITY OVER A 3 H INCUBATION PERIOD IN PLACENTAL AND ENDOMETRIAL TISSUE ON D 30(a.),60 (b.) AND 90(c.) OF GESTATION.

* INDICATES A SIGNIFICANT INCREASE IN T AROMATIZATION OVER THE 3 H INCUBATION PERIOD ($P<.001$).

approaching a plateau between 2 and 3 h. Day 60 tissue aromatized 619.34 pg T at 0 h and 1437.84 pg T after 3 h in incubation. On d 90, the increase in endometrial aromatase activity did not appear to be linear; 562.09 pg T were aromatized at 0 h and 1341.36 pg T were aromatized after 3 h in incubation. These results suggest in vitro endometrial tissue has equivalent aromatase capability on d 30, 60 and 90 of gestation. Endometrial aromatase activity was approximately two-fold greater than placental activity on all three days of gestation examined ($P \leq .002$; figure7).

In Vitro Sulfotransferase Activity on d 30, 60 and 90 of Gestation

Sulfotransferase activity is reported as mean percent sulfation of $^{3}H\text{-}E_1$ per sample adjusted for protein content of the sample (tables 9, 10, and 11). Because sulfotransferase activity was evaluated in a system utilizing tissue homogenates, apparent sulfotransferase activity, determined by formation of $^{3}H\text{-}E_1SO_4$, is influenced by simultaneous sulfatase activity within the tissue. Placental sulfotransferase activity was relatively stable over the three days of gestation examined (figure 9). Percent sulfation of $^{3}H\text{-}E_1$ by placental tissue was 10.75% on d 30, 8.22% on d 60, and 9.43% on d 90 (table 11). Endometrial activity decreased from 14.0% on d 30 to 3.65% on d 60 and 4.04% on d 90 (figure 9). Neither placental nor endometrial sulfotransferase activity was significantly affected by day of gestation. Gilt effects on sulfotransferase activity of both placental and endometrial tissue were significiant on d 30 and 60 of gestation ($P \leq .007$). There were no significant tissue responses in sulfotransferase activity to time in incubation (figure 10). There were no significant differences in the sulfotransferase

TABLE 9.

Placental Sulfotransferase Activity Over a Three Hour Incubation Period at 30, 60 and 90 Days of Gestation

	Day of Gestation		
Incubation Time (h)	30	60	90
0	1.14±6.15(6)*	7.04±4.45(7)	1.84±4.15(8)
0.5	6.73±5.69(7)	10.03±4.45(7)	9.69±4.15(8)
1	6.34±6.74(5)	3.64±4.16(8)	7.25±4.15(8)
2	19.31±6.15(6)	6.02±4.45(7)	15.87±4.15(8)
3	20.14±6.15(6)	16.19±4.80(6)	14.01±4.43(7)

*Mean percent activity adjusted for protein content per sample ± s.e.m. (n).

TABLE 10.

Endometrial Sulfotransferase Activity Over a Three Hour Incubation Period at 30,60 and 90 Days of Gestation

	Day of Gestation		
Incubation Time(h)	30	60	90
0	4.92±5.12(7)*	0±4.14(8)	9.43±3.95(8)
0.5	11.71±4.78(8)	0.03±4.78(6)	0.32±4.22(7)
1	23.52±4.78(8)	0±4.14(8)	2.41±3.95(8)
2	19.26±5.12(7)	10.15±4.14(8)	1.06±3.95(8)
3	9.63±5.12(7)	8.20±4.42(7)	6.87±3.95(8)

*Mean percent activity adjusted for protein content per sample ± s.e.m. (n).

TABLE 11.

Mean Sulfotransferase Activity of Placental and Endometrial Tissue at 30, 60 and 90 Days of Gestation

Tissue	Day of Gestation 30	60	90
Placenta	10.75 ± 6.78 (30)*	8.22 ± 6.28 (35)	9.43 ± 5.95 (39)
Endometrium	14.01 ± 4.63 (37)	3.65 ± 4.63 (37)	4.04 ± 4.51 (39)

*Mean percent activity adjusted for protein content per sample ± s.e.m. (n).

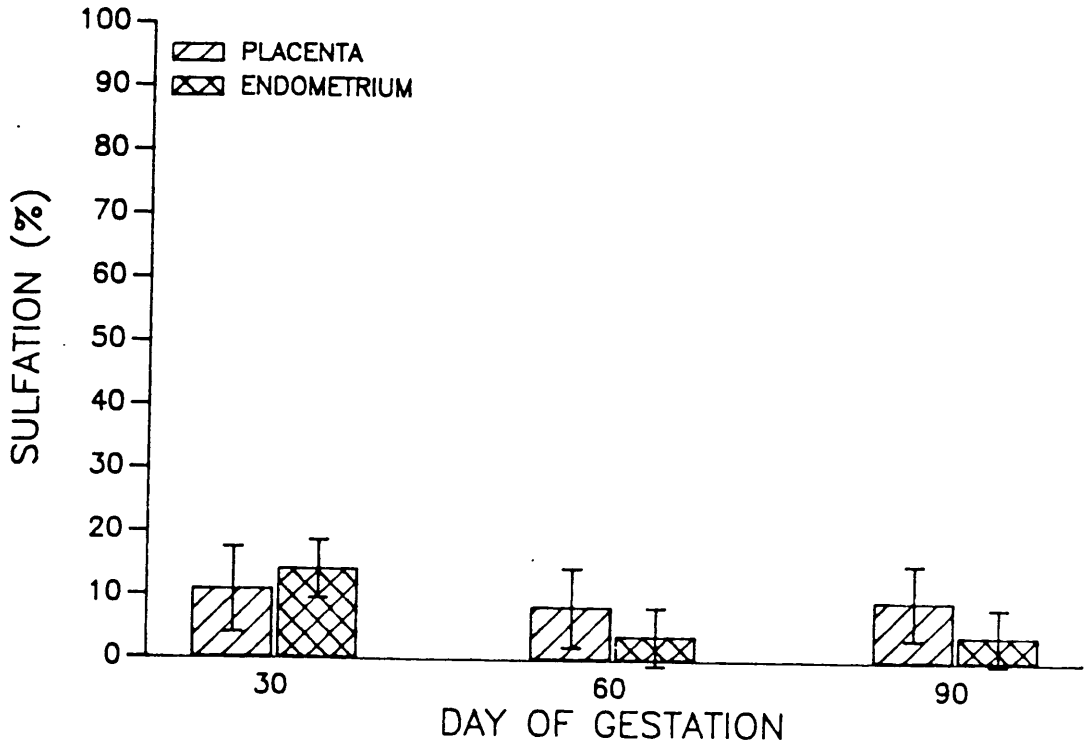

FIGURE 9. MEAN IN VITRO SULFOTRANSFERASE ACTIVITY IN PLACENTAL AND ENDOMETRIAL TISSUE ON D 30,60 AND 90 OF GESTATION.

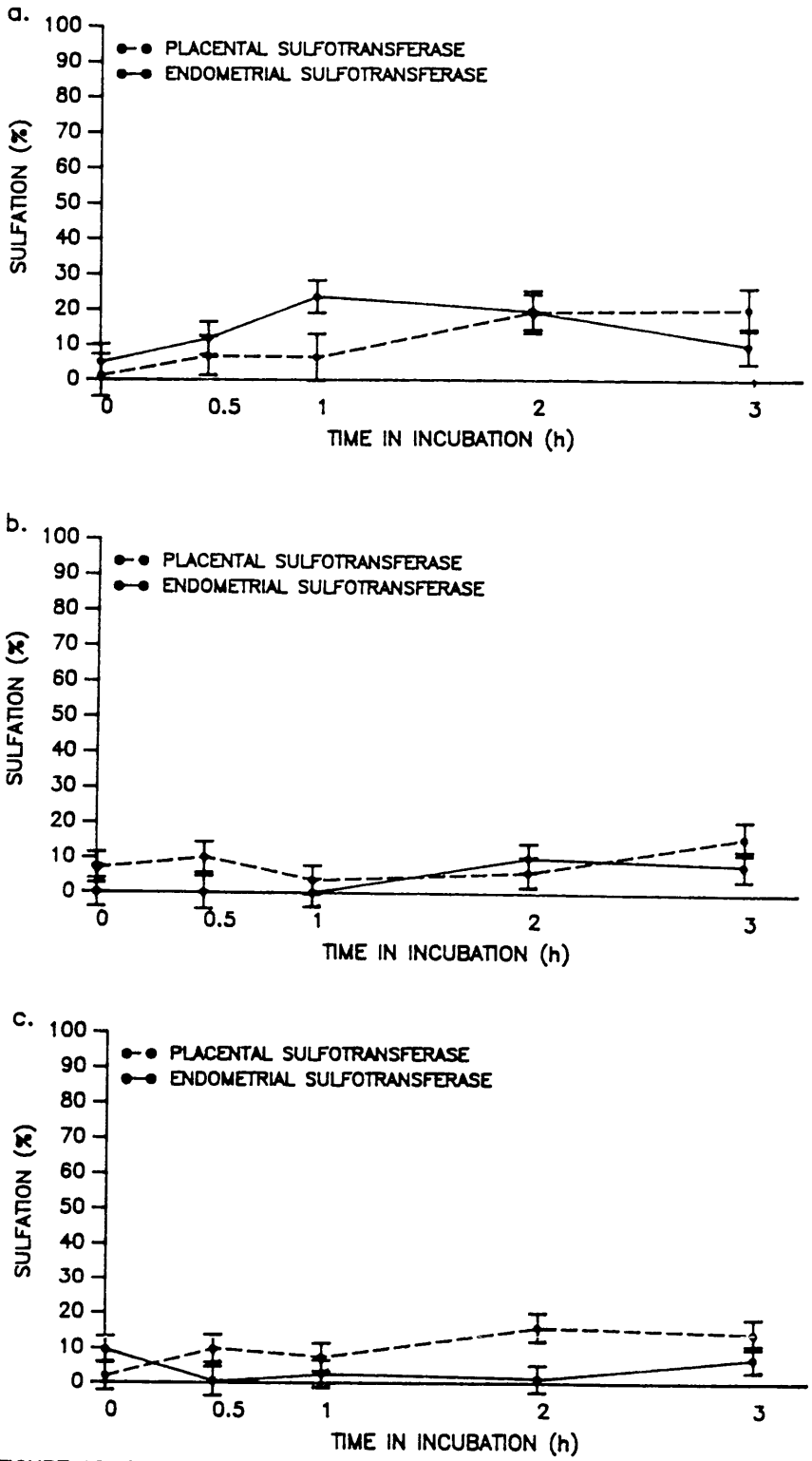

FIGURE 10. SULFOTRANSFERASE ACTIVITY OVER A 3 H INCUBATION PERIOD IN PLACENTAL AND ENDOMETRIAL TISSUE ON D 30(a.),60 (b.) AND 90(c.) OF GESTATION.

activity of placental tissue compared to endometrial tissue at any of the three days of gestation examined (figure 9). However, the ratio of means over the 3 h incubation period of placental to endometrial sulfotransferase activity underwent a reversal as gestation progressed changing from 1:1.3 on d 30 to 2.3:1 on d 60 and 90.

In Vitro Sulfatase Activity on d 30, 60 and 90 of Gestation

Sulfatase activity is reported as mean percent hydrolysis of $^{3}H\text{-}E_1SO_4$ per sample adjusted for protein content of the sample (tables 12, 13 and 14). Because sulfatase activity was evaluated in a system utilizing tissue homogenates, the apparent sulfatase activity, determined by formation of $^{3}H\text{-}E_1$ from $^{3}H\text{-}E_1SO_4$, is influenced by simultaneous sulfotransferase activity within the tissue. Mean placental sulfatase activity over the 3 h incubation period decreased from 32.84 % on d 30 to 28.13% on d 60 and to 20.79% d 90. In contrast to placental sulfatase activity, endometrial sulfatase activity increased from 51.00% on d 30 to 62.74% on d 60 and remained stable at 61.54% on d 90. Day of gestation effects were not significant on either tissue (figure 11). Gilt effects were significant on placental sulfatase activity on all three days of gestation examined ($P \leq .004$) and on endometrial sulfatase activity on d 30 and 90 ($P \leq .0003$). The gilt and time in incubation interaction was significant on both tissues on d 30 ($P \leq .005$), on d 60 placental tissue ($P \leq .002$), and on d 90 endometrial tissue ($P \leq .0002$). Time in incubation effects on sulfatase activity were significant on d 90 placental and endometrial tissue ($P \leq .05$, figures 11 and 12). Day 90 placental sulfatase activity exhibited a peculiar response to time in

TABLE 12.

Placental Sulfatase Activity Over a Three Hour Incubation Period at 30, 60 and 90 Days of Gestation

	Day of Gestation		
Incubation Time (h)	30	60	90
0	33.64±9.63(7)*	30.29±4.46(7)	15.34±3.80(8)^{b}
0.5	21.01±11.39(5)	29.58±4.46(7)	33.61±4.07(7)^{a}
1	25.81±9.63(7)	24.28±4.82(6)	14.34±3.80(8)^{b}
2	35.11±9.63(7)	26.37±4.82(6)	16.77±3.80(8)^{b}
3	47.31±10.40(6)	29.36±4.46(7)	25.52±3.80(8)^{ab}

*Mean percent activity adjusted for protein content per sample ± s.e.m. (n).

^{ab} Different superscripts within a column indicate significant differences ($P \leq .05$).

TABLE 13.

Endometrial Sulfatase Activity Over a Three Hour Incubation Period at 30, 60 and 90 Days of Gestation

	Day of Gestation		
Incubation Time (h)	30	60	90
0	40.53±6.74(8)*	56.42±10.05(8)	46.75±4.17(7)[a]
0.5	53.39±7.21(7)	56.05±10.05(8)	62.14±3.90(8)[b]
1	49.60±6.74(8)	68.61±10.74(7)	60.11±3.90(8)[b]
2	45.97±6.74(8)	64.77±10.74(7)	65.21±3.90(8)[b]
3	67.95±7.21(7)	68.85±10.05(8)	73.10±4.17(7)[c]

*Mean percent activity adjusted for protein content per sample ± s.e.m. (n).

[abc] Different superscripts within a column indicate significant differences ($P \leq .05$).

TABLE 14.

Mean Sulfatase Activity of Placental and Endometrial Tissue at 30, 60 and 90 Days of Gestation

	Day of Gestation		
Tissue	30	60	90
Placenta	32.84 ± 7.41 (32*)	28.13 ± 7.30 (33)	20.79 ± 6.71 (39)
Endometrium	51.00 ± 6.41 (38)	62.74 ± 6.41 (38)	61.54 ± 6.41 (38)

*Mean percent activity adjusted for protein content per sample ± s.e.m (n).

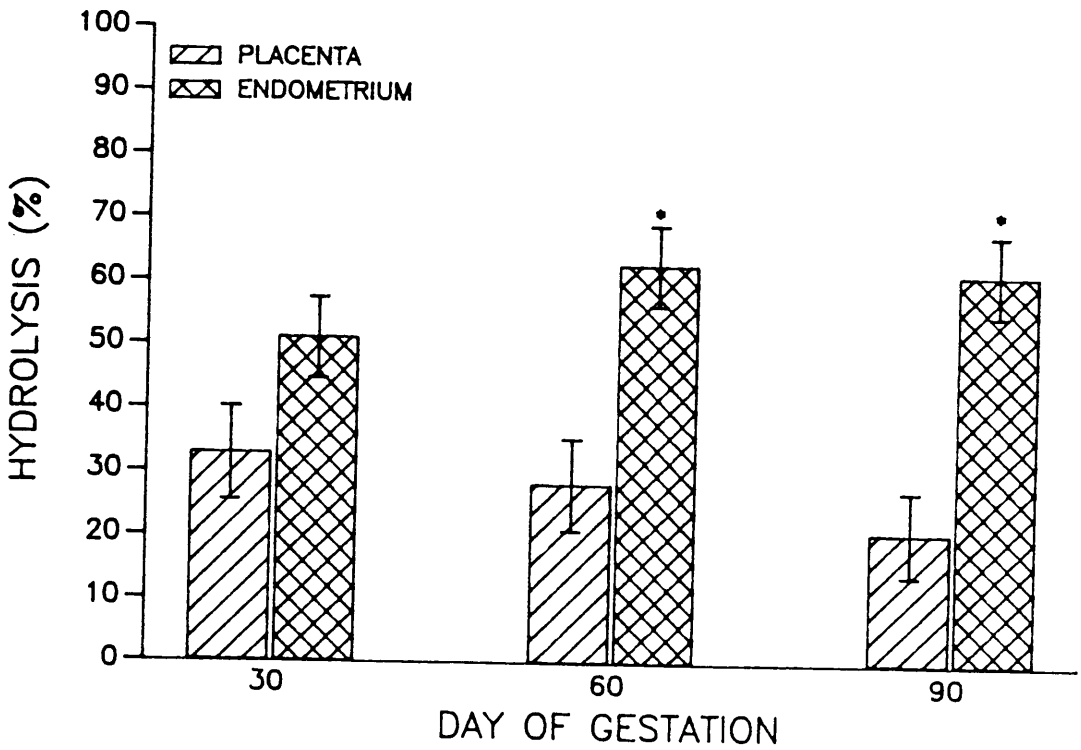

FIGURE 11. MEAN IN VITRO SULFATASE ACTIVITY IN PLACENTAL AND ENDOMETRIAL TISSUE ON D 30,60 AND 90 OF GESTATION.

* INDICATES SIGNIFICANT DIFFERENCES IN TISSUE SULFATASE ACTIVITY WITHIN DAY OF GESTATION ($P<.02$).

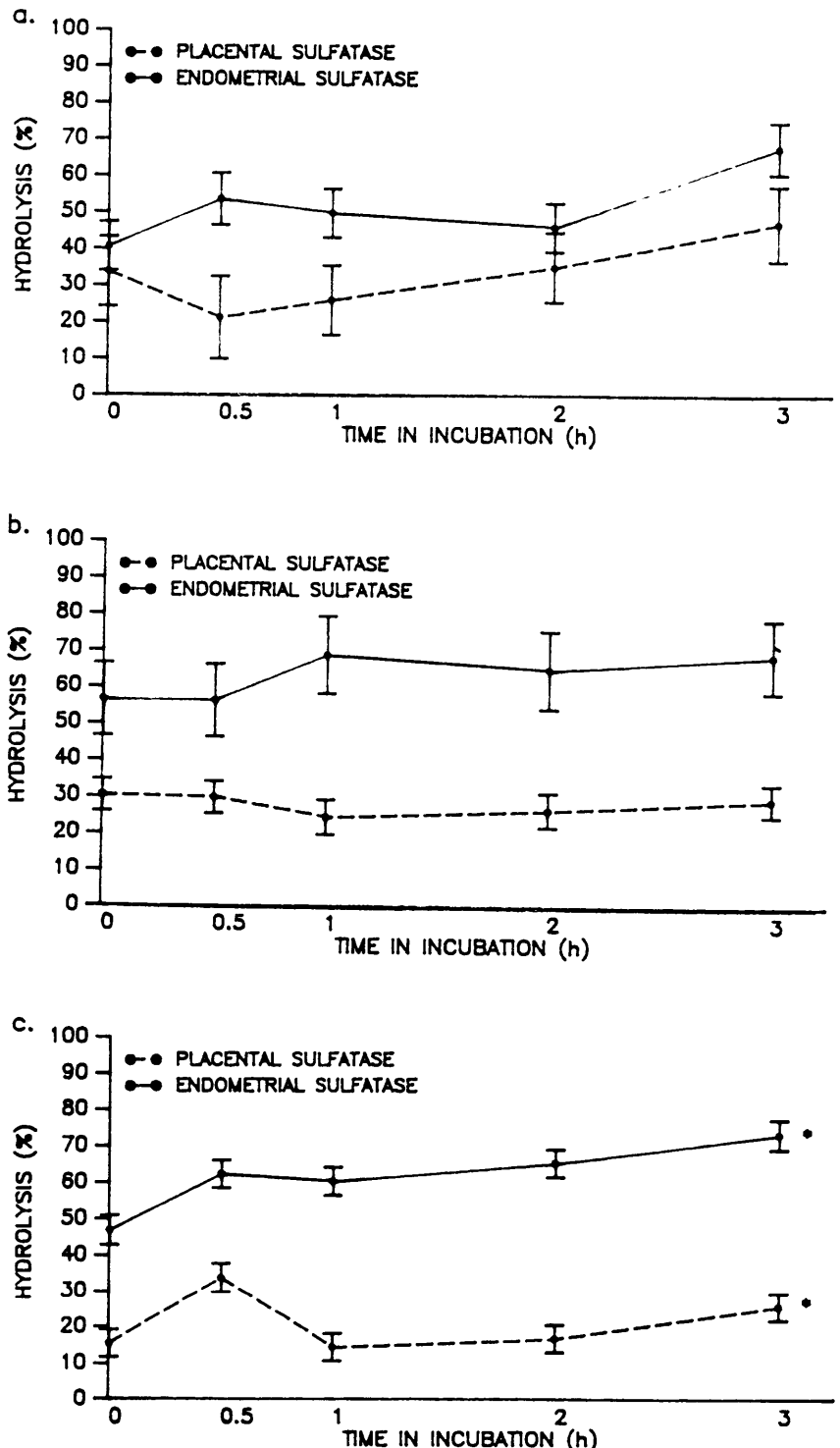

FIGURE 12. SULFATASE ACTIVITY OVER A 3 H INCUBATION PERIOD IN PLACENTAL AND ENDOMETRIAL TISSUE ON D 30(a.),60 (b.) AND 90(c.) OF GESTATION.

* INDICATES SIGNIFICANT TIME IN INCUBATION EFFECTS ON SULFATASE ACTIVITY ($P<.05$).

incubation, peaking at 0.5 h. Day 90 endometrial sulfatase activity increased from 46.75% at 0 h to 73.10% after 3 h in incubation. Endometrial tissue hydrolyzed approximately 25% more $^{3}H\text{-}E_1SO_4$ after 3 h in incubation. This suggests that by d 90 of gestation, endometrial tissue has the capacity for prolonged sulfatase activity in vitro. Endometrial sulfatase activity was greater than placental sulfatase activity on all days of gestation. This difference was significant on d 60 ($P \leq .02$), and on d 90 ($P \leq .002$). The ratio of placental to endometrial sulfatase activity changed over gestation as follows: 1:1.6, on d 30, 1:2.2 on d 60, and 1:3.0 on d 90. The change in the ratio, favoring endometrial sulfatase activity is primarily due to decreasing in vitro placental sulfatase activity over the three days of gestation examined.

Steroid Concentration in Maternal Plasma on d 30, 60, and 90 of Gestation

Testosterone

Within day of gestation, there were no significant differences in plasma T concentrations between the AVC, UA and UV (figure 13). Plasma T concentrations were highest on d 30 ($P \leq .001$): AVC = .126 ng/ml, UA = .146 ng/ml and UV = .162 ng/ml. In the AVC, plasma concentrations declined to .051 ng/ml on d 60 then increased to .084 ng/ml on d 90 ($P \leq .02$). In the UA and UV, plasma T concentrations dropped significantly between d 30 and 60 ($P \leq .0001$); there was no significant difference between d 60 and 90. A comparison of UA and UV plasma T concentrations revealed a negative UA-UV difference on d 30 and 90 which was not significant. No UA-UV difference was seen on d 60 (table 15).

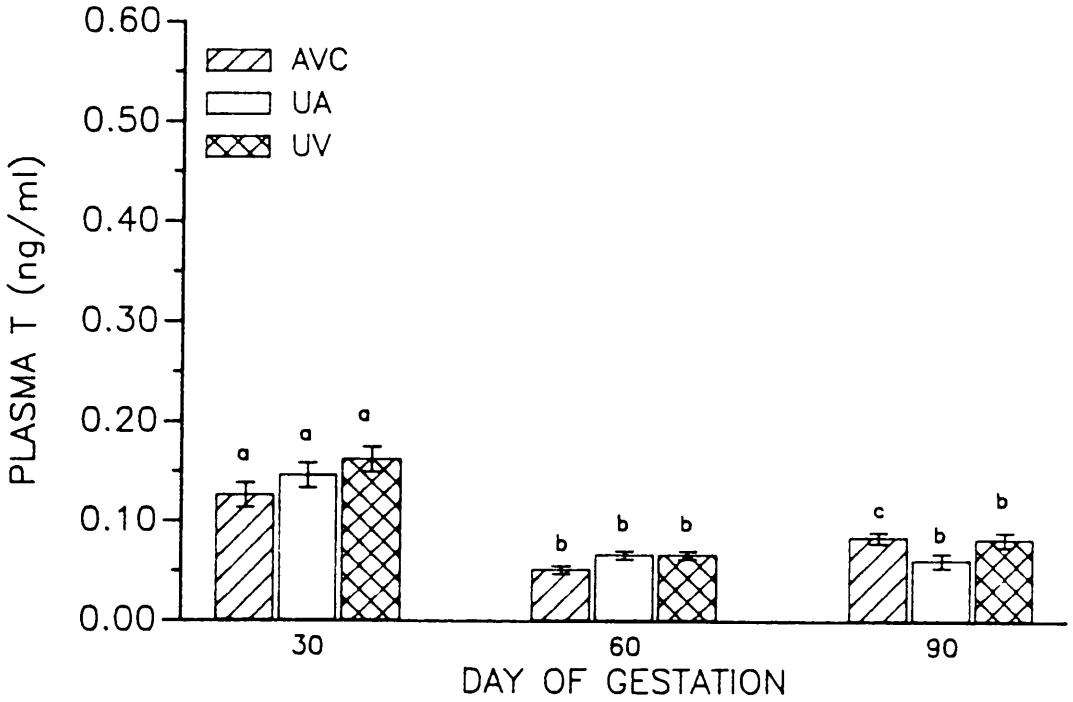

FIGURE 13. PLASMA TESTOSTERONE (T) CONCENTRATIONS WITHIN THE ANTERIOR VENA CAVA (AVC), UTERINE ARTERY (UA) AND UTERINE VEIN (UV) ON D 30,60 AND 90 OF GESTATION.

^{abc} INDICATE SIGNIFICANT DIFFERENCES WITHIN A VESSEL ($P \leq .02$).

TABLE 15.

Plasma Testosterone Concentrations in the Anterior Vena Cava (AVC), Uterine Artery (UA) and Uterine Vein (UV) at 30, 60 and 90 Days of Gestation

	Day of Gestation		
Vessel	30	60	90
AVC	.126±.0148*[a] n=4	.051±.0051[b] n=4	.084±.0067[c] n=4
UA	.146±.0148[a] n=4	.066±.0051[b] n=4	.061±.0087[b] n=3
UV	.162±.0148[a] n=4	.066±.0051[b] n=4	.082±.0087[b] n=3
UA-UV	-.016±.0112	0±.0112	-.021±.0129

*Least squared mean (ng/ml) ± s.e.m.

[abc] Different superscripts indicate significant differences within a row ($P \leq .02$).

Estrone

On d 30 and 60 of gestation, there were no significant differences in plasma E_1 concentration between the AVC, UA and UV (figure 14). The UA-UV difference on d 30 and 60 was negative, though not significant (table 16). On d 90, the UV E_1 concentration (.540 ng/ml) was significantly higher ($P \leq .05$) than either the AVC (.261 ng/ml) or the UA (.267 ng/ml). This significant negative UA-UV difference of .273 ng/ml is similar to that reported by Jeantet (1985)($P \leq .0001$). In all 3 vessels examined, plasma E_1 concentrations rose dramatically from d 30 and 60 to d 90 ($P \leq .001$; table 16). Plasma E_1 concentrations ranged from 2-to 4.5-fold less than those reported by Jeantet (1985) which could be due to differences in extraction procedures since the same batch of Ab was used for both experiments. Trends in changes of E_1 concentrations of the three vessels over the three days of gestation examined in both experiments were similar.

Estrone Sulfate

Within day of gestation there was no significant difference in plasma E_1SO_4 concentration among the 3 vessels examined (figure 15). The negative UA-UV difference apparent on all 3 days of gestation examined was significant on d 30 ($P \leq .005$; table 17). Among d 30, 60 and 90, plasma E_1SO_4 concentrations peaked in a biphasic pattern; E_1SO_4 concentrations were lowest ($P \leq .009$) on d 60 in all 3 vessels examined: AVC = .061, UA = .071, and UV = .074 ng/ml (table 17).

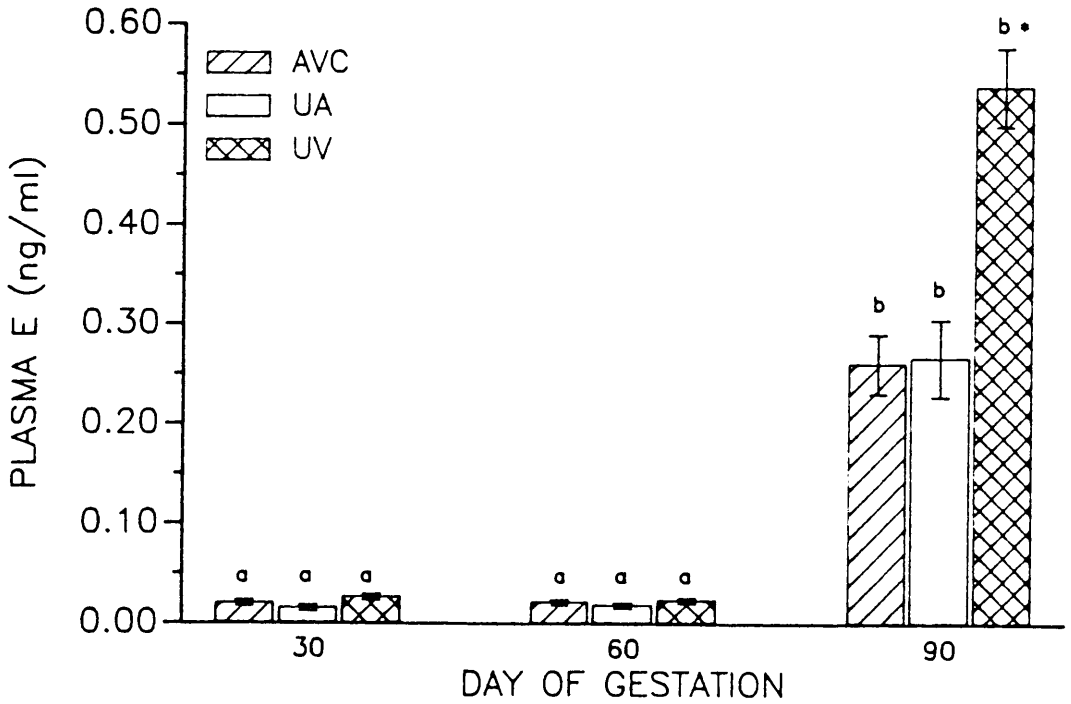

FIGURE 14. PLASMA ESTRONE (E) CONCENTRATIONS WITHIN THE ANTERIOR VENA CAVA (AVC) UTERINE ARTERY (UA) AND UTERINE VEIN (UV) ON D 30,60 AND 90 OF GESTATION.
abc INDICATE SIGNIFICANT DIFFERENCES WITHIN A VESSEL ($P \leq .001$).
* INDICATES SIGNIFICANT DIFFERENCES WITHIN A DAY OF GESTATION ($P \leq .05$).

TABLE 16.

Plasma Estrone Concentrations in the Anterior Vena Cava (AVC), Uterine Artery (UA) and Uterine Vein (UV) at 30, 60, and 90 Days of Gestation

	Day of Gestation		
Vessel	30	60	90
AVC	.020±.0031*[a] n=4	.021±.0029[a] n=4	.261±.0296[b] n=4
UA	.015±.0031[a] n=4	.018±.0029[a] n=4	.267±.0383[b] n=3
UV	.026±.0031[a] n=4	.023±.0029[a] n=4	.540±.0383[b+] n=3
UA-UV	-.011±.0123	-.005±.0123	-.273±.0142**

*Least squares means (ng/ml) ± s.e.m.

[ab] Different superscripts indicate significant differences within a row ($P \leq .001$).

+ Indicates this value is significantly different from the other values within the same column ($P \leq .05$).

**Indicates this value is significantly different from 0 ($P \leq .0001$).

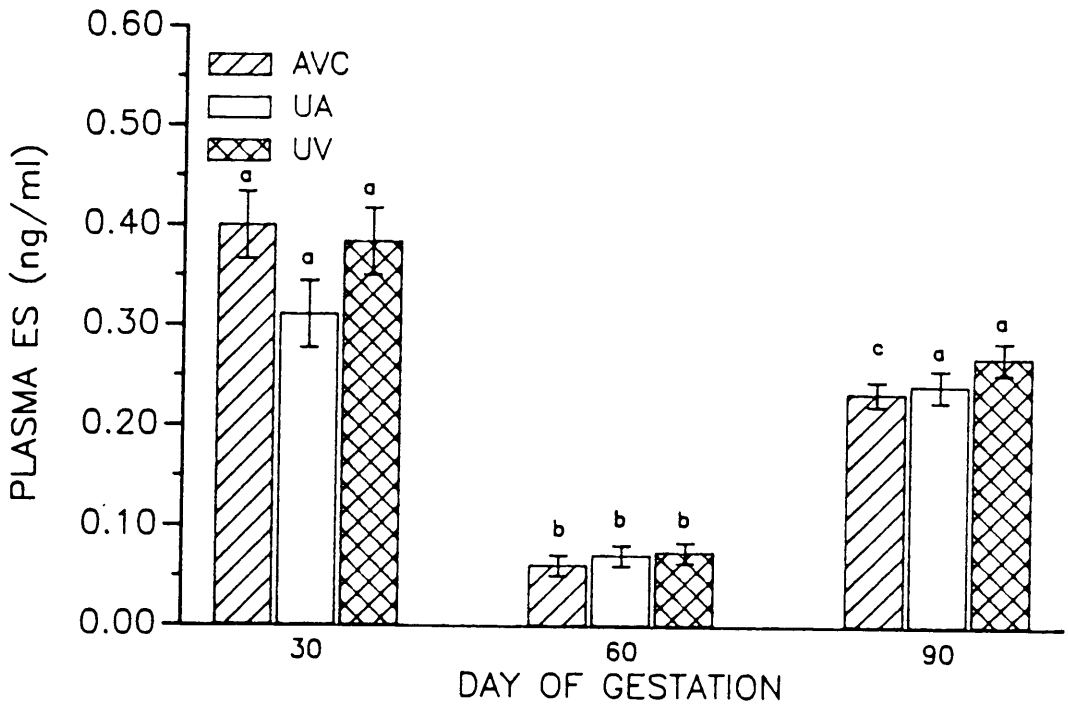

FIGURE 15. PLASMA ESTRONE SULFATE (ES) CONCENTRATIONS WITHIN THE ANTERIOR VENA CAVA (AVC) UTERINE ARTERY (UA) AND UTERINE VEIN (UV) ON D 30,60 AND 90 OF GESTATION.
^{abc} INDICATE SIGNIFICANT DIFFERENCES WITHIN A VESSEL ($P \leq .05$).

TABLE 17.

Plasma Estrone Sulfate Concentrations in the Anterior Vena Cava (AVC), Uterine Artery (UA) and Uterine Vein (UV) at 30, 60 and 90 Days of Gestation

	Day of Gestation		
Vessel	30	60	90
AVC	.400±.0551*[a] n=4	.061±.0172[c] n=4	.234±.0204[b] n=4
UA	.311±.0551[a] n=4	.071±.0172[b] n=4	.242±.0264[a] n=3
UV	.384±.0551[a] n=4	.074±.0172[b] n=4	.270±.0264*[a] n=3
UA-UV	-.073±.0160**	-.003±.0160	-.028±.0184

*Least squares means (ng/ml) ± s.e.m.

[abc] Different superscripts indicate significant differences within a row ($P \leq .009$).

**Indicates this value is significantly different from 0 ($P \leq .005$).

Peripheral plasma E_1SO_4 concentrations were much less than those reported by Robertson and King (1974) by 7.5 fold on d 30.

Comparison of Maternal Plasma Testoserone, Estrone and Estrone Sulfate

The ratio of plasma concentrations of E_1 to E_1SO_4 changed dramatically as gestation progressed (table 18). Estrone sulfate was the predominant plasma E when compared to E_1 on d 30 by a margin of approximately 20:1 in the AVC and UA and 15:1 in the UV. Estrone sulfate was three to four times greater than E_1 in all three vessels on d 60. By d 90, E_1 was equal to E_1SO_4 in the AVC and UA and was twice as great in the UV. The sum of E_1 and E_1SO_4 concentrations is indicative of total plasma E concentration. The ratio of T to $(E_1 + E_1SO_4)$ changed as gestation progressed. Plasma E concentrations were significantly greater than plasma T concentrations in the AVC, UA, and UV on all three days of gestation examined ($P \leq .02$) with the exception being in the UA on d 60. The ratio of T to E exhibited a biphasic pattern which is a reflection of the biphasic peaks seen in plasma E_1SO_4 concentration. Plasma E was 2 to 31 times greater than plasma T on d 30 and 1.5 times greater on d 60. On d 90, the ratio of plasma E to T was approximately 6:1 in the AVC, 8:1 in the UA and 10:1 in the UV (table 19).

TABLE 18.

Ratio of Plasma Estrone to Estrone Sulfate in the Anterior Vena Cava (AVC), Uterine Artery (UA) and Uterine Vein (UV) at 30, 60 and 90 Days of Gestation

	Day of Gestation		
Vessel	30	60	90
AVC	1:20.0*	1:2.9*	1.1:1
UA	1:20.7*	1:3.9*	1.1:1
UV	1:14.8*	1:3.2*	2.0:1*

*Indicates significant differences in steroid concentrations within the ratio ($p \leq .01$).

TABLE 19.

Ratio of Plasma (Estrone + Estrone Sulfate) to Testosterone in the Anterior Vena Cava (AVC), Uterine Artery (UA) and Uterine Vein (UV) at 30, 60 and 90 days of Gestation

	Day of Gestation		
Vessel	30	60	90
AVC	3.3:1*	1.6:1*	5.9:1*
UA	2.2:1*	1.4:1	8.3:1*
UV	2.5:1*	1.5:1*	9.9:1*

*Indicates significant differences in steroid concentrations within the ratio ($p \leq .03$).

DISCUSSION

In their investigation of in vitro aromatase activity in rabbit blastocysts, Hoversland et al. (1982) used ^3H_2O production from ^3H-T by boiled embryos as blank values. The blank values were used to correct aromatase activity expressed as pg T aromatized per embryo per h for ^3H_2O formation which was not due to aromatase activity. In the current investigation, placental and endometrial tissue minces were microwaved before weighing into the incubation media in order to generate blank values. There are two reasons why this system of incubating microwaved tissue minces was not used in the final analysis for correcting values of aromatase activity attained from live tissue. One reason is the degree of ^3H_2O production by the microwaved tissue over the incubation period which suggests that the aromatase enzyme system might have been active in the microwaved tissue. The second reason is the difference in affinity for ^3H_2O and ^3H-T between the unaltered tissue and the microwaved tissue. The microwaved tissue appears to have a higher affinity for both ^3H-T and ^3H_2O as suggested by the lower dpm recovered from the media incubated with the microwaved tissue. The problem of steroids being associated non-specifically with protein or lipid in the tissue preparation has been cited as a major difficulty in working with radioactive steroids in incubation due to the low solubility of unconjugated steroids in aqueous media (Bush, 1968). If one can assume the steroid-tissue affinity remains constant for a given tissue the

implications of this phenomenon are somewhat lessened. However, differential steroid affinity for a tissue or tissue type, in this case closer affinity of ^{3}H-T with microwaved tissue than with unaltered tissue, argues against using the microwaved tissue-containing tubes as controls.

The coexistence of sulfotransferase and sulfatase activity in the minces of placental and endometrial tissues is a handicap in determining levels of specific enzyme activity within a tissue. Neither the sulfotransferase nor the sulfatase reaction is reversible under near standard conditions; however, as sulfotransferase generates ^{3}H-E_1SO_4 from ^{3}H-E_1, provided as a substrate, sulfatase re-generates ^{3}H-E_1 from the newly formed ^{3}H-E_1SO_4. Alternately, as sulfatase generates ^{3}H-E_1 from ^{3}H-E_1SO_4, provided as a substrate, sulfotransferase regenerates ^{3}H-E_1SO_4 from the newly formed ^{3}H-E_1. Therefore, it is most appropriate to discuss the in vitro activity of these enzymes within a tissue as sulfotransferase activity coincident with sulfatase activity and, vice versa, as sulfatase activity coincident with sulfotransferase activity. When the activity of one of the two enzymes is apparently low, there may actually be relatively high activity of the other enzyme masking the activity of the enzyme in question. Relatively high activity levels determined for one of the two enzymes would be so despite the activity of the other enzyme. Activity of these two enzymes relative to each other can be reported in terms of net gain of either ^{3}H-E_1SO_4 or ^{3}H-E_1. It must be emphasized that the activity of sulfotransferase and sulfatase on each day of gestation as well as

for each time increment in incubation was determined with different tissue samples in different culture tubes. It is assumed that sulfotransferase and sulfatase activity are similar between samples cultured with $^{3}H\text{-}E_1$ and those cultured with $^{3}H\text{-}E_1SO_4$.

Incubation with nonlabelled precursors and subsequent RIA would be an alternate procedure for determining sulfotransferase and sulfatase activity which would also measure endogenous E_1 and E_1SO_4. Physical separation of the microsomal fraction, containing sulfatase, from the soluble cell fraction, containing sulfotransferase, or inhibition of one of the enzymes would be other alternatives for quantifying sulfatase or sulfotransferase activity without cross-contamination of enzyme systems. By incubating tissue in media containing $Na_2\ ^{35}SO_4$, sulfotransferase activity relative to sulfatase activity could be determined by following the ^{35}S label (Pack and Brooks, 1974). Simultaneous incubation with $^{3}H\text{-}E_1SO_4$ could be used to determine sulfatase activity by follwoing the ^{3}H label.

On d 30 of gestation, in vitro placental tissue that had been incubated for 3 h exhibited approximately 20.14% sulfation (n=6), indicative of sulfotransferase activity, coincident with approximately 47.31% hydrolysis (n=6), indicative of sulfatase activity. Since time in incubation had no effect on the activity of these enzymes in d 30 placental tissue, mean activity of the two enzymes over the 3 h incubation period can be compared. Mean placental enzyme activity was 10.75% sulfation (n=30) coincident with 32.84% hydrolysis (n=32). In both cases, there

was a net gain of approximately 25% free E_1 by placental tissue on d 30. Because time in incubation had no significant effect on d 30 endometrial enzyme activity, mean enzyme activity over the 3 h incubation period can be compared. Sulfation was 14.01% (n=37); hydrolysis was 51.00% (n=38). Net product formation favored ^3H-E_1 by 37%. After 3 h in incubation, endometrial tissue exhibited 9.63% sulfation (n=7) concurrent with 67.95% hydrolysis (n=7). Net product formation favored ^3H-E_1 by approximately 58% following 3 h in incubation. In a similar study, Dwyer and Robertson (1980) reported elevated sulfotransferase activity between d 12 and 28 of gestation sulfating approximately 78% concurrent with hydrolysis of approximately 20% resulting in product formation favoring ^3H-E_1SO_4 by 58%. The large discrepancy in results between the two studies is unclear since the procedure of the current investigation was based on that of Dwyer and Robertson (1980). In the current investigation, endometrial sulfotransferase activity was greatest after 1 h in incubation, sulfating approximately 25% (n=8) which is still substantially lower than that reported by Dwyer and Robertson.

On d 60 of gestation, time in incubation had no effect on enzyme activity of either placental or endometrial tissue. Enzyme activity of placental tissue resulted in 8.22% sulfation (n=35) concurrent with 28.13% hydrolysis (n=33) or a net gain of approximately 20% of ^3H-E_1 when examining mean placental enzyme activity over the 3 h incubation period. Mean endometrial enzyme activity over the 3 h incubation period on d 60 resulted in 3.65% sulfation (n=37) concurrent with

62.74% hydrolysis (n=38) resulting in net product formation of approximately 60% $^{3}H\text{-}E_1$. Following 3 h in incubation, endometrial sulfotransferase activity was 8.20% (n=7) concurrent with sulfatase activity of 68.85% (n=8). For comparison, Dwyer and Robertson (1980) reported approximate endometrial sulfotransferase activity of 20% and sulfatase activity of 60% on d 60 of gestation following 3 h in incubation resulting in net product formation of 40% $^{3}H\text{-}E_1$.

On d 90 of gestation, there was no significant time in incubation effect on sulfotransferase activity of either the placenta or the endometrium. There was a significant time in incubation effect on sulfatase activity of both the placenta and the endometrium. Following 3 h in incubation, placental sulfotransferase sulfated 14.01% (n=7) while placental sulfatase hydrolyzed 25.52% (n=8). Net steroid production of these two enzymes in the placenta favored $^{3}H\text{-}E_1$ by approximately 11%. Following 3 h in incubation, endometrial tissue exhibited 6.87% sulfation (n=8) concurrent with 73.10% hydrolysis (n=7). Net steroid production of sulfotransferase and sulfatase of the d 90 endometrium favored $^{3}H\text{-}E_1$ by approximately 66%. For comparison, Dwyer and Robertson (1980) reported d 90 endometrial sulfation of approximately 20% concurrent with hydrolysis of approximately 70% resulting in net product formation favoring $^{3}H\text{-}E_1$ by 50%.

Dwyer and Robertson (1980) reported in vitro endometrial sulfotransferase activity relative to sulfatase activity was extremely high before d 30 of gestation. They found net product formation favoring $^{3}H\text{-}E_1SO_4$ by approximately 60%.

Declining sulfotransferase activity coincident with sharply increasing sulfatase activity following d 30 of gestation resulted in product formation favoring $^{3}H\text{-}E_1$ by approximately 60% on d 60 and by 50% on d 90. In contrast to the above investigation, the current investigation determined $^{3}H\text{-}E_1$ was the major product formed by coincident sulfotransferase and sulfatase activity of both the endometrium and the placenta on d 30, 60 and 90 of gestation. Endometrial enzyme activity favored $^{3}H\text{-}E_1$ production over $^{3}H\text{-}E_1SO_4$ by 37% on d 30, 60% on d 60 and 66% and d 90. Placental enzyme activity favored $^{3}H\text{-}E_1$ production by 25% on d 30, 20% on d 60 and 11% on d 90. Endometrial in vitro $^{3}H\text{-}E_1$ production relative to $^{3}H\text{-}E_1SO_4$ increased from d 30 to d 60 and 90 of gestation, placental $^{3}H\text{-}E_1$ production relative to $^{3}H\text{-}E_1SO_4$ production decreased from d 30 to d 90.

In a general comparison of the results obtained in the current investigation to those obtained by Dwyer and Robertson (1980), Dwyer and Robertson obtained more generous values of in vitro endometrial sulfotransferase activity, especially on d 30 of gestation. The low in vitro sulfotransferase activity of both placental and endometrial tissue on d 30, 60 and 90, which were obtained in the current investigation could be due, in part, to the low solubility of $^{3}H\text{-}E_1$ in the aqueous incubation media. The low aqueous solubility limits the availability of $^{3}H\text{-}E_1$ as a substrate to sulfotransferase within the tissue. However, Dwyer and Robertson did not report encountering such a problem using a similar procedure.

In the current investigation, the ratios of sulfatase to sulfotransferase activity of the endometrium illustrate, along with predominant ^{3}H-E_1 formation, that in vitro sulfatase activity is greater on all three days of gestation examined by a margin of 3.5:1 on d 30, 17:1 on d 60 ($P \leq .0010$) and 15:1 on d 90 ($P \leq .0202$). The ratio of in vitro placental sulfatase to sulfotransferase activity was 3:1 on d 30, 3.5:1 on d 60 and 2:1 on d 90. These ratios suggest that endometrial sulfatase activity is only slightly greater than sulfotransferase activity on d 30. On d 30, mean endometrial sulfotransferase activity is at its highest level and mean endometrial sulfatase activity is at its lowest level. The dramatic increase in the ratio of sulfatase to sulfotransferase activity on d 60 and 90 is reflective of the increase in endometrial sulfatase activity coincident with a decline in endometrial sulfotransferase activity. The change in the enzyme activity ratio of placental tissue reflects a slight decline in mean sulfatase activity between d 30, 60 and 90 coincident with a slight decline in placental sulfotransferase activity on d 60 when compared to d 30 and 90.

Time in incubation had no effect on placental aromatase activity on d 30 and 60, but did have a significant effect on d 90. Placental tissue aromatized an additional 16% of the ^{3}H-T provided over the 3 h incubation period on d 30 and 60 and aromatized an additional 47% over the 3 h incubation period on d 90. Time in incubation had a significant effect on endometrial aromatase activity on all three days of gestation examined. Three h in incubation resulted in the additional aromatization of 75% and d 30, 57% on d 60 and 58% on d 90.

There were no significant time in incubation effects on sulfotransferase activity

in either placental or endometrial tissue on any of the three days of gestation examined. However, placental tissue exhibited an increase in sulfation of 20% on d 30 over the 3 h incubation period as compared to approximately 8% on d 60 and 12% on d 90. Coincident placental sulfatase hydrolyzed approximately 14% over the 3 h incubation period on d 30, 0% on d 60 and showed a significant increase over the 3 h period of approximately 10% on d 90. Initial $^{3}H\text{-}E_1$ production by placental tissue was higher than $^{3}H\text{-}E_1SO_4$ production on all three days of gestation examined. Day 30 placental sulfatase and sulfotransferase responded to prolonged incubation with increased product formation, whereas enzyme activity on d 60 and 90 was relatively stable over the 3 h incubation period, excepting the substantial and unexpected increase in d 90 placental sulfatase activity after 0.5 h in incubation.

As in placental tissue, time in incubation effects on endometrial tissue were only significant on d 90 sulfatase activity. Endometrial tissue exhibited an increase in sulfation of approximately 5% on d 30 following 3 h in incubation; d 30 sulfation peaked after 1 h in incubation, sulfating approximately 19% over 0 h tubes. Endometrial tissue on d 60 sulfated approximately 8% over the 0 h tubes. On d 90, endometrial sulfotransferase activity was highest at 0 h , sulfating 9.43% (n=8); at 0.5 h, sulfation was approximatley 0%; after 3 h in incubation, sulfation was 6.87% (n=8). Coincident sulfatase activity of the endometrium resulted in the hydrolysis of approximately 20% of the $^{3}H\text{-}E_1SO_4$ over the 3 h incubation period on d 30, 12% hydrolysis on d 60 and 26% hydrolysis on d 90. The ability of

endometrial sulfotransferase to sulfate E_1 in vitro is greatest on d 30. On d 60, sulfotransferase activity increases slightly after 1 h in incubation. Day 90 endometrial sulfotransferase activity in vitro is near 0. Initial $^3H\text{-}E_1$ production was substantially greater than $^3H\text{-}E_1SO_4$ production on all three days of gestation examined. Endometrial sulfatase activity increased on all three days. The response to prolonged time in incubation with increased sulfatase activity was greatest on d 90, the day when in vitro endometrial sulfotransferase activity was fairly random over the 3 h incubation period.

Although there were no day of gestation effects on enzyme activity of placental or endometrial tissue, overall results of time in incubation effects indicate that there are differences in the response of tissue enzymes to prolonged in vitro incubation conditions within day of gestation. By d 90 of gestation, placental aromatase and sulfatase responded to prolonged periods of incubation with increased product formation. Endometrial aromatase exhibted a significant response to increased time in incubation with increased aromatization on all three days of gestation examined; however, the response was greatest on d 30. Endometrial sulfatase response to time in incubation exhibited a significant increase on d 90. Placental and endometrial sulfotransferase responded most to increased time in incubation with increased $^3H\text{-}E_1SO_4$ on d 30.

Incubating larger tissue samples in a greater volume of incubation media and taking aliquots at the desired time intervals would reduce the influence of tissue

differences between samples on enzyme activity over time in incubation. Time 0 activity would not be affected by continued enzyme activity of the tissue as it freezes. Removing media aliquots at consistent time intervals, every half hour for example, would make analysis of time in incubation effects by orthogonal contrasts possible. Also, taking aliquots from media plus substrate which is subjected to identical incubation conditions, with no tissue sample, would generate control values for each time interval to be examined.

Maternal plasma concentrations of T, E_1 and E_1SO_4 in the AVC, UA and UV were significantly affected by day of gestation. Plasma T concentrations were greater than plasma E_1 concentrations on d 30 and 60 of gestation. Plasma T and E_1SO_4 concentrations were greatest on d 30; corresponding E_1 concentrations were remarkably low. Plasma T and E_1SO_4 concentrations in all three vessels dropped significantly between d 30 and 60. Plasma T concentrations remained low on d 90 while plasma E_1SO_4 increased to concentrations equivalent to those of d 30. Plasma E_1 remained low on d 60, and was increased on d 90 in all three vessels examined; however, the increase of E_1 in the UV of greater than 20-fold was the most dramatic. Although there were no significant differences in plasma concentrations of T or E_1SO_4 on d 30, 60 and 90, or of E_1 on d 30 and 60, the UA-UV difference was negative, except for T on d 60, when the difference was 0. The negative UA-UV difference of E_1SO_4 concentration on d 30 and of E_1 concentration on d 90 was

significant. These results suggest that on d 90, some element within the uterus,(either the fetus, placenta or endometrium), is releasing significant concentrations of E_1SO_4 into the circulation on d 30 and is releasing significant concentrations of E_1 ond 90.

Furthermore, they suggest that some element of the uterus is releasing E_1 on d 30 and 60 and T and E_1SO_4 on d 60 and 90 into the circulation to a lesser extent. Robertson and King (1974) reported high concentrations of E_1SO_4 in the UV as well as allantoic fluid but not ovarian venous blood between d 20 and 30 of gestation and suggested that E_1SO_4 present at this time was from the conceptus. The E_1SO_4 could subsequently be hydrolyzed to biologically active E by the placenta or endometrium. The negative UA-UV difference apparent on d 30, 60 and 90 is in agreement with the results of Knight et al (1977), Kukoly (1984) and Jeantet (1985). The magnitude of the UA-UV difference of E_1 concentrations of -.011 ng/ml on d 30 and -.005 ng/ml on d 60 are similar to those reported by Knight (1977) and Jeantet (1985). However, the UA-UV E_1 concentration difference on d 90 of -.273 ng/ml is much less than previously reported. For all three steroids, the UA-UV difference was lowest on d 60, corresponding to low steroid concentrations on d 60. These results suggest that the lower circulating levels of T, E_1 and E_1SO_4 on d 60 could be due to slower release rates from the uterus due either to decreased production or increased in utero metabolism. After d 60 of gestation, the porcine placenta will grow very

little in terms of length and weight (Knight et al., 1977). However, the most rapid increases in fetal growth occur after d 50 (Knight et al., 1977). The increase of circulating E_1 and E_1SO_4 on d 90 could be due to increased release from the uterus due either to increased production, decreased in utero metabolism, decreased metabolic clearance rate, a combination of these three possibilities. Increased in utero production would be due to increased enzyme capability, not increased placental size.

The total of E_1 and E_1SO_4 concentrations within a vessel is indicative of total plasma E concentration. Concentrations of E_2 were not determined in this study. Robertson and King (1974) reported that E_2 concentrations followed the same trends as E_1 concentrations in maternal plasma, but were approximately six-fold lower. Others have also reported E_1 to be the predominant E of pregnancy in swine (Velle, 1960; Raeside, 1963; Wetteman, et al., 1974; Knight et al., 1977). On d 30, the ratio of maternal plasma E to T was approximately 3:1 in the AVC, 2:1 in the UA, and 2.5:1 in the UV. The ratio of maternal plasma E_1SO_4 to E_1 on d 30 was 20:1 in the AVC, 21:1 in the UA and 15:1 in the UV. Robertson and King (1974) report a much more dramatic ratio of E_1SO_4 to E_1 in porcine maternal peripheral plasma of 200:1 between d 23 and 30 of gestation. While the ratios of E_1SO_4 to E_1 are not as high in the current investigation, they do indicate that E_1SO_4 is the predominant circulating E on d 30 of gestation. Despite the fact that plasma T concentrations are

greater than E_1 concentrations on d 30, they are less than half that of total circulating E concentrations. There is no clear indication in this study that circulating T is being utilized by the uterus on d 30 for E synthesis as the E to T ratio is relatively stable across the three vessels examined. The data do suggest that the decrease in the E_1SO_4 to E_1 ratio apparent in the UV on d 30 is the result of increased E_1 concentrations leaving the uterus by the UV.

On d 60 of gestation, the E to T ratio was approximately 1.5:1 in all three blood vessels examined. The significant decrease in this ratio from d 30 is the result of the dramatically reduced plasma E_1SO_4 concentrations apparent on d 60. Although circulating T concentrations are significantly lower on d 60 than on d 30, there is no significant difference between vessel T concentration. Therefore, as on d 30, the uterus does not appear to be utilizing circulating T for E metabolism. The decrease in plasma E_1SO_4 concentrations is again reflected in the decreased E_1SO_4 to E_1 ratio of 3:1 in the AVC, 4:1 in the UA and 3:1 in the UV. Estrone sulfate is the predominant plasma E on d 60 as on d 30; however, the magnitude of the difference in E_1SO_4 and E_1 concentrations is not as great.

On d 90 of gestation, the maternal plasma steroid profile changes dramatically. Increasing concentrations of both E_1 and E_1SO_4 coupled with static T concentrations result in the maternal plasma E to T ratio of 6:1 in the AVC, 8:1 in the UA and 10:1 in the UV. The increase in the ratio of the UV is due to a significant increase in E_1

concentration of the UV, not decreased T release from the uterus. Therefore, this study indicates a lack of uterine metabolism of T from the general circulation on d 30, 60 and 90 of gestation. The ratio of maternal plasma E_1SO_4 to E_1 was 1:1 in the AVC and UA but in the UV, E_1 was the predominant E by a margin of 2:1. The reversal is the result of the increase in the E_1 concentration with no compensatory decrease in E_1SO_4 concentration in the UV. This suggests the increase in E_1 release from the uterus is not the result of uterine hydrolysis of E_1SO_4 from in the general circulation. Robertson and King (1974) report the ratio of E_1SO_4 to E_1 in the peripheral plasma approaches unity by d 70 of gestation, seen here on d 90. At term, Robertson and King (1974) reported E_1 was the predominant E of peripheral circulation by a margin of 2:1. The results of the current study suggest the increase of E_1 in the peripheral circulation near term is being introduced by the UV, once again suggesting uterine involvement in E_1 production.

Analysis of T, E_1 and E_1SO_4 concentrations in allantoic and amniotic fluid samples froma representative number of fetuses per gilt, in adition to the data on maternal plasma steroid concentrations, would have been useful in yielding a more representative profile of steroid production and metabolism by the conceptus. This additional information might have made recognition of the primary steroid producing tissues within the uterus, possible. Knowledge of corpora lutea number, per ovary, at time of Hyst-X would have been a useful addition to this study. Number of corpora

lutea per gilt may have had a significant affect on circulating steroid concentrations.

It is impossible to directly correlate in vitro enzyme activity, resulting in E_1 and E_1SO_4 production, with plasma concentrations of T, E_1 and E_1SO_4. However, comparing in vitro results to endogenous steroid concentrations can give some indication of the applicability of the results from in vitro conditions to the in vivo situation. The negative UA-UV difference of endogenous steroids suggests the uterus produces some T on d 30 and 90, and E_1SO_4 and E_1 on d 30, 60, and 90. The significant, negative UA-UV difference of E_1SO_4 on d 30 suggests that the uterus is producing E_1SO_4. Apparent uterine production of E_1, evidenced by great increases in UV E_1 concentrations, was significant on d 90.

Net production of E , in vitro, was determined by evaluating aromatase activity, and sulfatase activity relative to sulfotransferase activity of endometrial and placental tissue in culture. No significant day of gestation effects on the activity of aromatase, sulfotransferase or sulfatase in placental or endometrial tissue were found in this investigation. Placental aromatase was most active over a 3 h incubation period in converting T to E on d 90; while endometrial aromatase was most active on d 30. Assuming that the enzyme activity of the placenta and endometrium would be additive under conditions of coincubation, the percentage of ^{3}H-T aromatized by both tissues over a 3 h incubation period by both tissues together would be 91% on d 30, 73% on d 60, and 105% on d 90. Of course 105% enzyme activity is not possible, but the concept illustrates that d 30 and 90 in vitro

aromatase activity of placenta and endometrium combined is greater than that of d 60. If this were to be the case in vivo, it would be the basis for the increased E concentrations apparent in maternal plasma on d 30, as E_1SO_4, and on d 90, as E_1 and E_1SO_4.

In this investigation, sulfotransferase coincident with sulfatase activity resulted in the net production of free E_1 by both placental and endometrial tissue on d 30, 60 and 90. Net E_1 production following 3 h in incubation was greatest in placental tissue on d 30 and was greatest in endometrial on both d 60 and 90. Once again making the assumption that enzyme activity between the two tissues is additive, the net E_1 versus E_1SO_4 production would be 62% on d 30, 80% on d 60 and 77% on d 90. If this were to be the case in vivo, it would suggest net E_1 production by the uterus would be greater than E_1SO_4 production on d 30, 60 and 90 of gestation. Net E_1 production by the uterus in vivo is evidenced by the negative UA-UV difference which is significant on d 90 of gestation. The magnitude of change in E_1 release from the uterus is not reflected by a surge in in vitro sulfatase activity relative to sulfotransferase activity. While the ratio of sulfotransferase to sulfatase activity does favor sulfatase, there is measurable ^3H-E_1SO_4 production by the placenta and the endometrium on all three days of gestation. In vitro sulfotransferase activity of both the placenta and endometrium is slightly greater on d 30 than on d 60 and 90.

Since the in vivo peak of plasma E_1SO_4 occurs between d 23 and 30, perhaps in vitro sulfotransferase activity in d 30 placental and endometrial tissue may be low due to negative feedback. However, Dwyer and Robertson (1980) report high endometrial sulfotransferase activity on d 30. Days 30, 60 and 90 of gestation were chosen for monitoring enzyme activity since they had been shown by earlier investigations to be times of substantial changes in in vitro E_1 production by placental and endometrial tissue. The enzyme activity responsible for these changes may be appearing before the manifestation of these changes in plasma E concentration. Examination of in vtro enzyme activity in tissue from days before the production maxima might yield more dramatic changes in enzymatic activity. Examination of enzyme activity on additional days of gestation might also reveal whether fluctuations in plasma steroid concentrations are due to changes in production or changes in metabolic clearance rate.

Utilizing RIA procedures, Kukoly (1984) and Jeantet (1985) observed in vitro placental E_1 production to be biphasic, peaking on d 30 and d 90 to term. Jeantet (1985) further reported that in vitro endometrial E_1 production followed the same pattern, although the peak at d 90 was less dramatic than that produced by placental tissue. Results from the current investigation suggest that in vitro aromatase, sulfotransferase and sulfatase activities within both the placenta and endometrium on d 30, 60 and 90 are not responsible for the peaks of E_1 production observed in vitro on d 30, and 90. A major difference between these three studies is that while Kukoly

(1984) and Jeantet (1985) measured actual steroid concentration in the incubation media as evidence of tissue steroid production, the current investigation examined the interconversions of tritium-labelled substrates to tritium-labelled products as evidence of tissue enzyme activity. There is no way in the current investigation to account and correct for the levels of endogenous substrates or inhibitors which may be affecting the formation of the tritium-labelled products.

Definitive reasons for the dissimilarity in response of placental versus endometrial in vitro enzyme activity on d 30, 60 and 90 are beyond the scope of the present investigation. However, the possible explanations for the differential behavior are multifold. Placental tissue exhibited no response in aromatase activity to prolonged time in incubation on d 30 and 60. Placental tissue on d 90 and endometrial tissue on all three days exhibited a significant response to prolonged time in incubation by aromatizing greater amounts of ^{3}H-T. Incubation conditions were similar for both tissues, suggesting there is something inherently different between the aromatase system in d 30 and 60 placental tissue and that in d 90 placental tissue and endometrial tissue. Placental aromatase activity was significantly lower than endometrial aromatase activity suggesting an inherent difference in enzyme activity between the two tissues.The first most obvious explanation would be differences in enzyme quantity between the two tissues. Tissue differences in endogenous precursors, androstenedione and T, could lead to misleading conclusions concerning tissue activity. If placental tissue did contain greater amounts of these precursors competing with ^{3}H-T for substrate binding sites, it would result in less apparent in vitro aromatase activity than is actually occurring

due to less $^{3}H_2O$ formation. Physiologically, this explanation is unlikely since the concentration of T added to the incubation medium was much greater than circulating T concentrations. Assuming plasma T concentrations are similar to intracellular T concentrations, the concentration of exogenously supplied ^{3}H-T was great enough to make effects of endogenous substrates negligible. Tissue differences in endogenous concentrations of the cofactor NADPH could be involved and/or tissue differences in the metabolism of NADP, a potent inhibitor formed during the aromatase reaction could be involved in the differences in aromatase activity between tissues and between placental activity on d 30 and 60 versus d 90. Oxygen is another cofactor necessary to the aromatase reaction. Because it was supplied continuously to the culture tubes, it should not have been a factor limiting aromatase activity in either tissue.

Like aromatase activity, sulfatase activity was greater in endometrial tissue than in placental tissue on all three days of gestation examined. This difference may be due to different amounts of sulfatase in the two tissues. Greater concentrations of endogenous sulfoconjugated E and/or DHEAS competing with ^{3}H-E_1SO_4 for binding sites on the enzyme would result in less apparent sulfatase activity. Differential amounts of free steroids including androgens and E which have an inhibitory effect on sulfatase activity could be responsible for the differences in tissue activity. Day 90 sulfatase activity of both tissues was significantly affected by time in incubation, suggesting that d 90 sulfatase capability is different than that on d 30 or 60.

Although there were no significant differences in in vitro sulfotransferase

activity between placental and endometrial tissue, placental sulfotransferase was more active than endometrial sulfotransferase on d 60 and 90 due to a decrease in apparent endometrial sulfation. Placental sulfotransferase activity remained constant over the three days of gestation while endometrial sulfotransferase activity declined after d 30. Tissue differences in activity could be due to tissue differences in the amount of 1) E_1 and E_2 competing with ^3H-E_1 for substrate binding sites, 2) magnesium ion which is necessary as a cofactor, 3) activated sulfate with which the steroid is conjugated or 4) the inhibitors, deoxycorticosterone and T.

Record of the litter size per gilt would have been a useful addition to this investigation. There was a large degree of gilt-to-gilt variation which permeated every aspect of this study. Awareness of significant differences in litter size might have accounted for some of this variation.

When the enzyme activity patterns were examined by gilt, it became apparent that tissues from some gilts were more responsive to in vitro conditions than tissues from other gilts. Examination of the in vitro enzyme activity of placental and endometrial tissue from a larger number of gilts at each day of gestation might have allowed for categorization of gilts in terms of high or low in vitro enzyme activity. When coupled with data on litter size per gilt, this information might have provided a basis for a connection between in vitro enzyme activity and prenatal survivability in swine.

CONCLUSION

In this study, the activities of three enzymes: aromatase, sulfotransferase and sulfatase in the porcine placenta and endometrium of d 30, 60 and 90 of gestation were examined in vitro. Mean activity of each of these enzymes was not significantly different among the three days of gestation examined. The lack of change in mean in vitro enzyme activity over the three days of gestation suggests that the activity of aromatase, sulfotransferase and sulfatase in the porcine placenta and endometrium may not be responsible for regulating in utero E production or in determining circulating steroid concentrations. However, the capability of tissue to maintain enzymatic activity over the 3 h incubation period varied depending upon the type of tissue, the gestational age of the tissue and the enzyme in question. Overall, placental enzymes did not respond consistently to increased time in incubation with increased product formation. Endometrial aromatase activity of d 30, 60 and 90, and endometrial sulfatase of d 90 responded to increased time in incubation with increased product formation. These results suggest that there are differences in enzyme integrity, maturation, enzyme substrate and/or enzyme inhibitor concentrations both between tissue types and among gestational age groups, which affect enzyme productivity. Possibly, these qualitative differences in enzyme activity seen in vitro are reflective of in vivo differences in enzyme activity which could be responsible for the change in circulating steroid concentrations as gestation progresses.

Differences in plasma steroid concentrations among the AVC, UA, and UV suggest

that some factor(s) within the uterus is involved in T, E_1, and E_1SO_4 production since concentrations leaving the uterus via the UV were greater than those entering the uterus via the UA. This UA-UV difference was dramatic for plasma E_1SO_4 on d 30 and for plasma E_1 on d 90. Plasma T and E_1SO_4 concentrations were greatest on d 30; plasma E_1 concentrations were greatest on d 90. The results of plasma steroid concentrations indicate a biphasic trend in peaking steroid concentrations when comparing d 30, 60 and 90 of gestation. These changes illustrate a physiological response on the part of the mother to the demands of pregnancy.

This investigation confirms the existence of aromatase, sulfotransferase and sulfatase activity in the porcine endometrium, and provides evidence of the existence of these three enzymes in the porcine placenta. While the precise role of these enzymes in determining in vivo E production and activity has not been determined, the potential for these enzymes to play a role in the regulation of E production and activity has been demonstrated. Further investigation into the roles of aromatase, sulfotransferase and/or sulfatase play in regulating E production and activity may shed some light on the elusive cause of prenatal mortality in swine and point towards some method for alleviating the problem of placental insufficiency.

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APPENDICES

APPENDIX A

Compositon of Medium

Medium 199 powder mix (Gibco Laboratories, Grand Island, NY)	9.9 g/pkg
Sodium Bicarbonate ($NaHCO_3$)	2.2 g
Penicillin	100,000 I.U.
Streptomycin	100,000 ug
Amphotericin B (Fungizone)	250,000 ug
Deionized Water	Total volume 1000 ml

pH was adjusted such that following filtration through a pressurized millipore filter (.45 um), pH was 7.0. Medium was stored at 4 C until use.

APPENDIX B

Tritium labelled steroids: [2,4,6,7 - 3H(N)]-Estrone, [6,7-3H(N)]-Estrone sulfate and [1ß,2ß-3H(N)]-Testosterone (New England Nuclear Division of DuPont, Boston, MA) were used as sent by the manufacturer. This author recommends chromatographing these steroids for purity before use in subsequent investigations. One uCi 3HE1 or 1uCi (10 ul) 3H E1S04 suspended in ethanol was added to the appropriate 16 x 100mm polypropylene culture tubes. The ethanol was permitted to evaporate before addition of 3 ml M199 to each tube. In order to evaluate aromatase activity, 0 .5 uCi T in ethanol was added to each of the appropriate tubes. The ethanol was permitted to evaporate before addition of 3ml M199 containing non-radioactive T (0.5 ug/ml; Sigma Chemical Co., St. Louis, MO) to each of these tubes.

APPENDIX C

Scintillation Cocktails

1. Recovery Scinitillation Cocktail

Toluen	4	L
2a70 Preblend: 98% PPO,, 2% MSB	15.75	g

2. Dioxane-based Scintillation Cocktail (Rabe et al., 1982).

2a70 Preblend 98% PPO, 2% MSB	25	g
Napthalene	400	g
Xylene	1650	g
Dioxane	3,300	ml

Yield = 4.98 L

3. Assay Scintillation Cocktail

Toluene	1500	ml
Triton X-100 (reagent grade)	500	ml
2a70 Preblend 98% PPO, 2% MSB	15.75	g

Chemicals:

2a70 Preblend 98% [2,5-diphenyloxazole](PPO)
2% [p-bis (o-methylstryl)-benzene] (MSB)
(Research Products International Copr., Elk Grove Village, IL)

Dioxane (Mallinckrodt, Inc., Paris, KY)
Napthalene (Scintanalyzed, Fischer Scientific Co., Fairlawn, NJ)
Toulene (Scintanalyzed, Fischer Scientific Co., Fairlawn, NJ)
Triton X-100 (Research Products International Corp., Elk Grove Village, IL)
Xylene (Mallinckrodt, Inc., Paris KY)

APPENDIX D

Preparation and Utilization of Androstenedione Quench Curve

Quench Curve = relationship between counting efficiency of an isotope and a quench parameter. The quench parameter in this case is the Samples Channels Ratio (SCR).

Counting Efficiency = $\frac{\text{measured counts per minute (cpm)}}{\text{known disintegrations per minute (dpm)}}$

Rationale: Counting efficiency of a sample is affected by factors such as choice of scintillation cocktail or trace impurities. As the counting efficiency is affected, the cpm is affected but not the dpm. Therefore, dpm can be compared between experiments.

The quench curve consisted of glass scintillation vials each containing 43,663 dpm [1, 2, 6, 7, - ^{3}H(N)]-Androst-4-ene-3, 17-dione (^{3}H-Androstenedione, New England Nuclear Division of DuPont, Boston, MA). The vials also contained either 90, 80, 70, 60, 50, 40, 30, 20, 10 or 0 ul methylene chloride (HPLC grade, Fischer Scientific Co., Fairlawn NJ). as a quenching agent. Each quenched sample was replicated 4 times.

In order to determine dpm of ^{3}H-T and $^{3}H_2O$, the quench curve was loaded and stored internally by a LKB Wallac Rackbeta Scintillation Counter which then output sample data as dpm.

In order to determine dpm of ^{3}H-E_1 and ^{3}H-E_1SO_1 from the incubation medium, the quench curve was entered into BSTAT, a statistical package for IBM personal computers, and fitted by a quadratic regression. Another program for the personal computer developed in this laboratory (G.L. Johnson, 1985, unpublished) used this regression to convert cpm and SCR to dpm.

APPENDIX E

Developing X-Ray Film [ab]

Solution	Time in Solution
Kodak GBX Developer (#1900943)	4 min
1% Acetic Acid Stop Bath	30 sec
Kodak GBX Fixer (#190 1875)	2.5 min
Cold, moving tap water	final rinse

Film was hung to dry.

[a] Kodak X-Omat AR-5 film.

Kodak Products (Eastman Kodak Co., Rochester, NY)

[b] Developed under filtered safety light

APPENDIX F

Dye for Protein Assay

Coomassie Brilliant Blue Dye (G-250)	100 mg.
95% Ethanol	50 ml
85% (w/v) Phosphoric acid	100 ml
Deionized water	total volume 1L

The dye mixture was filtered using .45 um filter.

Special Instructions: Dissolve dye in Ethanol before mixing phosphoric acid or deionized water.

APPENDIX G

Testerone and Estrone Antibody Crossreactivity

A. Crossreactivity of Testosterone Antibody with Various Steroids

Steroid	Percent Cross Reaction (at 1000 pg)
Androstenedione	6.6
Dehydroisoandrosterone	2.7
5 Dihydrotesterone (5 Androstan 17-ol-3-one)	71.0

B. Crossreactivity of Estrone Antibody with Various Steroids [a]

Compound	Percent Cross Reaction
E_1	100
E_1SO_4	11.2
16-Ketoestradiol	<.1
16-Epiestriol	<.1
Pregnanetriol	<.1
Reichsteins Substance	<.1
17 -Hydroxyprogestrone	<.1
5ß-pregnan-3,20-dione	<.1
Cortisone	<.1
16-17, Epiestriol	<.1
Δ^4 Androsten-3,17-dione	<.1
5 α-pregnane-3,20-dione	<.1
Dehydroepiandrosterone	<.1
17 -Ethynlestradiol	<.1
Dihydrotestosterone	<.1
19-Nortestosterone	<.1
Estriol	<.1
17 -Estradiol	<.1
D-Equilenin	<.1

[a] from Jeantet, 1985.
Ab dilution = 1:8500. Mass E, required to inhibit 50% binding divided by mass of steroid required to inhibit 50% binding x 100.

APPENDIX H

Preparation of Phosphate Buffered Saline (PBS-Ga) Solution

Reagents

1. Solution A (0.2 M)

NaH_2PO_4 H_2O	27.6 g
Distilled H_2O	1,000 ml
Sodium Azide	2.0 g

2. Solution B (0.2 M)

$Na_2H_2PO_4 - 7H_2O$	53.6 g
Distilled H_2O	1,000 ml
Sodium Azide	2.0 g

3. Phosphate Buffer (PBS-a), pH 7.0

Sodium Cholride	9 g
Solvent A	195 ml
Solvent B	305 ml
Distilled H_2O	500 ml

This buffer may be kept at room temperature for approximately 1 mo.

4. 0.1% Gelatin in Phosphate buffer (PBS-Ga)

Gelatin (Knox, unflavored)	100 mg
PBS-a	100 ml

Warm if necessary to get into solution.
Adjust to pH 7.0.
Store at 4 C.

Chemicals:
Sodium Azide (Sigma Chemical Co., St. Louis, MO)
Sodium Chloride (Centrified: Fischer Scientific, Fairlawn, NJ)
Sodium Phosphate (Monobasic Crystal and Anhydrous-Dibasic: Fischer Scientific, Fairlawn, NJ)

APPENDIX I

Dextran Coated Charcoal Solution

Charcoal	250	mg
Dextran T-70	25	mg
Distilled Water	50	ml

APPENDIX J

Improving Experimental Design

If this project were to be redone and expanded upon, there are a number of procedural changes to be made.

1. Add gilts on d 25, 55 and 85 to the study. Days 30, 60 and 90 were chosen originally as representative times of dynamic changes in plasma estrogen concentrations and in vitro placental and endometrial estrogen production. The enzyme activity for these changes may be developing before the appearance of changes in plasma estrogen concentrations.

2. Record corpora lutea number per ovary at the time of Hyst-X. The large gilt to gilt variation permeates every aspect of this study. Awareness of significant differences in litter size might have accounted for some of this variation.

3. Collect allantoic and amniotic fluid samples from a representative number of fetuses per gilt to analyze for T, E_1 and E_1SO_4. In conjunction with plasma concentrations of these steroids, awareness of fetal fluid steroid concentrations would give a more representative profile of steroid production and metabolism by the conceptus than plasma steroid concentrations alone.

4. Incubate a larger tissue sample in a greater volume of incubation media and take aliquots at the desired time intervals. Enzyme activity over time in incubation would not be influenced by tissue differences between samples as they are in the current procedure where separate tissue samples are incubated for each time

interval. Time 0 activity would not be affected by continued enzyme activity of the tissue as it freezes. Removing media aliquots at consistent time intervals, every half hour for example, would make analysis of time in incubation effects by orthogonal contrasts possible. Also take aliquots from media plus substrate which is subjected to identical incubation conditions, with no tissue sample to generate control values for each time interval to be examined.

5. Change proecedure for determining sulfatase and sulfotransferase activity.

Without a TLC plate scanner, quantification of $^{3}H\text{-}E_1$ and $^{3}H\text{-}E_1SO_4$ on the TLC plates is a long and tedious procedure subject to a substantial degree of human error when removing the radioactive bands from the plates. Incubation with nonlabelled precursors and subsequent RIA would be an alternative which would also measure endogenous E_1 and E_1SO_4. Physical separation of the microsomal fraction, containing sulfatase, from the soluble cell fraction, containing sulfotransferase, or inhibition of one of the enzymes would be alternate methods of quantifying sulfatase or sulfotransferase activity without cross-contamination of enzyme systems.

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